Centro de Investigación Científica y de Educación Superior de Ensenada, Baja California

Doctorado en Ciencias en Acuicultura

Optimization of alternative ingredients in low fishmeal diets using functional nutrients in *Totoaba macdonaldi* juveniles

Tesis
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Abstract of the thesis presented by **José Pablo Fuentes Quesada** as a partial requirement to obtain the Doctor of Science degree in Aquaculture.

Optimization of alternative ingredients in low fishmeal diets using functional nutrients in *Totoaba macdonaldi* juveniles

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The use of fishmeal (FM) in diets for carnivorous fish is one of the main concerns of the growing aquaculture industry. The constant increase in cost, the limited supply and sustainability issues related to its use in fish diets rather than for human consumption, urgently warrant reducing FM dependence in carnivorous fish diets and increase the use of more sustainable and cost-effective ingredients. Therefore, the objectives of this study were to evaluate the inclusion of alternative ingredients in low FM diets and the supplementation with the prebiotic agavin and the functional nutrient glutamine as a nutritional strategy to improve intestinal health and optimize the use of more sustainable diets resulting in better growth at a lower cost in cultured *Totoaba macdonaldi* juveniles. For this, four consecutive bioassays were performed in totoaba juveniles fed to apparent satiation three times a day. For the first bioassay, (fish initial weight (IW) 72 g), the effect of increased inclusion levels of soybean meal (SBM) in the diet (i.e., 0 %, 22 %, 44 %, and 64 %) was evaluated during 56 days. Increasing the level of SBM showed a significant negative relationship on growth, digestive capacity, and distal intestine integrity. Additionally, a significant decrease in the thermal growth coefficient, condition factor, protein efficiency ratio with an increase in the feed conversion ratio and alteration of the intestinal integrity associated with enteritis were observed on inclusion levels above 22 % SBM. Fish fed with the 44 % and 64 % SBM diets resulted in the most severe damage in the distal intestine, including intestinal atrophy. For the second bioassay, (IW 61 g), a practical diet was formulated with poultry by-product meal and FM in a 2: 1 ratio with SBM included at 24 %. Supplementation with the prebiotic agavin at 2 %, increased growth and feed utilization, decreased the dependence for FM, reduced the costs of producing a kilogram of farmed totoaba and counteracted the negative effects of SBM by maintaining the intestinal integrity compared with fish fed the SBM diet without agavin, and did not result in significant differences compared to the fish fed the FM diet in all parameters evaluated after 44 days of feeding. In the third bioassay (IW 29 g) using the same formulation of the previous bioassay that included 24 % SBM, three levels of agavin (i.e., 1 %, 2 %, and 3 %) were evaluated to estimate the minimum quantity of the prebiotic in the diet that maximizes growth performance and gut health. The inclusion of agavin in the diet, independently of the prebiotic level, resulted in better growth, similar feed utilization in terms of nutrient digestibility and prevented alterations of the distal intestine associated with SBM induced enteritis after 56 days of feeding. The use of 1 % agavin is suggested as the recommended minimum level in diets with 24 % SBM inclusion. For the fourth and last bioassay (IW 168 g), the supplementation of 1.5 % glutamine, 1 % agavin and the combination of both in practical diets containing 24 % SBM were evaluated for 56 days. In contrast to previous results, there was no significant difference in growth, feed utilization and intestinal integrity between the control diet with SBM and diet supplemented with agavin; these results suggest that fish with larger initial size are less susceptible to SBM induced adverse effects. On the other hand, glutamine significantly increased growth, feed utilization, reduced the FM dependency ratio and lowered the cost of producing a kilogram of farmed totoaba when using low FM diets. Since the fish fed with the diet supplemented with glutamine + agavin resulted in intermediate values for the parameters evaluated, it is recommended to use longer bioassays and possibly perform some immunological or environmental challenge to aid in detecting any potential additional benefits of these functional nutrients and their synergetic effects.

Keywords: gut health, intestinal integrity, alternative ingredients, enteritis, functional nutrients, prebiotic, glutamine, *Totoaba macdonaldi*

Resumen de la tesis que presenta **José Pablo Fuentes Quesada** como requisito parcial para la obtención del grado de Doctor en Ciencias en Acuicultura.

Optimización de ingredientes alternativos en dietas bajas en harina de pescado usando nutrientes funcionales en juveniles de *Totoaba macdonaldi*

Resumen aprobado por:

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Dr. Juan Pablo Lazo Corvera

Director de tesis

El uso de harina de pescado (FM) en dietas para peces carnívoros es una de las principales preocupaciones de la industria acuícola en continuo crecimiento. El aumento del costo de la FM, la limitación en la oferta y cuestionamientos de sustentabilidad sobre su uso como un recurso que puede ser utilizado para consumo humano, hace urgente reducir la dependencia de este recurso marino en las dietas para peces carnívoros y reemplazarlo por ingredientes de menor costo. Por lo tanto, los objetivos del presente estudio fueron evaluar la inclusión de ingredientes alternativos en dietas bajas en FM y la suplementación con el prebiótico, agavina, y el nutriente funcional, glutamina, como estrategia nutricional para mejorar la salud intestinal y optimizar la utilización de dietas que permitan un crecimiento adecuado a menor costo en juveniles de *Totoaba macdonaldi*. Se realizaron cuatro bioensayos consecutivos en juveniles de totoaba alimentados a saciedad aparente tres veces al día. En el primer bioensayo (peso inicial del pez (IW) 72 g), se evaluó el incremento de la inclusión de harina de soya (SBM) en dieta al 0 %, 22 %, 44 % y 64 % durante 56 días. El aumento del nivel SBM mostró una relación negativa en el crecimiento, capacidad digestiva e integridad del intestino distal. A partir del 22 % de inclusión de SBM se observó una disminución en el coeficiente térmico de crecimiento, factor de condición, tasa de eficiencia proteica, aumento de la tasa de conversión alimenticia y cambios en la integridad intestinal asociados a enteritis. Los peces alimentados con 44 % y 64 % de SBM fueron los que presentaron los daños más severos en el intestino distal (i.e., enteritis). En el segundo bioensayo (IW 61 g), se formuló una dieta con harina de subproducto de ave y FM en una relación 2:1, y con 24 % SBM. La suplementación con el prebiótico agavina al 2 %, incrementó el crecimiento y la utilización del alimento, disminuyó la dependencia por la FM, lo que redujo los costos de producir un kilogramo de totoaba cultivada y contrarrestó los efectos negativos de la SBM manteniendo la integridad intestinal comparado con los peces alimentados con la dieta con SBM y sin agavina, y sin cambios significativos con los peces alimentados con la dieta de FM después de 44 días de alimentación. En el tercer bioensayo (IW 29 g), con la misma formulación del bioensayo anterior incluyendo 24 % SBM, se evaluaron tres niveles de agavina en dieta (i.e., 1 %, 2 % y 3 %) para definir un valor mínimo de uso del prebiótico que maximice el crecimiento y mantenga la salud intestinal. La inclusión de agavina, independientemente del nivel, resultó en mejor crecimiento, mantuvo la utilización del alimento sin cambios en la digestibilidad de nutrientes y previno cualquier alteración en el intestino distal causada por la SBM durante 56 días de bioensayo. Se sugiere el uso de 1 % de agavina como el nivel mínimo en dietas con 24 % de inclusión de SBM. En el cuarto bioensayo (IW 168 g), se evaluó la suplementación de 1.5 % de glutamina, 1 % de agavina y la combinación de ambos en dietas prácticas con 24 % SBM por 56 días. En contraste con los resultados anteriores, no se encontró diferencia en el crecimiento, la utilización del alimento e integridad intestinal entre los peces alimentados con la dieta control con SBM y la suplementada con agavina, lo cual sugiere que los peces de mayor tamaño son menos susceptibles a los efectos adversos de la SBM. Por su parte, la glutamina incrementó significativamente el crecimiento y utilización del alimento, reduciendo la dependencia por la FM y el costo de producir un kilogramo de totoaba cultivada con dietas bajas en FM. Por su parte, la dieta suplementada con glutamina + agavina presentó valores intermedios, por lo que se recomienda el desarrollo de experimentos de mayor duración y/o retos a cambios en las condiciones de cultivo o retos inmunológicos para detectar los beneficios potenciales adicionales de los nutrientes funcionales y sus efectos sinérgicos.

Palabras clave: salud intestinal, integridad intestinal, ingredientes alternativos, enteritis, nutrientes funcionales, prebiótico, glutamina, *Totoaba macdonaldi*

Dedication

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Chapter 1. General introduction

1.1 Replacement of fishmeal in marine fish diets

The demand for seafood products by the growing world population and the stagnant catch of fisheries has led to the constant growth of the aquaculture industry, going from producing 32 million tons in 2000 to 80 million tons in 2016 and is expected to increase by more than 30 million tons to meet the human demand for foods in 2030 (FAO, 2018). It is a given fact that the aquaculture industry will play an important role in global food security in the near future. However, almost 70 % of cultured aquatic animals depend on external feeds, particularly the culture of marine finfish (Tacon and Metian, 2015). One of the main limitations to continue with the expansion of the aquaculture industry is the availability of sufficient adequate protein raw materials for the manufacture of aquafeeds (Naylor et al., 2009; Pelletier et al., 2018). Marine fish production represents 8 % (i.e., 6.6 million tons) of the total aquaculture production due in part to the main bottlenecks in the culture of marine fish larvae, the limited number of species with a constant production of juveniles and the high investment capital required for the used marine facilities. In recent years, the collaboration between academia and private companies has sought to identify and develop marine finfish aquaculture in a sustainable way (e.g., https://www.diversifyfish.eu/).

Fishmeal (FM) and fish oil (FO) have been the most important ingredients in the formulation of aquafeeds due to the excellent results in most species cultured which resulted in a high dependency of the aquaculture industry for these finite marine resources (Olsen and Hasan, 2012). Fishmeal has a high digestibility, good palatability and the amino acid composition supplies enough of these major essential nutrient requirements for fish (NRC, 2011). These nutrient quality characteristics results in the aquaculture industry consuming 68 % of the FM produced worldwide each year (Mallison, 2013). In particular, the culture of carnivorous species, such as marine fish that demand high crude protein content (40-55 %) in their diets (NRC, 2011; Tacon and Metian, 2015). Nevertheless, most of the FM and FO come from the capture of forage fish that could instead be used for human consumption, and the catches of this forage fish are at the maximum (if not higher) than their sustainable limits (Cashion et al., 2017).

In the last two decades, the stability in the production of FM from 4.5 to 6 million tons per year (IFFO, 2018) and the continuous growth of aquaculture industry have reduced its availability and increased its cost more than two-fold (Fig. 1). If this trend continues, in the coming years the demand from the aquaculture industry for fishmeal will surpass the annual production and drive the feed manufacturers to

gradually reduce the dependence of these commodities using alternative cost-effective protein sources (Silva et al., 2018). For 2020, it is expected a reduction of FM use from 24 % to 12 % inclusion levels in commercial diets for marine fish (Tacon et al., 2011). Thus, the substitution of the FM is a priority in the aquaculture industry to reduce the cost of feed with a less expensive species-specific formulation that maintains efficient growth at a lower cost per unit gain (Hardy, 2010).

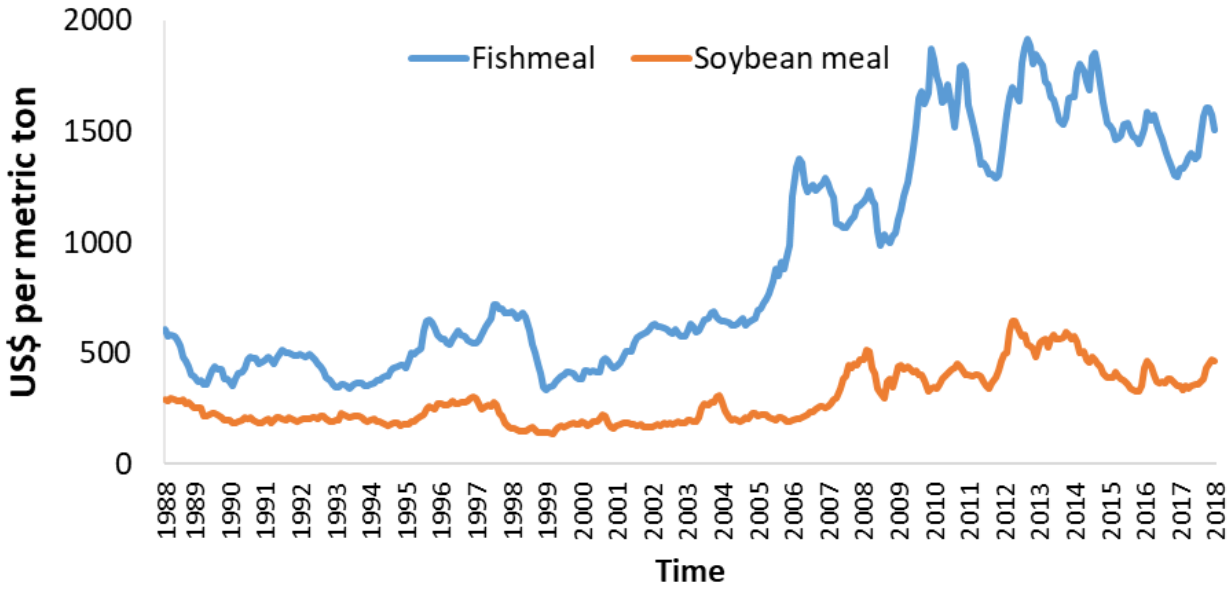

Figure 1. Fishmeal (Perú Fish meal 65 % protein) and soybean meal 44 % (any origin) prices per year. Modified from Indexmundi.com. Consulted August 22, 2018. https://www.indexmundi.com/commodities/?commodity=fish-meal&months=360

The formulation of low FM diets is essential to achieve sustainability in the aquaculture industry in the long term and as a net producer. Marine fish farming has been considered as a high consumer of fishery resources, because it consumes more fish (FM and FO) than they produce (Naylor et al., 2000). Indicators of fisheries resource utilization such as the fish in: fish out (FIFO) ratio or the marine protein (or oil) dependency ratio (MPDR or MODR) have been used to calculate the amount of FM and FO to produce 1 kg of farmed fish (Tacon and Metian, 2008; Crampton et al., 2010; Sarker et al., 2013). For example, in the Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* farming, Tacon and Metian (2008) calculated FIFO ratios that resulted in 4.9 and 3.9, respectively. In addition, Naylor et al. (2009) reported a FIFO ratio of 5.0 for farmed salmon. Nonetheless, for the same species, Sarker et al. (2013) reports that current commercial diets can significantly reduce the dependency for FM due to increasing inclusion levels

of non-marine proteins and reported a value for MPDR equal to 0.77 kg marine proteins to produce 1 kg of protein from farmed salmon.

Among the alternative protein sources to substitute FM, rendered animal by-product proteins and vegetable proteins are economic commodities for the formulation of cost-effective aquaculture feeds (Bureau, 2006; Brown et al., 2008). Rendered animal by-product proteins as ingredients alone or in combination are good sources of digestible energy and protein, essential amino acids, fatty acids, minerals and are highly palatable for most aquaculture species (Bureau, 2006; Gasco et al., 2018). In the last years, new production technologies have increased the quality of these animal by-product ingredients (Sarker et al., 2013). The main products of the rendering process are meat and bone meal, blood meal, hydrolyzed feather meal, plasma hydrolysate and poultry meal, with a protein content ranging from 50 % to 85 %, and have been shown to satisfactorily replace up to 20-40 % of the FM protein in the diets, corresponding to 15-60 % of dietary inclusion (Oliva-Teles et al., 2015). The typical incorporation levels of meat meal and poultry by-product in practical diets for carnivorous fish are from 10 % to 30 % (Tacon et al., 2009). On the other hand, the oilseed plants such as cottonseed meal, rapeseed meal, sunflower meal and soybean meal (SBM) are the main vegetable ingredients available to replace FM (Gatlin et al., 2007).

Oilseed meals have relatively low protein content (i.e., compared to animal products) ranging from 38 % to 52 %, but with a competitive price (Oliva-Teles et al., 2015). Research has shown that FM can be replaced by plants meals in fish diets with typical levels of 20 % to 40 %, corresponding to an inclusion level of 15-30 % of the diet (Oliva-Teles et al., 2015). In particular, the incorporation of oilseed meals in practical diets for carnivorous fish, is 10 % to 20 % (Tacon et al., 2009). The presence of certain antinutritional components among other factors is associated with the lower inclusion levels typically obtained with plant protein ingredients in marine fish diets (Krogdahl and Bakke-Mckellep, 2015).

The blend of several alternative protein ingredients has been shown to be a better strategy to replace the FM in marine fish diets compared to using only one ingredient. Diet formulation using two or more ingredients may reduce the ash content of the diet, balance amino acids deficiencies or fatty acids profiles allowing the formulation of practical diets at lower cost. Studies evaluating the combination of several plant and animal protein ingredients have resulted in diets formulations with FM inclusion lower than 20 % in species such as Australian snapper *Pagrus auratus* (Booth et al., 2012), Senegalese sole *Solea senegalensis* (Cabral et al., 2013), rainbow trout (Burr et al., 2012; Bruce et al., 2018), white seabass *Atractoscion nobilis* (Jirsa et al., 2015) and Atlantic salmon (Torstensen et al., 2008; Burr et al., 2012).

1.2 Alternative protein sources to fishmeal

1.2.1 Poultry by-product meal (PBM)

The poultry industry produced 22.4 million tons of offal in 2016 (http://www.fao.org/faostat/en/#data/QL). These inedible parts of poultry carcasses including, heads, necks, undeveloped eggs, feet, gizzards and intestine without their content, are rendered to produce the poultry by-product meal. The PBM is considered a good substitute to FM, since it has high protein content (60-65 %) with adequate levels and balance of amino acids, is well-digestible, has lower price and can be incorporated into the feed industry due the large quantities produced around the world (Bureau et al., 1999; Metts et al., 2011; NRC, 2011). Two types of PBM are available in the market, feed-grade (PBM-F) and pet-food grade (PBM-P). The PBM-F has a larger variability in its proximate composition (crude protein 49.3-63.7 %, lipids 10.5-24.5 %, ash 12.8-20.6 %), while PBM-P typically has a higher crude protein content (66.1 ± 1.9 %) with greater amino acid digestibility and lower lipid content (12.6 ± 1.6 %) and ash (15.1 ± 1.6 %), but with a higher cost (Dozier et al., 2003). In 2017, the average price of PBM with 67 % and 57 % crude protein was around $688 USD and $306 USD per metric ton, respectively (Swisher, 2018).

Studies evaluating the inclusion of PBM as a replacement for FM can be found with several species with promising results, such as gibel carp *Carassius auratus gibelio* (Yang et al., 2004), Black Sea turbot *Psetta maeotica* (Yigit et al., 2006), humpback grouper *Cromileptes altivelis* (Shapawi et al., 2007) and malabar grouper *Epinephelus malabaricus* (Li, K et al., 2009). An important concern when using PBM in fish diets is the lower levels of methionine, lysine and taurine that result when try to formulated diets with low FM content (Table 1). This warrants the supplementation of these indispensable amino acids and taurine to optimize fish growth (Rossi and Davis, 2012).

Table 1. Amino acids content in fishmeal and poultry by-product meal

Amino acid (%)	Fishmeal (FM)	Poultry By-product meal (PBM)	Dif. with FM
Methionine	1.70	1.13	-0.57
Cystine	0.57	0.76	0.19
Lysine	5.17	3.51	-1.66
Tryptophan	0.67	0.53	-0.14
Threonine	2.49	2.41	-0.08
Isoleucine	3.64	2.25	-1.39
Histidine	1.53	1.37	-0.16
Valine	3.26	5.40	2.14
Leucine	4.69	4.39	-0.30
Arginine	3.73	4.08	0.35
Phenylalanine	2.68	2.46	-0.22

Source: Evonik Industries, 2010.

1.2.2 Soybean meal (SBM)

The soybean (*Glycine max*, Linnaeus) is the most cultivated oilseed in the world with an estimated production of 347 million tons in 2016 (http://www.fao.org/faostat/en/#search/Soybean%20meal). The yield of soybean meal after oil extraction is 75 % (Hardy, 2010) resulting in a gross production of 231 million tons of SBM in 2016. A wide range of products are obtained from the bean, such as soy flour, soybean meal, full-fat SBM (heat-treated whole beans), soy protein concentrate (SPC) and soy protein isolate (SPI) (Erickson, 1995; Gatlin et al., 2007). Soybean meal is probably the most evaluated alternative ingredient in fish feeds, either dehulled (48 % crude protein, CP) or with hulls (44 % CP) (Gatlin et al., 2007; NRC, 2011). Among the soybean products, SBM is the most economical feed ingredient with high protein content, constant amino acid profile and large availability (Brown et al., 2008; Zhou, Z et al., 2017).

The use of SBM in marine fish diets has resulted in some nutritional deficiencies that should be addressed to optimize its use. Soybean meal has a lower concentration of some essential amino acids compared to FM, mainly in lysine, methionine, and threonine content, but can be solved with adequate amino acid supplementation (Table 2). In addition, the presence of several antinutritional factors (ANFs) that have been indicated as responsible for the adverse effects when SBM is included in fish diets (Francis et al., 2001a; Krogdahl and Bakke, 2015). For this reason, an adequate thermal process is used to reduce or inactivated the thermolabile ANFs in SBM, such as lectins, proteases inhibitors, phytic acid, antivitamins and antigenic proteins (Witte, 1995; Lusas and Rhee, 1995; Zhou, Z et al., 2017). Moreover, the processing of SBM (i.e., steaming, roasting, acid/alcohol percolation) to produce SPC and SPI considerably reduces the content of the thermostable ANFs (e.g., glucosinolates, oligosaccharides, non-starch polysaccharides, phytoestrogens, saponins) and increases the availability of some essential amino acids close to those found in FM (Lusas and Rhee, 1995; Buentello et al., 2015). But this refinement process increases the cost and limits its use in large-scale aquafeeds (Gatlin et al., 2007; Zhou, Z et al., 2017). Therefore, SBM is possibly one of the most cost-effective ingredients largely available with a reasonable nutritional value that could be used to reduce the use of FM in new emerging aquaculture fish species. However, for each species it is necessary to evaluate the effects on growth performance and proposed a maximum level of inclusion in diets.

Table 2. Amino acids content in fishmeal, soybean meal and soy protein concentrate

Amino acid (%)	FM	SBM	Dif. with FM	SPC	Dif. with FM
Methionine	1.70	0.64	-1.06	0.82	-0.88
Cystine	0.57	0.69	0.12	0.86	0.29
Lysine	5.17	2.92	-2.25	3.81	-1.36
Tryptophan	0.67	0.64	-0.03	0.77	0.10
Threonine	2.49	1.86	-0.63	2.40	-0.09
Isoleucine	3.64	2.17	-1.47	2.81	-0.83
Histidine	1.53	1.26	-0.27	1.61	0.08
Valine	3.26	2.26	-1.00	2.91	-0.35
Leucine	4.69	3.63	-1.06	4.73	0.04
Arginine	3.73	3.49	-0.24	4.51	0.78
Phenylalanine	2.68	2.42	-0.26	3.11	0.43

Source: Evonik Industries, 2010.

1.3 Antinutritional factors (ANFs) in soybean meal

Plants produce secondary metabolites that are used as a defense mechanism against animals, insects, fungi or bacteria (Bennett and Wallsgrove, 1994). These compounds are called ANFs or antinutrients since they reduce or prevent the use of one or several nutrients and/or alter the biochemical, physiological or immunological response of the animals that use plants or parts of them as a feed source (Makkar, 1993). Nonetheless, the high demand for formulated feeds and the restrictive use of FM due to its costs have increased the levels of vegetal proteins used in marine fish diets. Interestingly, research has shown that some of these ANFs when ingested in low amounts have a beneficial effect on the organism, and can serve as prebiotics, antioxidants, growth promoters or have immunostimulatory effects (Liu et al., 2003; Virgili and Marino, 2008; Chakraborty and Hancz, 2011; Chakraborty et al., 2014).

It is well known that inclusion of plant ingredients in fish diets, including SBM, may have adverse effects such as, reduced palatability, less feed utilization, inhibition of growth, intestinal dysfunction, modified gut microbiota, immune modulation, goitrogenesis, pancreatic hypertrophy, altered plasma biochemistry or liver damage (Francis et al., 2001a; Krogdahl et al., 2010; Krogdahl and Bakke, 2015; Bonvini et al., 2018; Fuentes-Quesada et al., 2018). The response and possible negative effects of plant feedstuffs in the animals may be modified by inclusion level, origin of the meal, fish species, age, feeding behavior, state of health, addition of functional nutrients or external digestive enzyme in diets, and environmental and farms management stressors (Urán et al., 2008a, 2008b; Krogdahl et al., 2003; 2010). The principal ANFs found in the SBM, adverse effects and mechanism of action are described below;

1.3.1 Lectins

Often called phytohaemagglutinins, lectins are glycoproteins with high specificity for recognizing carbohydrates, can agglutinate cells and precipitate glucoconjugate complexes without self-modifying (Kumar et al., 2013; He et al., 2015). In the epithelium of the small intestine there are many membrane receptors that have chains rich in carbohydrates. The lectins can recognize and bind to these receptors changing the intestinal cell membrane homeostasis, reducing the absorption of nutrients, increasing the permeability of the intestinal walls and increasing the number of mucous cells (Banwell et al., 1983; Menard et al., 2010).

1.3.2 Protease inhibitors

The Kunitz soybean trypsin inhibitor (KTI) and the Bowman-Birk protease inhibitor (BBI) are the two most characterized inhibitors in soybeans, the BBI being more thermostable than KTI (Norton, 1991). These compounds reduce the activity of pancreatic proteases by competitive inhibition in the intestinal chime and thus reduce digestibility of the diet. KTI has a single active site, binding more strongly to trypsin than to chymotrypsin. Otherwise, BBI has two active sites that can bind two molecules of the same enzyme or two different enzymes (Habib and Fazili, 2007). The reduction of the proteolytic activity in the intestine increases the loss of nitrogen in the feces. In addition, this reduction in digestive enzyme activity can cause pancreatic hypertrophy, since the hormone cholecystokinin responsible for the detection of trypsin levels stimulates the secretion of more proenzymes in the pancreas to compensate for the decrease activity (Krogdahl et al., 2010).

1.3.3 Phytic acid

The myoinositol hexakisphosphate (IP6) or phytic acid is the largest reserve of phosphorus and minerals of plants and can chelate essential minerals such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{3+} and Fe^{3+}, decreasing the absorption and bioavailability of these ions in monogastric animals that are feeding with high plant inclusion. In addition, the inability to hydrolyze phytic acid reduces the availability of phosphorus and the others divalent ions, which can lead to mineral deficiencies and limitations in the performance of animal growth (Bretti et al., 2012; Kumar et al., 2012).

1.3.4 Glucosinolates

Glucosinolates are secondary metabolites known as thioglucosides and are separated from the enzyme myrosinase in different compartments within the plant cell. When this barrier breaks down and come into contact, the enzyme hydrolyzes the glucosinolates, and toxic volatile compounds are generated such as, Isothiocyanates, oxazolidine- 2-thiones, nitriles, thiocyanates, that reduced feed intake, produce intestinal mucosal irritation, iodine deficiency and hypertrophy in liver, thyroid, and kidney (Tripathi and Mishra, 2007).

1.3.5 Antivitamins

Other antinutritional factors identified in soybean are the antivitamins A, D, E and B_{12}, which act by competition when having an analogous structure, by binding and by inactivation (NRC, 2011). Vitamin deficiencies affect a large number of physiological processes since they are cofactors in the intermediary metabolism, reducing the synthesis of neurotransmitters, affecting the function of organs such as the eyes, increase the incidence of anemia and mortality under stressful situations (Shiau and Lin, 2015).

1.3.6 Oligosaccharides

Oligosaccharides are water-soluble sugar compounds. The most important oligosaccharides found in soybean meal are sucrose (6-7 %), stachyose (5-6 %), raffinose (1-2 %) and verbascose, which represents between 12-15 % of total soluble carbohydrates (Francis et al., 2001a). These compounds are not digested by monogastric animals due to deficiency of the enzyme alpha-1,6-galactosidase, but upon reaching the colon can be fermented by bacteria and during the hydrolysis release gases such as carbon dioxide, methane, hydrogen (Mussato and Mancilha, 2007), which causes flatulence, increased intestinal motility and diarrhea (Krogdahl et al., 2010).

1.3.7 Non-starch polysaccharides

The non-starch poliysaccharides (NSP) are complex carbohydrates (e.g., pectins, galactans, celluloses, lignin, arabinoxylans, galactomannans) that are part of the cell wall of grains and cereals such as soybean (Saini, 1989). Although the exact mechanism of how they act is still unknown, it is believed that NSPs create a barrier to the action of digestive enzymes, bind minerals and bile salts, reducing the digestion of nutrients (Storebakken et al., 1998; Refstie et al., 1999). In addition, they are osmotically active compounds that can cause diarrhea (Refstie et al., 1997).

1.3.8 Phytoestrogens

The main phytoestrogens in soybean are isoflavones, which bind to estrogen receptors and other nuclear hormone receptors present in the cell in various tissues such as intestine and liver (Krogdahl and Bakke, 2015), which have antagonistic or synergistic effects with estrogens and are classified as endocrine disruptors (Kostelac et al., 2003). These compounds can interfere with reproduction regulation, affect feed intake, and modified the lipid and insulin metabolism (Mauvais-Jarvis, 2011, Caiozzi et al., 2012).

1.3.9 Antigenic proteins

The glycinin and beta-conglycinin are reserve proteins and have been identified as the main allergenic factors, which can trigger a nonspecific and specific response in the immune system (Maruyama et al., 2003; Li et al., 2017). In mice, they have been shown to modulate key transcription factors involved in the regulation of lipid metabolism (Moriyama et al., 2004). These proteins in monogastric animals can cross the mucosal epithelium of the intestine increasing permeability and altering intestinal motility, which decreases the absorption of nutrients, increases the predisposition to diarrhea and over time suppresses the immune system (Chen et al., 2011; Li et al., 2017).

1.3.10 Saponins

Saponins are glycosides of steroids and triterpenoids that have amphipathic properties (Faizal and Geelen, 2013) and have the ability to bind cholesterol to form insoluble complexes (Malinow et al., 1977). The amphipathic characteristic of saponins allows them to form micelles that can be interspersed with the cholesterol of the intestinal membrane, forming holes that modify the permeability of the enterocytes and facilitate the entry of bacteria or other compounds that would not normally enter or be absorbed (Krogdahl and Bakke, 2015).

1.4 Intestinal morphological changes in fish fed with diets containing SBM

The presence of one or more ANFs from vegetable ingredients, especially in diets containing SBM have been tipically asociated with intestinal inflammation in fish, often named soybean meal-induced enteritis (SBMIE) (Bakke-Mckellep et al., 2000; Gu et al., 2016). Enteritis is associated with an intestinal disorder or alteration of the intestinal mucosa integrity that was first described in the Atlantic salmon (*Salmo salar*) by van den Ingh et al. (1991, 1996), later defined as non-infectious inflammation of the distal intestine (Baeverfjord and Krogdahl, 1996) and which can be reversed by eliminating dietary SBM (Bakke et al., 2010). Recently, Atlantic salmon fed with increasing levels of semi-purified saponins from soybean in diets with fish meal, resulted in fish with inflammation of the distal intestine and has thus been proposed as the main causal agent of enteritis (Krogdahl et al., 2015).

The morphofunctional changes are more evident in the distal intestine (DI) than in the proximal intestine (van den Ingh et al., 1991; Burrells et al., 1999; Buttle et al., 2001). The DI gut segment is considered more sensitive to enteropathies caused by food because it is the major site of endocytosis of intact proteins (Bakke-Mckellep et al., 2000). The histological alterations typically observed and associated with the process of developing enteritis are the following: shortening of the mucosal folds (MF), reduction in the number of supranuclear vacuoles (SNV) of the enterocytes, widening of the lamina propria (LP) and the sub-epithelial mucosa (SM), increasing number of goblet cells (GC) in the intestinal epithelium and infiltration of inflammatory cells (e.g., lymphocytes, macrophages, eosinophilic granulocytes and neutrophils) in the SM and LP that decrease the capacity DI to digest and absorb nutrients (van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996; Bakke-McKellep et al., 2000; Refstie et al., 2000; Urán et al., 2008a, 2008b; Penn et al., 2011; Gu et al., 2016; Fuentes-Quesada et al., 2018).

1.5 Intestinal health of cultured fish

Intestinal health is a term that has recently entered the spotlight in aquaculture nutrition. The gastrointestinal tract (GIT) is responsible for vital physiological processes such as osmoregulation, feed digestion, absorption of nutrients, endocrine regulation, immune response, elimination of toxic metabolites and constitutes a barrier of defense against pathogens (Ray and Ringø, 2014). The intestinal mucosa is an interface between the external natural environment and internal fish environment and is the place where symbiont and pathogenic microorganisms colonize (Romero et al., 2014; Kiron, 2015) and the majority of this complex community of microorganisms resides within the GIT (Tremaroli and Bäckhed, 2012). The complex community of bacteria, yeasts, viruses, archaea and protozoa that inhabit the surface of the mucous membranes of the body of animals and that form commensal or mutualistic relations with the host is named microbiota (Bäckhed et al., 2005; Merrifield and Rodiles, 2015).

Undoubtedly, this close relationship goes back to thousands of years of coevolution (Ley et al., 2008), where the survival and numerous metabolic processes of the host can be carried out in their entirety or facilitated by these communities of microorganisms (Llewellyn et al., 2014). The importance of the role of these microorganisms in the GIT, mainly in the intestine, is due to the fact that the symbiotic relationship that prevails between the microbiota and the host is key to maintain fish homeostasis (Gatlin et al., 2015). Imbalances of the microbiota that modify its composition can alter the functionality of the normal or healthy condition of the host and is called dysbiosis.

In fish, the microbiota plays a vital role in the early development and maturation of the GIT and the immune system (Rawls et al., 2004, Bates et al., 2006; Pérez et al., 2010). In gnotobiotic zebrafish (*Danios rerio*), the microbiota is involved in the expression of 212 intestinal genes related to those that stimulate epithelial proliferation, nutrient metabolism, and the innate immune response (Rawls et al., 2004). In humans, Bäckhed et al., (2005) reported that the microbiota modulates the proliferation and differentiation of intestinal cells, which is fundamental to maintain the integrity of the barrier of the intestinal mucosa.

In the case of the immune system, the microbiota is in continuous contact with the gut-associated lymphoid tissues (GALT), which is composed of a large number of immune cells; lymphocytes, granulocytes, macrophages and immunoglobulins (Pérez et al., 2010; Rombout et al., 2011). The continuous stimulation of commensal bacteria modulates the maturation of GALT and allows the recruitment of immune cells in the intestinal mucosa and submucosa without mediating an inflammatory

process, this is done through a variety of pattern recognition receptors that recognize the antigens between commensal and pathogenic bacteria (Sanz and De Palma, 2009, Ignacio et al., 2016). The microbiota is part of the first line of defense within the intestine, regulate the paracellular permeability, the expression of mucin genes by goblet cells that produce a biofilms formed by glycoproteins and the secretion of antimicrobial peptides (e.g., defensins and angiogenins) by Paneth cells, all these mechanisms prevent establishment of pathogenic bacteria (Laparra and Sanz, 2010; Gomez et al., 2013).

The contribution of the microbiota associated with the GIT with exogenous enzymes to the host for the digestion of nutrients in the diet has been documented in several species. Ramírez and Dixon (2003) found the activity of carbohydrases, phosphatases, esterases, lipases and peptidases in intestinal anaerobic bacteria isolated from the oscar fish *Astronotus ocellatus*, angelfish Pt*erophyllum scalare,* and the southern flounder *Paralichthys lethostigma*. Ray et al. (2012) isolated and identified the enzyme-producing microbiota and reported the activity of chitinases, cellulases, and phytases that enhance the digestive processes in the GIT of fish. In addition, the microbiota can supply essential vitamins (e.g., cobalamin). For example, bacteria such as *Cetobacterium somerae* present in rainbow trout (Kim et al., 2007) and tilapia *Oreochromis niloticus* (Tsuchiya et al., 2008) have been shown to produce cobalamin. Interestingly, in other species such as the channel catfish *Ictalurus punctatus* and the Japanese eel *Anguilla japonica,* in which this bacterium has not been reported, have a requirement for this vitamin (Romero et al., 2014).

The diet can modify the abundance and richness of the microbiota according to the quantity and types of ingredients, and levels of nutrients (Navarrete et al., 2013; Romero et al., 2014). Changes in the microbiota by diet have been observed in Atlantic salmon with high and low levels of fishmeal (Zarkasi et al., 2016), with different mixtures of alternative ingredients to fishmeal (Gajardo et al., 2017) and with 100 % vegetable protein diets (Schmidt et al., 2016). The latter studies found that the diversity and the abundance of acid-lactic bacteria increased in fish fed vegetable protein sources and that Bifidobacteria and lactobacilli were the most predominant bacteria in comparison with those found in fish fed fish meal based diets and were considered beneficial for the health (Ventura et al., 2009).

Reveco et al. (2014) in Atlantic salmon found a reduction in the diversity of bacterial populations in fish that presented inflammation of the distal intestine when fed with soybean meal based diets. These authors concluded the intestinal bacterial population was highly affected by dietary constituents and may play a role in the development of SBM-induced enteritis. In a recent study with sea bream fed diets with a 100 % vegetable protein sources, the intestinal microbiota profile was highly altered and a high mortality was observed in fish fed the 100 % vegetable diet compared to the fish fed fishmeal diet. The authors suggested

that lower survival was due the vegetable ingredients in the diet which produced imbalances in bacterial populations (Estruch et al., 2015).

The gastrointestinal tract of carnivorous marine fish is not morphologically and physiologically adapted to use relatively high levels of alternative protein sources (i.e., soybean) in commercial diets. Additionally, the intensification of the culture systems, have resulted in many cases with increased use of antibiotics, reduced feed efficiency, low growth and massive mortalities under these relatively stressful situations. The development of cost-effective diets with alternative ingredients is mandatory for the next years if we are to be sustainable, and the use of functional nutrients will be help to optimize feed utilization, reduce antibiotic use and improve growth performance if we achieve to maintain healthy intestines in fish using these new practical diets.

1.6 Functional nutrients in fish nutrition

The research to improve growth performance and gut health in fish, mainly when high levels of plant ingredients are included in the diet, has promoted the use of nutrients, supplements or compounds with a biological function that help protect the intestine and reduces the presence of enteropathies. These molecules of biological origin, which may be nutrients or not, that have a selective effect on one or several functions and that confer benefits to the health of the organism are named bioactive or functional compounds. Among these compounds there are prebiotics, probiotics, nucleotides, organic acids and amino acids such as glutamine, arginine, and taurine.

1.6.1 Prebiotics

Research with prebiotics in aquaculture has resulted in a milliard of benefits in a wide range of species triggering the number of studies in the last years (Dimitroglou et al., 2011a; Ringø et al., 2010, 2014, 2016; Torrecillas et al., 2014; Akhter et al., 2015; Gatlin et al., 2015; Hoseinifar et al., 2016a; Guerreiro et al., 2017). For a compound or nutrient to be classified as a prebiotic several requirements have to be met; 1) no hydrolysis can occur of the compound in the stomach or anterior or middle intestine, 2) can be fermented by a group of bacteria present in the gut and 3) this group of bacteria stimulates fish growth or

their activity confers a benefit to the host (Gibson et al., 2004). Therefore, the definition of prebiotic refers to non-digestible food ingredients that selectively stimulate the growth and/or activity of one or a limited number of bacteria in the gastrointestinal tract that confer benefits on the welfare and health of the host (Gibson and Roberfroid 1995; Gibson et al., 2004; Ringø et al., 2016; Akhter et al., 2015).

Prebiotics are non-digestible carbohydrates that can be classified according to their molecular size or degree of polymerization as monosaccharides, oligosaccharides or polysaccharides (Akhter et al., 2015). Among the polysaccharides we find inulin and β-glucans, while in the oligosaccharides the most studied are mannanoligosaccharides (MOS), galactooligosaccharides (GOS), fructooligosaccharides (FOS), short-chain fructooligosaccharides (scFOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS), isomaltooligosaccharides (IMO), transgalactooligosaccharides (TOS) and commercial mixtures (Ringø et al., 2014; Guerreiro et al., 2017). Some commercial products containing prebiotics have recently been evaluated with interesting results in fish such as Previda ™, Bio-MOS ® and Grobiotic-A (Song et al., 2014; Gatlin et al., 2015; Guerreiro et al., 2017).

The positive effects of the prebiotics reported include growth promoters (Zhou, et al., 2010), immune system response (Torecillas et al., 2015; Carbone and Faggio, 2016), feed utilization (Buentello et al., 2010), nutrient digestibility (Burr et al., 2008), activity of digestive enzymes (Soleimani et al., 2012), survival (Ibrahem et al., 2010), antioxidant potential (Guerreiro et al., 2015a), intermediary metabolism (Torrecillas et al., 2015; Guerreiro et al., 2015b, 2015c, 2015b), microbiota modulation (Dimitroglu et al., 2010b; Guerreiro et al., 2018b), modification of the gastrointestinal integrity (Torrecillas et al., 2013; Dimitroglu et al., 2009, 2010a; Bai et al., 2017) and reduce negative effects of SBM-induced enteritis (Resftie et al., 2010, Bai et al., 2017). Nonetheless, some studies have reported no effects when using prebiotics (Welker et al., 2007; Grisdale-Helland et al., 2008, Piccolo et al., 2011; Hoseinifar et al., 2014, Guerreiro et al., 2015b, 2015c, 2015d, 2016b, 2018a) and some studies even reported adverse effects on growth (Reza et al., 2009; Hoseinifar et al., 2011) and intestinal damage (Olsen et al., 2001; Ferrara et al., 2015). The effectiveness of prebiotics may depend on several factors such as the processing technology, level of supplementation, composition of the diet, culture conditions, species and the age of the organisms been fed the prebiotic (Guerreiro et al., 2017).

Based on the definition of prebiotics, the suggested mechanisms of action in fish include changes in the structure of bacterial communities, resulting in an increase in beneficial bacteria that promote the production of inhibitory compounds against harmful bacteria, competition for nutrients and adhesion sites, inhibition of virulent gene expression or disruption of quorum sensing mechanism of harmful

bacteria (Merrifield et al., 2010; Ringø et al., 2010; Akhter et al., 2015). Other mechanisms of action reported include beneficial bacterial end-products from the fermentation of carbohydrates. For example, the *Bifidobacteria* and *lactobacilli* are two groups of bacteria that can produce short-chain fatty acids (SCFAs), principally, butyrate, propionate, and acetate, that can decrease the pH of the intestine reducing the pathogen bacteria colonization and can modulate the host energetic metabolism (Gibson et al., 2004; Roberfroid et al., 2010; Slavin et al., 2013). In addition, SCFAs interact with pattern recognition receptors in the intestine epithelium and immune cells. These interactions induce mechanisms that play a key role in maintaining intestinal homeostasis (Sivaprakasam et al., 2016).

1.6.1.1 Fructans

One type of highly studied polysaccharides as prebiotic are the well know fructans. Fructans have been recognized as health-promoting food ingredients (Franco-Robles and López, 2015) and are classified according to the structural bonds in inulin, levans, graminans, and neoseries fructans (Vijn and Smeekens, 1999). Among these, the inulin-type fructans is a common studied prebiotic in fish (Ringø et al. 2010) and consists in a linear β(1-2) linked fructosyl chain (Ritsema et al., 2003). The length of fructosyl chains or degree of polymerization (DP) varies greatly in plants, in general this number is between 30 to 50 fructosyl residues (Vijn and Smeekens, 1999). Fructans chain lengths are considered small when this number is between 2 to 4 DP, medium from 5 to 10, and large from 11 to 60 fructose units. Inulin has a DP from 3 to 60 (Niness, 1999), the oligofructoses (OF) from 3 to 10 DP derived from native inulin (Roberfroid, 2007) and fructooligosaccharides (FOS) are a small chains fructans with a DP from 3 to 5 derived from sucrose (Carabin and Gary-Flamm, 1999). The linkages structure of fructans makes them indigestible for mammals and fish endogenous digestive enzymes. Fructans can reach without degradation the last portion of the intestine in fish or caecum and colon in humans, and can be fermented by the intestinal microbiota in these regions producing SCFAs (Kolida and Gibson, 2007).

The fructans are synthesizes in 12-15 % of higher plants as the main source of carbohydrates (Cairns et al., 2000) and can be isolated from edible fruits and plants such as wheat, onions, leeks, garlic, asparagus, artichokes, chicory roots, bananas and agave stem (Roberfroid, 1993; Van Loo et al., 1995). *Agave* genus is an economically important plant used as the raw materials on the tequila and mezcal manufacture in México. In the last years, the agave fructans coming from the *Agave* and *Dasylirion* species have been investigated for the prebiotics effects that benefits the host health by providing specific changes in the

composition and activity of the gut microbiota (Gibson et al., 2010; Huazano-García et al., 2017). *Agave* fructans can contain an external glucose, characteristic of graminans, and an internal glucose, characteristic of neofructans, and this type of fructans has been called “agavins” (Mancilla-Margalli and López, 2006). Agavins have a highly branched structure, which contain a complex mixture of β(1-2) and β(2-6) linkages (López et al., 2003), and have a DP from 6 to 32 that can vary according to the age, species and climate environment of the plant (Mancilla-Margalli and López, 2006). Although, agavin is not *per se* a natural fiber in fish diets, the prebiotic potential of this type of fructan may have interesting application in aquaculture and should be evaluated.

1.6.2 Glutamine

Glutamine is considered a conditional amino acid under certain physiological conditions such as malnutrition, infection or inflammation in vertebrates (Wu, 2009, 2010; Wu et., 2013). However, Windmueller and Spaeth (1980) showed the glutamine was the primary metabolic energy source of the small intestine of rats and is considered an essential nutrient for this organ. In vertebrates, it is the largest source of energy for the cells of the immune system and in rapid proliferation as the enterocytes (Burrin and Stoll, 2009; Pohlenz et al., 2012b). Increasing concentrations of intracellular glutamine stimulates protein synthesis and inhibits proteolysis in muscle and enterocytes, and thus it is considered to be a modulator of cell proliferation, differentiation, migration, and metabolism (Rhoads and Wu, 2009; Xi et al., 2011). In addition, it prevents the apoptosis of intestinal cells, by increasing the expression of antioxidant genes such as glutathione peroxidase, improving the ability to withstand epithelial lesions and is necessary for tight junction stabilization of the enterocytes (Rhoads and Wu, 2009; Xie et al., 2016; Kim and Kim, 2017).

The supplementation of glutamine in fish diets improves the overall zootechnical performance (Oehme et al., 2010; Cheng et al., 2011, 2012; Pereira et al., 2017), by increasing the response to immunological challenges (Pohlenz et al., 2012b, 2012c), enhancing digestive enzymatic activity (Yan and Zhou, 2006), resistance to hypoxia stress (Liu et al., 2015), improving the digestion process, nutrient absorption and intestinal integrity (Qiyou et al., 2011; Pohlenz et al., 2012a). Likewise, the incorporation of glutamine in diets with SBM or purified ANFs has been shown to have a protective effect on the intestine integrity and functionality with a reduction of the inflammation process (Cheng et al., 2011, 2012; Jiang et al., 2015; Gu et al., 2017; Liu et al., 2018).

1.7 *Totoaba macdonaldi*

Totoaba macdonaldi (Gilbert, 1891) is an endemic species of the Gulf of California (Fig. 2) and is distributed from the Colorado River delta to Mulegé, Baja California Sur on the west coast and to the Fuerte River, Sinaloa on the east coast (Jordan and Evermann, 1896; Flanagan and Hendrickson, 1976). Totoaba is the largest Sciaenidae and can reach more than 2 m in length and over 100 kg of weight (Berdegué, 1956; Cannon, 1966).

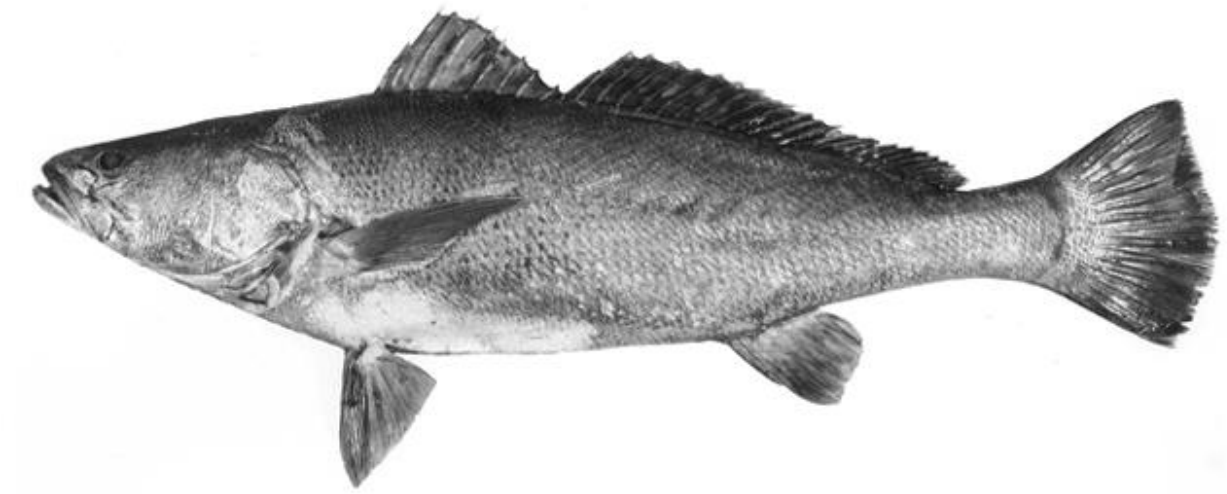

Figure 2. *Totoaba macdonaldi.*

The totoaba was an important sport and commercial fishery in the early twentieth century, with a maximum catch of 2300 metric tons in 1942 (Rosales-Juárez and Ramírez-González, 1987). Thereafter, due to overfishing and habit alteration catches dropped drastically to 59 metric tons in the early 70s (Lecardi and Chávez, 2007). Since 1975, the Mexican government declared total moratoria on fishing totoaba and in 1976 it was placed on the endangered list (Appendix I, threatened with extinction) of the Convention on International Trade in Endangered Species (CITES) (Román-Rodríguez and Hammann, 1997; CITES, 2018). Several authors report that the overfishing and the ecological alterations of breeding and nursery areas in the upper Gulf of California as the causes of population depletion (Flanagan and Hendrickson, 1976; Lecardi and Chávez, 2007). Currently, the totoaba continues to be place in the red list of the International Union for Conservation of Nature as a critically endangered species (Findley, 2010), mainly due the poaching fueled by the Chinese culture and market that believes the dried swim bladder often

called “maw” has lifesaving properties and is an afrodisiac. The latter believes and traditions have increase the black market were one kilogram or a single maw can cost up to $30 000 USD (Smith, 2017).

In the last two decades as part of the efforts to increase the population in the wild, a protocol for reproduction in captivity was developed (True et al., 1997, 2001), allowing the control production of eggs, larvae and juveniles resulting in the controlled release of juveniles into the wild for restocking purposes and helped develop the aquaculture industry of this promising species. Totoaba is considered a candidate for commercial aquaculture in the Baja California region, were under culture conditions can reach weights of up to 2-3 kg in one year and 6 kg in two years (Juarez et al., 2016). Today, there is a certified company for the cultivation of totoaba in La Paz, Baja California Sur and it is possible to buy meat for legal consumption, but only in Mexico.

Despite being a relatively new species for aquaculture, there is value information with respect to embryonic and larval development (Escuredo-Vielba et al., 2018), digestive ontogeny, larval culture and weaning (Galaviz et al., 2013; Mata-Sotres et al. 2015; Fuentes-Quesada et al., *unpublished data*), nutritional requirements and the use of alternative ingredients for grow-out diets (López-Rueda et al., 2011; Minjarez-Osorio et al., 2012; Badillo et al., 2014; Satriyo et al., 2017; González-Félix et al., 2018; Fuentes-Quesada et al., 2018; Mata-Sotres et al., 2018; Madrid et al., 2019).

1.8 Justification

The replacement of fishmeal from marine finfish diets is essential to maintain the sustainability of aquaculture in the long term. For the totoaba, a carnivorous endemic species with high potential for aquaculture in Mexico, it is necessary to carry out studies to provide species-specific diets with a better feed efficiency and more cost-effective.

The incorporation of plant proteins and rendered by-products as potential substitutes for fishmeal in marine fish diets should be evaluated in totoaba assessing safe inclusion levels in the diet that will allow the formulation of species-specific diets which do not compromise the animal performance and welfare, even under stressful culture conditions. To date, for totoaba under cultured conditions, the effects of dietary soybean meal and poultry by-product meal on the overall health, but in particular on the intestine

integrity and physiology are unknown and could hindered the possible cost-effective benefits of using these ingredients.

The use of functional nutrients in diets with low fishmeal to enhance the performance and intestinal health is a new research field that needs to be studied in emerging aquaculture marine fish species. In totoaba, very little is known with respect to the use of prebiotics on growth and intestinal health of the fish and in the case of glutamine, the possible benefits are still unknown.

1.9 Hypothesis

- The increasing levels of soybean meal in *Totoaba macdonaldi* diets should induce enteritis and increase intestinal disorders and lower growth performance.

- The supplementation of a prebiotic and glutamine in diets for *Totoaba macdonaldi* juveniles will reduce the adverse effects of the antinutritional factors in soybean meal and would allow including low or intermediate levels to reduce fishmeal without compromise the growth and intestine health.

1.10 Objectives

1.10.1 General objective

To evaluate the inclusion of alternative ingredients in low fishmeal diets for *Totoaba macdonaldi* juveniles and to improve growth performance, feed utilization and intestinal health using functional nutrients in the diets.

1.10.2 Specific objectives

- To evaluate the effect of graded levels of soybean meal in diets for *Totoaba macdonaldi* juveniles on growth and intestinal integrity of the animals to determine an optimal inclusion level.

- To evaluate the use of a prebiotic agavin as a functional nutrient to prevent intestinal damage in diets including soybean meal and poultry by-product meal for *Totoaba macdonaldi* juveniles.

- To determine the optimum inclusion level of prebiotic agavin in diets with soybean meal and poultry by-product meal for *Totoaba macdonaldi* juveniles.

- To evaluate the use of glutamine, and the possible synergistic effect with a prebiotic agavin to prevent intestinal damage in diets including soybean meal and poultry by-product meal for *Totoaba macdonaldi* juveniles.

Chapter 2. Enteritis induced by soybean meal in *Totoaba macdonaldi* diets: effect on growth performance, digestive capacity, distal intestine, and liver integrity

2.1 Introduction

The increase in SBM inclusion in the carnivorous fish diet is related to the occurrence of enteritis (Bakke-McKellep et al., 2000; Krogdahl et al., 2003), which is defined as non-infectious inflammation of distal intestine (Baeverfjord and Krogdahl, 1996) and can be reversed by eliminating SBM from the diet (Bakke, 2011). It is well known the SBM contains a high content of antinutritional factors associated with a damage of the mucosal integrity, decreased pancreatic and mucosal enzymes, loss nitrogen in the feces, thyroid hormone suppressors, lower mineral absorption, reduced palatability and suppression of the immune system (Francis et al., 2001a; Krogdahl et al., 2010). Recently, the saponins were suggested to be responsible for enteritis in Atlantic salmon (Krogdahl et al., 2015). Distal intestine associated with enteritis exhibited the following histological features: shortening of mucosal folds (MF), reduction in 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).

The SBM increase inclusion (0-30 %) in Atlantic salmon diets generates adverse effects even at 10 % (Krogdahl et al., 2003), similarly in turbot with levels ranging from 26-54 % (Gu et al., 2016) and 18-30 % in gilthead sea bream *Sparus aurata* (Bonaldo et al., 2008), whereas for European sea bass *Dicentrarchus labrax* up to 34 % is suggested without histological damage in the intestine (Bonaldo et al., 2008). Recently, Minjarez-Osorio et al. (2016) on two species of Sciaenidae and using a non-genetically modified soybean variety containing lower values of antinutritional oligosaccharides (i.e. stachyose, raffinose and trypsin inhibitors) succeeded in replacing up to 75 % of FM with SBM in juveniles of red drum *Sciaenops ocellatus* and 50 % in Shortfin weakfish *Cynoscion parvipinnis*.

Totoaba is a carnivorous species with a high requirement of >50 % crude protein (Rueda-López et al., 2011), so there is an interest in reducing the amount of FM in its diet. Of the soybean derivatives, only the soybean protein concentrate (SPC) has been evaluated in totoaba. The inclusion of taurine in Totoaba diets with 30 and 60 % SPC improves hematological parameters, growth and feed efficiency, however, the hepatic damage was reported in all diets with SPC (Lopéz et al., 2015). Likewise, Bañuelos-Vargas et al. (2014) mentions that taurine is an important modulator of the intermediate metabolism of the liver when

SPC is included in the totoaba diets and, Trejo-Escamilla et al. (2016) concludes that up to 34 % of SPC can be included in the totoaba diet without affecting growth.

The SPC has the advantage of containing lower levels of antinutritional factors (Hardy, 2010) but a higher cost than SBM. Also, in the previous works, it is suggested that the addition of taurine would allow increasing the level of inclusion of SPC in the diet, but there is not yet information related to histological changes in the intestine that may affect fish performance and welfare. Therefore, as the 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, distal intestine and liver integrity in juveniles of *Totoaba macdonaldi*, that may result in enteritis with detrimental outcomes in fish of commercial size.

2.2 Materials and methods

2.2.1 Diets 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 soybean meals (SBM and SPC at 4:1 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 an 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 were 0, 22, 44 and 64 % (Table 3).

Table 3. Formulation of *Totoaba macdonaldi* diets containing increasing levels of SBM. The dietary formulation is presented as g kg^{-1} on as fed basis and proximate composition in g kg^{-1} on a dry matter basis.

	Experimental Diets			
Ingredients (g kg^{-1} DM)	**0 % SBM**	**22 % SBM**	**44 % SBM**	**64 % SBM**
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).

2.2.2 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 ± SD) 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 1/2hp 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 total ammonia nitrogen, nitrite-nitrogen, and nitrate-nitrogen 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). 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 56 days. Daily, all uneaten feed was removed within an hour of feeding, and dry weighed to determine the most accurate feed consumption rates possible.

2.2.3 Sampling

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

Thermal growth coefficient (TGC) = $[(\text{final weight}^{1/3} - \text{initial weight}^{1/3}) / (T°C \times D_{days})] \times 1000$. (1)

Feed Conversion Ratio (FCR) = total feed consumed / wet weight gained. (2)

Condition Factor (CF) = final body weight x $(\text{body length})^3$ x 100 (Hardy and Barrows, 2002). (3)

Protein Efficiency Ratio (PER) = weight gain / protein intake. (4)

Feed Intake = FI (%/day) = 100 x (total amount of the feed consumed per fish / ((initial body weight + final body weight) / 2) / days). (5)

Hepatosomatic Index (HSI) = (hepatopancreas weight / total body weight) x 100. (6)

Viscerosomatic Index (VSI) = (viscera weight / total body weight) x 100. (7)

Intestinal Somatic Index (ISI) = (intestine weight / total body weight) x 100. (8)

For analytical analyses samplings were performed at 28 and 56 days, using one fish from each tank (three fish per treatment). Fish were fasted for 18 hours, and then were 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. The distal intestine was located between the intestinal constriction and the anus (Fig 3). 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.

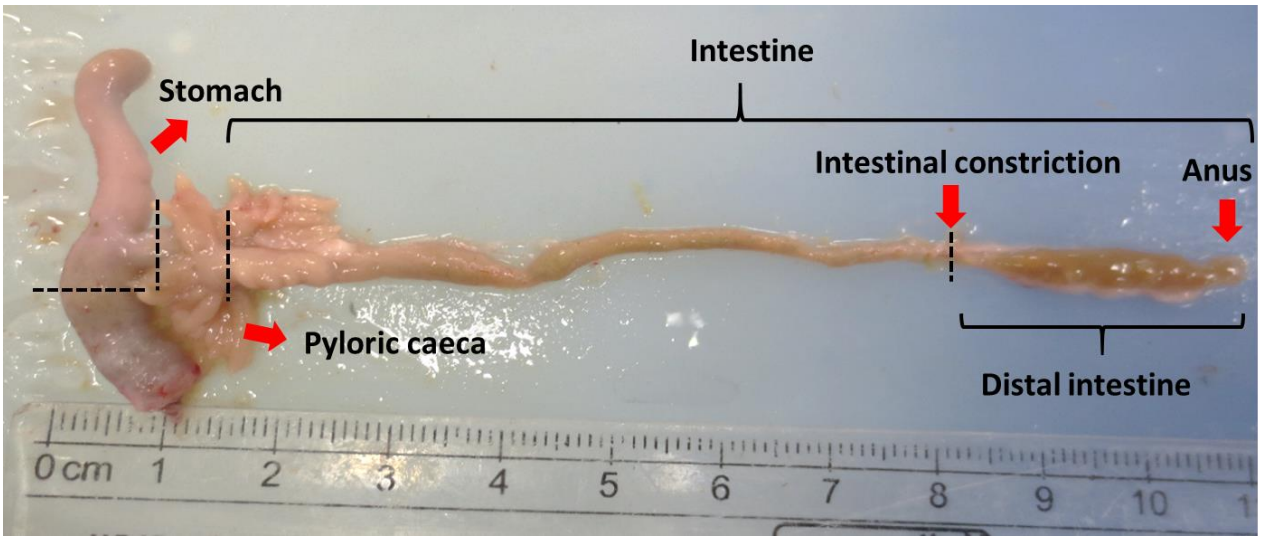

Figure 3. Illustration of sampling areas for histology and digestive enzyme assays.

Samples for digestive enzyme activity were taken only at the end of the experiment (56 days). The stomach, pyloric caeca, and intestine were dissected and stored separately from each experimental unit (Fig. 3). 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 28 and 56 days, while visceral and intestinal indexes only at 56 days. One sample from each liver lobule was taken (Fig. 4) at the end of the experiment (56 days), and histological samples following the same procedure described for the intestine fixation.

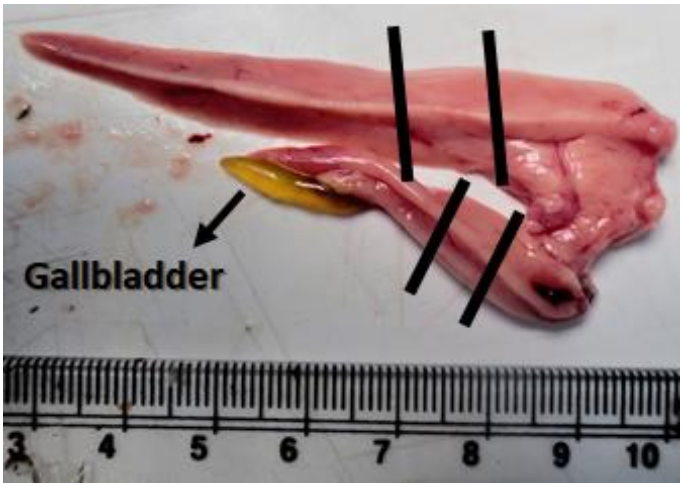

Figure 4. Sections for liver samples for histological evaluation.

2.2.4 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 (2015). 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 eight h. Crude protein (N x 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).

2.2.5 Intestine and liver histology

The figures 5 and 6 shows a distal intestine of totoaba fed with commercial diet as a reference for the normal intestinal histological arrangement and used to identify each structure assessed in the experimental trials of this thesis.

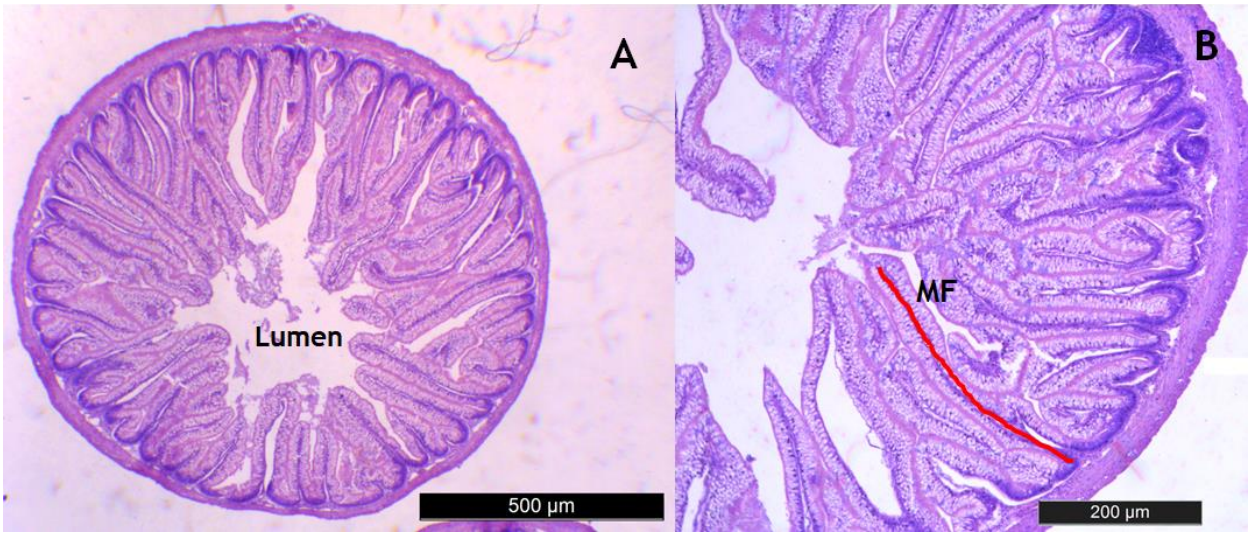

Figure 5. Totoaba distal intestine transversal section (Fig. 5A) and mucosal folds (Fig. 5B) fed with a commercial diet. MF = mucosal folds

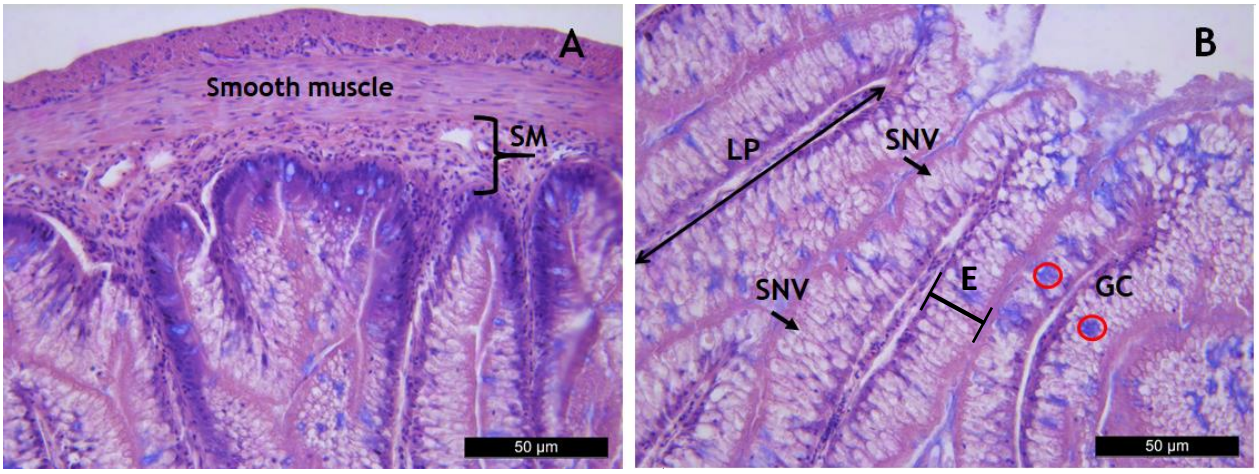

Figure 6. Totoaba sub-epithelial mucosa (SM, Fig. 6A) and, lamina propria (LP), supranuclear vacuoles (SNV), enterocyte (E) and goblet cells (GC) (Fig. 6B) fed with a commercial diet.

Per fish, samples of the distal intestine were 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 and placed into one slide for histological measurements using three replicates (n=9). 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 the 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 each intestinal section (Fig. 7A) length of complete MF (Fig. 8), enlargement of the lamina propria (LP) and sub-epithelial mucosa (SM) (Fig. 9A and B) and enterocyte height (EH) (Fig. 7B). The MF was count from the basal area and when it was visualized the continuity of the SM into the LP (Fig. 7B). All the MF were count by each intestinal section.

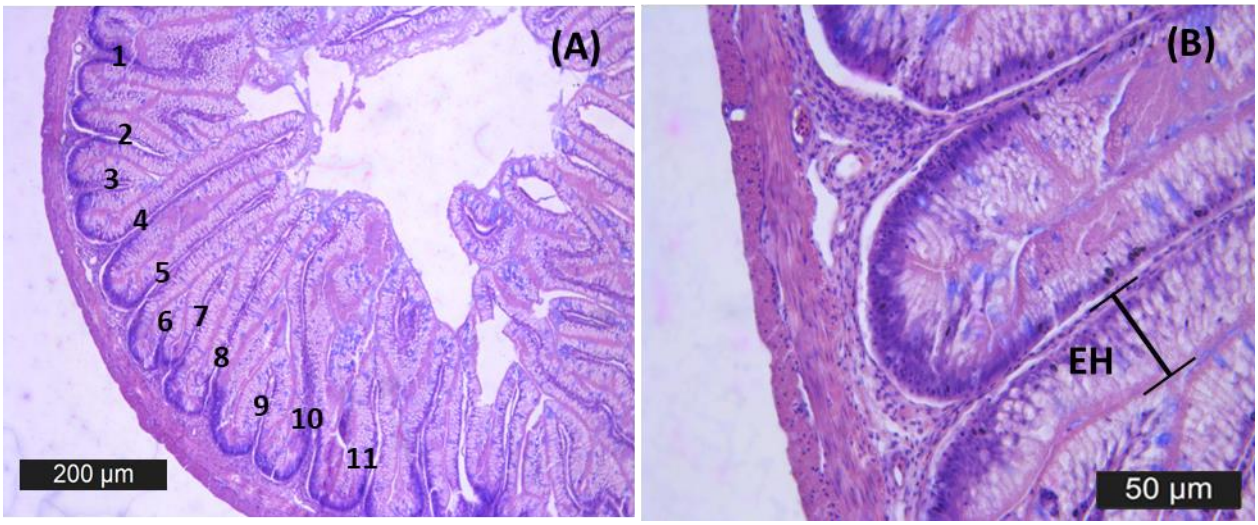

Figure 7. Illustration of the mucosal folds counting per intestinal section (A) and the measurement of enterocyte height (B).

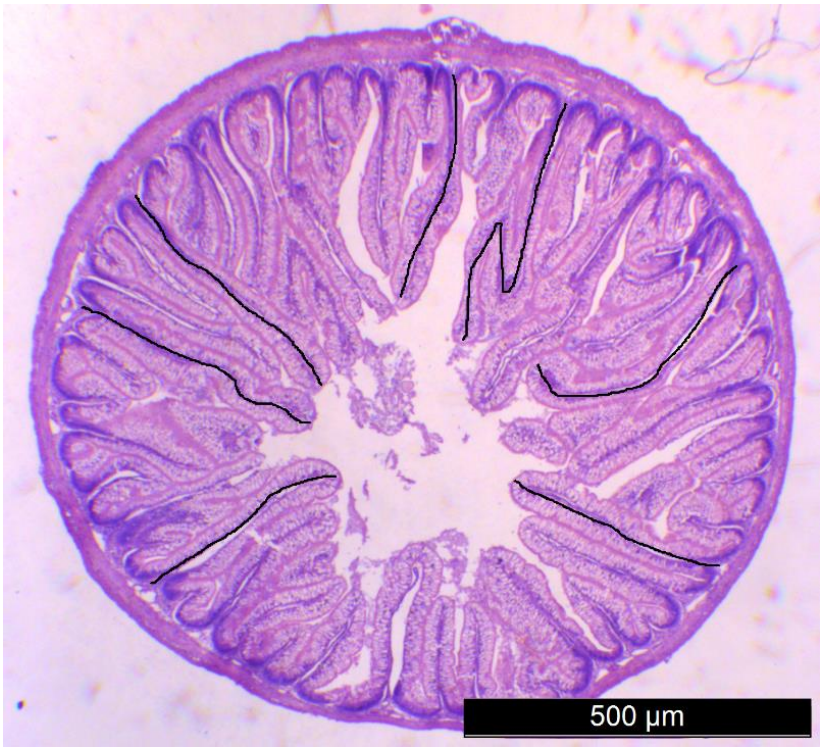

Figure 8. Measurement of the mucosal fold length in one section of the distal intestine of *Totoaba macdonaldi*.

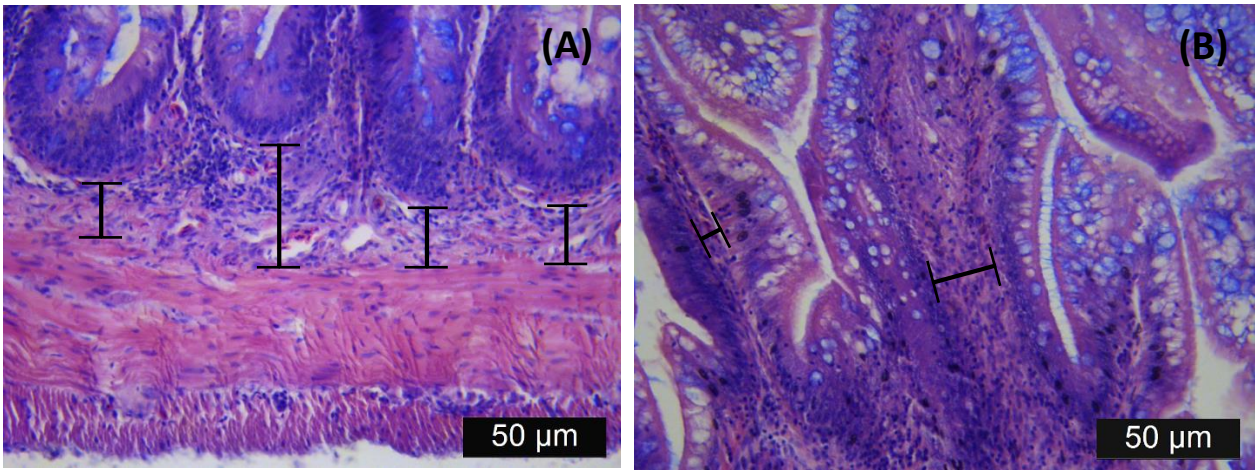

Figure 9. Illustration of the measurements of the sub-epithelial mucosa (A) and lamina propria (B) in the distal intestine of *Totoaba macdonaldi*.

Based on the results obtained at the end of the experiment, new thinner cuts of 3 µm from the same samples (56 days) were made to be able to observe in a better way the damage caused by the SBM inclusion. Liver histology alteration in terms of nuclear displacement, cytoplasm vacuolization, and infiltration of peripancreatic fat was assessed at the end of the experiment, based on the characterization of Caballero et al. (2004).

2.2.6 Digestive enzyme activity

Enzymatic extracts were obtained from whole organs by cutting them into pieces and homogenizing the tissues in 5 mL of cold distilled water for 2 min with a tissue grinder (POLYTRON® PT 1200, Kinematica AG, Switzerland) and centrifuged at 21000 g for 45 min at 4 °C (5417R, Eppendorf, USA). The 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 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. Each enzymatic assay includes a blank sample using distilled water instead of the homogenate and a positive control with commercial enzymes at a concentration of 1 mg mL^{-1}.

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 a substrate in 500 µL of DMSO. The reaction conditions were 50 mM Tris-HCl buffer, 20 mM CaCl2, 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 minutes 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 13110-F) as substrate in 100 mM Tris-HCl buffer, 25 mM CaCl2, pH 7.8 and methanol 2.5 % (v/v) at 37 °C. The reaction was recorded every minute for 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-leucine-P-nitroanilide (Sigma, L-9125) as a substrate in 50 mM HCl-Tris buffer, pH 8.0 at 37 °C. The reaction was incubated for 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 a substrate in 50 mM HCl-Tris buffer, 10 mM $CaCl_2$, 9.0 pH at 37 °C. The reaction was incubated for 10 min and stopped with 10 % trichloroacetic acid. The samples were centrifuged (21000 g, 5 min, 4 °C) and the absorbance of the 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 3 min, 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 the regression equation the micromoles of maltose released in each sample. The following formula was used to calculate the amylase activity; Unit/mg = (micromole maltose released / mg enzyme in reaction mixture x 3 (min)). All the samples were diluted 10 times in a final volume reaction of 1.2 mL to allow readings within the range of the standards.

Lipase activity was estimated according to 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 hemoglobin (Spectrum Chemical, HE120) in 200 mM, pH 2 at 37 °C. The reaction was incubated for 10 min and stopped with 5 % trichloroacetic acid. The samples were centrifuged (21000 g, 5 min, 4 °C) and the absorbance of the 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).

2.2.7 Statistical analyses

The assumptions of normality and homogeneity of variances were evaluated by the Shapiro-Wilks and Barlett test respectively (Zar, 2010). Significant differences in performance indexes, somatic indexes, and enzyme activities levels were analyzed by one-way ANOVA, followed by post-hoc Tukey rank test. The effect of SBM inclusion and time of MF number and length, EH, LP and SM were analyzed by two-way ANOVA. For all cases, statistical significance was set at $P<0.05$. Statistical analysis was performed using the software STATISTICA 8.0™ (StatSoft, Inc. USA).

2.3 Results

2.3.1 Growth performance and somatic indexes

At 56-days growth and production performance of juvenile totoaba was significantly affected by dietary SBM inclusion level (Table 4). 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 ($P<0.05$) affected TGC (1.18), FCR (1.11) and 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).

Table 4. Growth performance of *Totoaba macdonaldi* fed diets containing different levels of SBM at 56 days. Values are presented as means ± standard deviation (SD) and different letters represent significantly different values (P<0.05) within the same row.

	0 % SBM	22 % SBM	44 % SBM	64 % SBM	P value
TGC[1]	1.61 ± 0.13^{a}	1.39 ± 0.14^{ab}	1.30 ± 0.05^{ab}	1.21 ± 0.19^{b}	0.029
FCR[2]	0.82 ± 0.03^{b}	1.03 ± 0.07^{a}	1.06 ± 0.09^{a}	1.11 ± 0.07^{a}	0.004
CF[3]	1.70 ± 0.06^{a}	1.52 ± 0.09^{b}	1.51 ± 0.02^{b}	1.50 ± 0.02^{b}	0.007
PER[4]	2.50 ± 0.09^{a}	2.00 ± 0.13^{b}	1.94 ± 0.18^{b}	1.87 ± 0.11^{b}	0.002
FI[5] (% day^{-1})	0.87 ± 0.05	0.84 ± 0.04	0.98 ± 0.15	0.95 ± 0.07	0.260

[1] TGC (Thermal Growth Coefficient) = [(final weight^{⅓} - initial weight^{⅓}) / (T°C x D)] x 1000 (Jobling, 2003).
[2] FCR (Feed Conversion Ratio) = total feed consumed / wet weight gained.
[3] CF (Condition Factor) = final body weight x (body length)3 x 100 (Hardy and Barrows, 2002).
[4] PER (Protein Efficiency Ratio) = weight gain / protein intake.
[5] FI, Feed Intake = FI (%/day) = 100 x (total amount of the feed consumed per fish / ((initial body weight + final body weight) / 2) / days).

At 28 days, a significant reduction in the HSI was observed in fish fed with 44 % and 64 % SBM inclusion diets. At 56 days 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>0.05) were found for VSI and ISI (Table 5).

Table 5. Somatic indexes of *Totoaba macdonaldi* fed diets containing different levels of SBM at 56 days and HSI at 28 days. Values are presented as means ± standard deviation (SD) of three replicates, and different letters represent significantly different values (P<0.05) within the same row.

	0 % SBM	22 % SBM	44 % SBM	64 % SBM	P value
HSI %[1] at 28 days	1.71 ± 0.32^{a}	1.23 ± 0.33^{ab}	0.99 ± 0.15^{b}	1.04 ± 0.11^{b}	0.026
HSI %[1] at 56 days	1.48 ± 0.30^{a}	1.35 ± 0.28^{a}	0.87 ± 0.16^{b}	1.06 ± 0.29^{ab}	0.003
VSI %[2]	4.50 ± 0.39	4.18 ± 0.45	3.58 ± 0.46	3.57 ± 0.51	0.946
ISI %[3]	0.85 ± 0.10	0.83 ± 0.15	0.80 ± 0.10	0.71 ± 0.14	0.881

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

2.3.2 Distal intestine and liver morphology

Based in the control treatment fish fed with 0 % SBM did not show any sign of morphological change in the DI at 28 and 56 days (Table 6. Fig. 10. A). At 28 days the fish fed with SBM develop the characteristics associated with mucosal inflammation and the intestinal damage increased as the level of SBM increased in the diet. The 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. 10.B), increased the enlargement of LP (Fig. 10. E to H) and SM (Fig. 10. I to L) with some cellular infiltrations of leucocyte and eosinophilic granulocytes. Likewise, the GC shows hypertrophy and as the SBM level increased in the diet hyperplasia of these GC was observed towards the apexes of MF (Fig. 10. C and D).

Table 6. Distal intestine measurements to illustrate the degree of enteritis in *Totoaba macdonaldi* fed diets containing different SBM levels. Values are presented as means ± standard deviation (SD) of three replicates. P values resulting from a two-way ANOVA test.

Time	**28 days**				**56 days**				**Anova[7]**					
SBM	**0 %**	**22 %**	**44 %**	**64 %**	**0 %**	**22 %**	**44 %**	**64 %**	**Time (days)**	**Diet (%)**				**Int[6]**
									28 vs. 56	**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	>	*				*

[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

a, b, c, for parameters with a significant effect of diet and no interaction, values without a common letter are different (an indicated the highest value; P<0.05).

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<0.05).

7 NS, non-significant; *, P<0.05. For variables with a significant effect of time (P<0.05) and no interaction, < or > indicates whether the values measured at 28 days was less than or greater than that measured at 56 days.

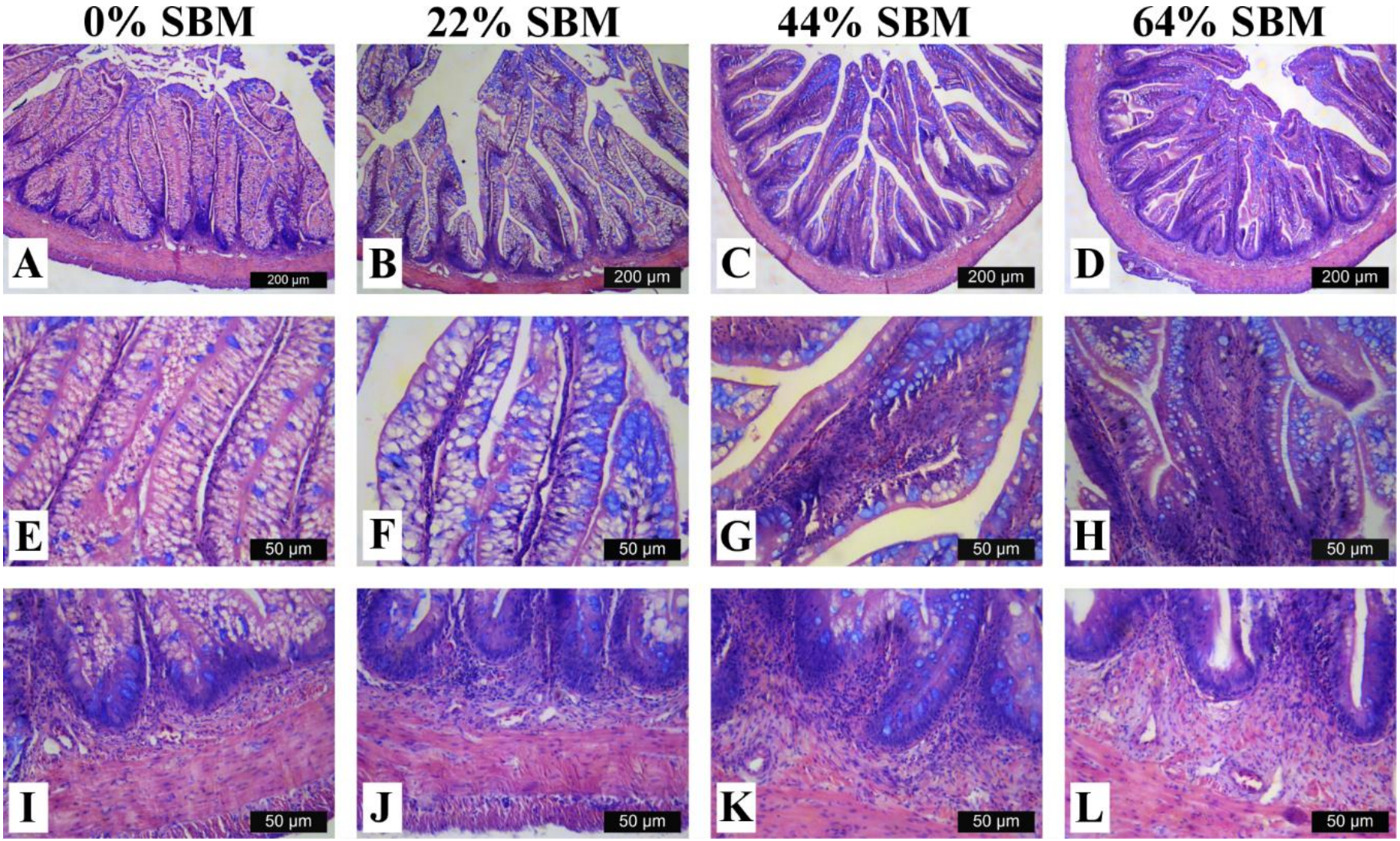

Figure 10. Light microscopic images depicting morphological changes in distal intestine associated to inflammatory process in *Totoaba macdonaldi* fed with 0 % SBM (A, E, I), 22 % SBM (B, F, J), 44 % SBM (C, G, K) and 64 % SBM (D, H, L) at 28 days. Mucosal folds length trend to decreases as SBM inclusion level increases (A to D, Bar = 200 µm); reduction of the SNV, markedly increase in the width of LP and hyperplasia of GC (blue dots) in relation with SBM inclusion level (E to H, Bar = 50 µm); SM directly enlarged as SBM inclusion levels increased (I to L, Bar = 50 µm).

At 56 days, the severity of the histological changes increased over time in fish fed with SBM (Fig. 11). The SBM diet reduces the number of MF from 55 and 51 to 46 MF in 22 % and 44 % SBM inclusion diets, respectively (Table 6). This reduction in the number of MF increased the intermucosal fold space. Fish fed with SBM present enlargement of the apical zone of the MF with lymphocyte infiltration and vascular congestion (Fig. 11F). In 44 % and 64 % SBM diets, the MF presents an atrophic process associated with a reduced number of cells like GC, decreased in the length and subsequent loss of absorption surface. The EH decreased especially in the 22 % SBM diet from 25 to 20.5 µm (Table 6) and an evident reduction of the SNV to almost extinction in 44 % and 64 % of SBM diets (Fig. 11G and H). Although the width of the LP decreased in comparison to the 28 days, the LP of fish fed with SBM are greater than fish fed SBM-free diet (Table 6). Contrarily to the 28 days, the SM decreased in fish fed with increasing levels of SBM at 56 days and showed a profuse infiltration of inflammatory cells like lymphocytes and eosinophilic granulocytes that migrate into the LP (Fig. 12). The distal intestine from totoabas fed with fish meal (upper intestine in the Fig. 13) can be seen the constriction that separated the midgut and the distal intestine, the diameter of the distal intestine increases in size and is darker, while the intestine of the fish fed with SBM (bottom intestine in the Fig. 13) is not distinguished clearly the constriction area, the diameter of the distal intestine is reduced and has a pale coloration.

The liver histology at 56 days in fish fed with 0 % SBM diet showed large vacuoles of lipid with a displacement of the nucleus of the hepatocytes from the central position and reduction of the sinusoid space (Fig. 14A to D), characteristic of a fatty liver, typical of this species. Fish fed with increasing levels of SBM diets present a reduction in cytoplasmic vacuolization with a displacement of the nucleus to the central position and increase of the sinusoid space (Fig. 14E to H). In the case of 44 % and 64 % of SBM, it was observed focal larges lipid vacuoles (Fig. 14G and H). The increase of SBM in the diet increased infiltration of lipid vacuoles in the pancreatic tissue, fish fed with 44 % and 64 % SBM diets shows large accumulation of vacuoles replaced part of the pancreatic tissue (Fig. 14I and L). Likewise, as dietary SBM content increased the pancreatic acini increased the eosinophilic coloration (Fig. 14I and L).

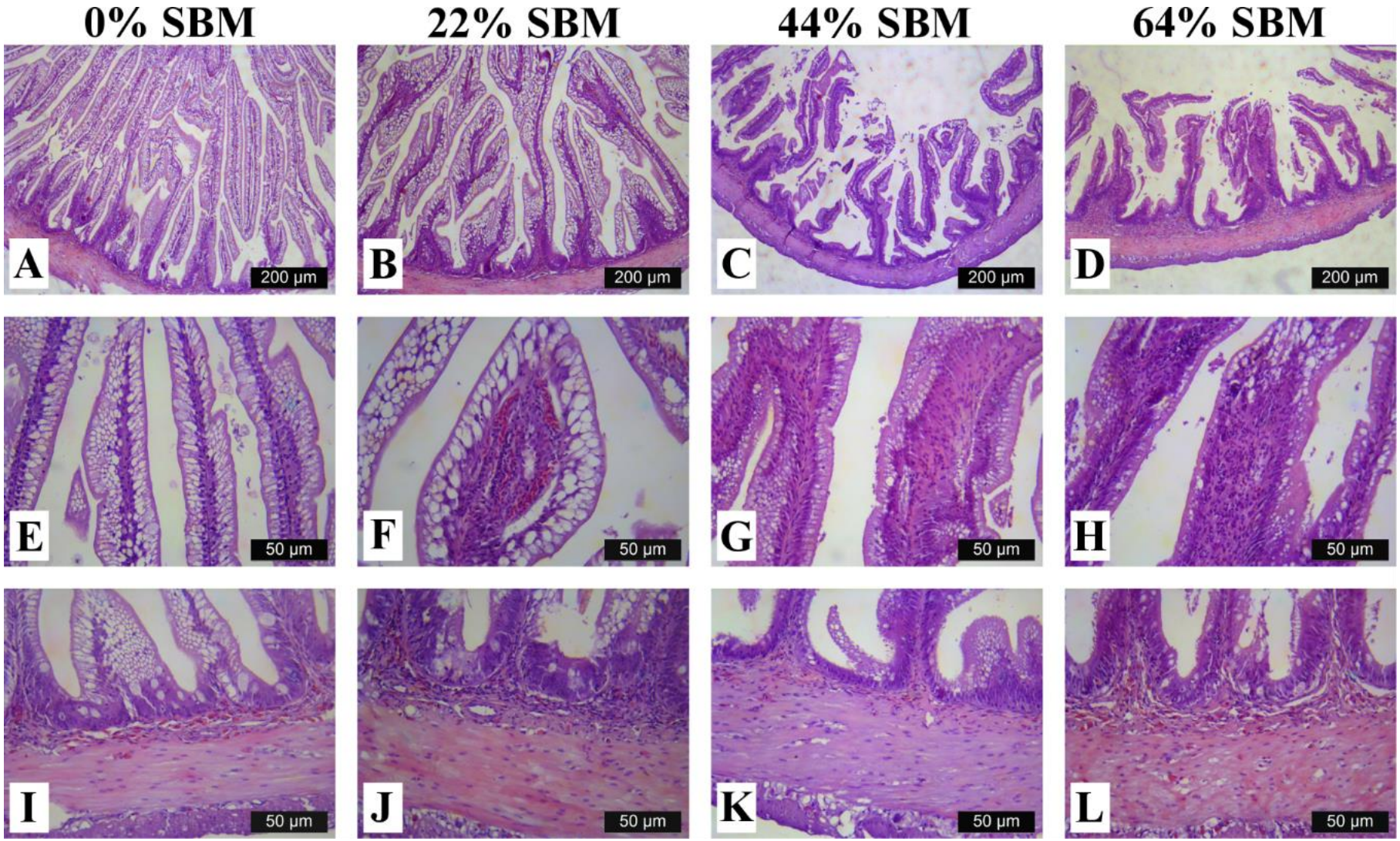

Figure 11. Light microscopic images depicting morphological changes in distal intestine associated to inflammatory process in *Totoaba macdonaldi* fed with 0 % SBM (A, E, I), 22 % SBM (B, F, J), 44 % SBM (C, G, K) and 64 % SBM (D, H, L) at 56 days. Mucosal folds number and length trend to decreases as 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); SM contrary to 26 days, were shrink as SBM inclusion levels increase (I – L, Bar = 50 µm).

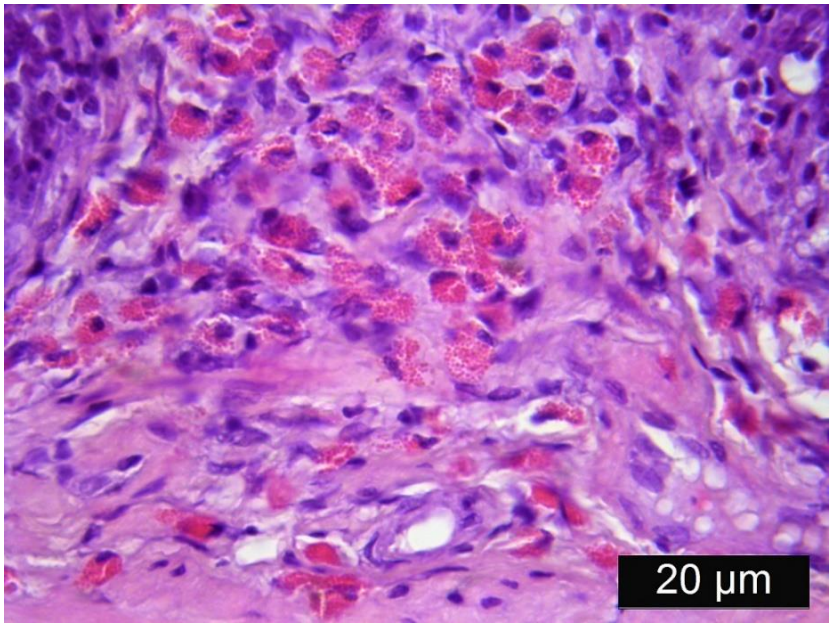

Figure 12. Profuse infiltration of inflammatory cells (eosinophilic granulocytes) at 56 days due to the inclusion of SBM in *Totoaba macdonaldi* diet.

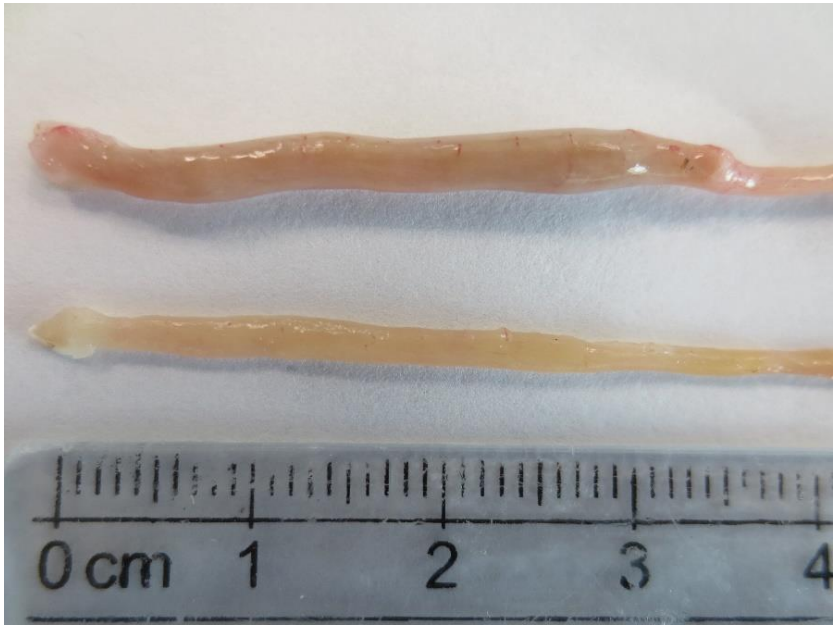

Figure 13. Distal intestine of *Totoaba macdonaldi* fed with the fishmeal based-diet (upper) and those fed with 64 % of soybean meal inclusion (bottom).

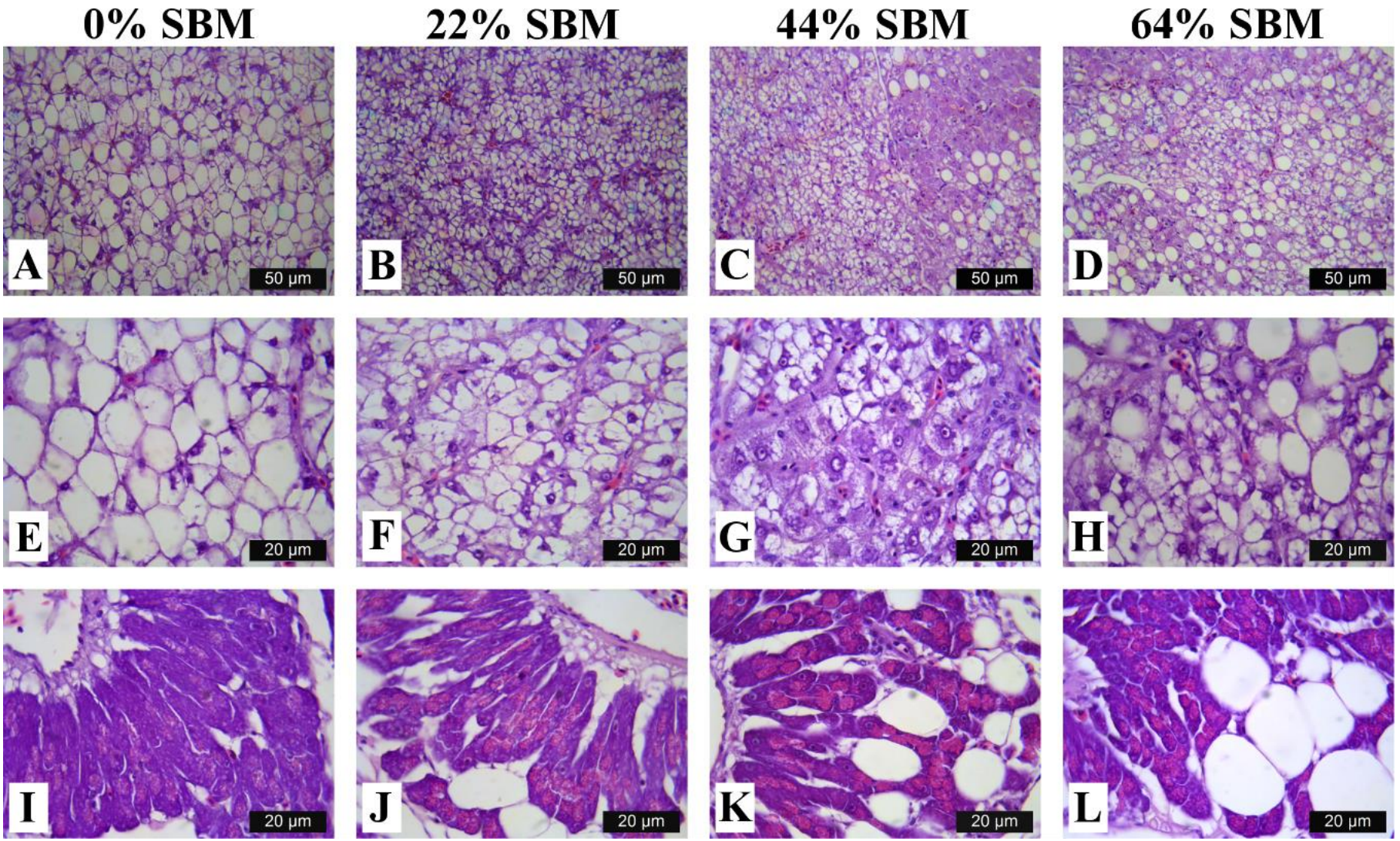

Figure 14. Light microscopic images depicting morphological changes in *Totoaba macdonaldi* liver fed with increasing levels of SBM in the diet at 56 days. A reduction in cytoplasmic vacuoles (Fig. 4A to D, Bar = 50 µm) with a displacement of the nucleus to the central position and increase of the sinusoid space (Fig. 4E 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. 4I to L, 20 µm) and in 44 % and 64 %, large accumulation of vacuoles replaced part of the pancreatic tissue (Fig. 4K and L). Likewise, the eosinophilic coloration increased in the pancreas acini in SBM diets (Fig. 4J, K, L).

2.3.3 Digestive enzymatic activity

The pepsin activity did not show any significant differences (P=0.751) among treatments (Fig. 15). In relation to alkaline digestive enzyme, the activity of pancreatic proteases (trypsin and chymotrypsin) and total alkaline proteases showed a gradual decrease as the level of SBM in the diet increased, with higher values for fish fed with 0 % SBM compared to the other treatments (Fig. 16). The same trend was observed for amylase activity (P<0.05). 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. 16).

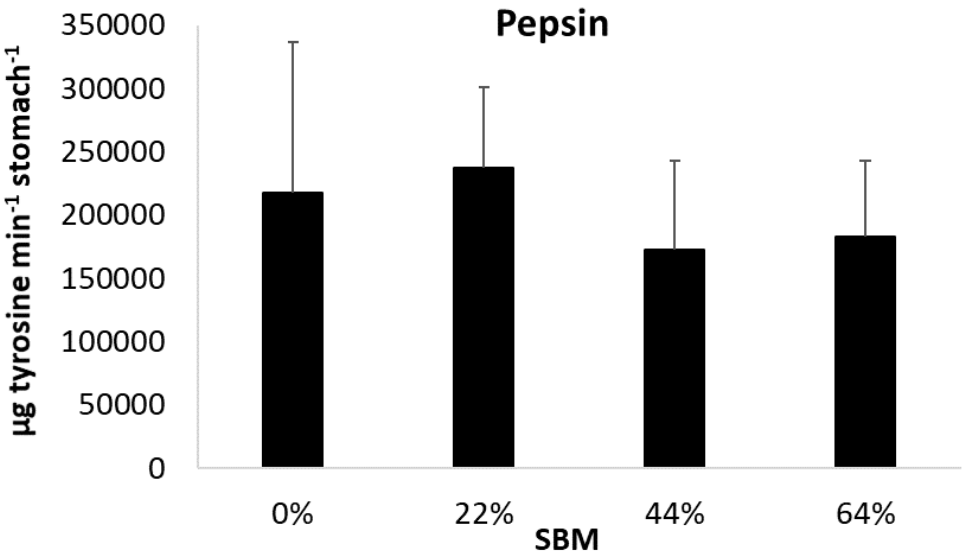

Figure 15. Acid protease (pepsin) activity with different SBM inclusion levels in the diet of *Totoaba macdonaldi* at 56 days fed diets containing different SBM levels.

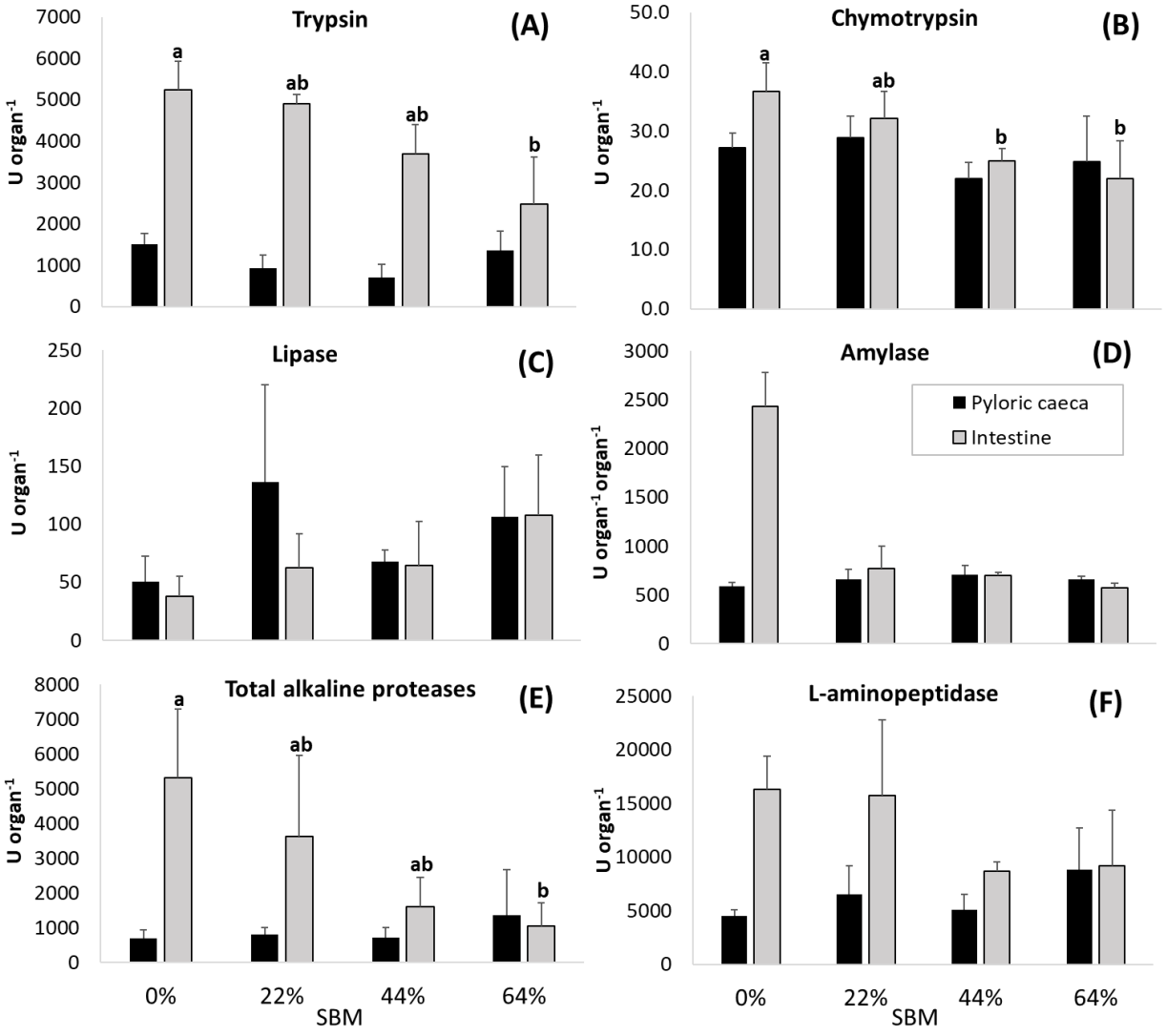

Figure 16. Total enzyme activity (U organ^{-1}) per pyloric caeca and intestine for trypsin (A), chymotrypsin (B), lipase (C), amylase (D), total alkaline proteases (E) and L-aminopeptidase (F) in *Totoaba macdonaldi* at 56 days fed diets containing different SBM levels. Different letters represent significantly different values ($P<0.05$) within the same organ (n=3).

2.4. 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 in juvenile totoabas fed with SBM. The fish performance resulted in a negative dose-dependent effect with dietary SBM inclusion. A gradual 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 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. 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 the fish diet (NRC, 2011). Some authors have suggested that reduce feed intake is due to the poor palatability of SBM caused by the bitter taste of saponins (Bureau et al., 1998; Chikwati et al., 2012). In the present study, FI 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 an 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 Mediterranean yellowtail 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 integrity 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 leads to 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 28 days, 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 28 days recovery from a case of enteritis was observed. In the present study, totoaba fed with SBM for 56 days

showed an increased in the severity of intestinal damages. Fish fed with SBM diets exhibited more branched folds in the intestine that had not been observed at 28 days (Fig 2. B, C and G). The most severe cases were found in fish fed with 44 % and 64 % SBM diets that showed a markedly reduced number and length of MF, increase fold fusion, revealing 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 56 days (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 126 days (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 higher sensitivity to dietary SBM.

The structural changes observed in the DI at 56 days 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) documented at 56 days. Moreover, during this sampling date, it was noticed that the intestine was flaccid and thinner in particular with the intermediate and high SBM inclusion levels. The integrity of the mucosal barrier is crucial in maintaining tissue homeostasis against pathogens and feed antigens, the mucus secreted from GC provides the first barrier of intestinal protection, and the integrity of mucosal layer 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 L.F. 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 mucosal folds integrity with sloughing of mucosa and cellular infiltration (i.e., lymphocites) into the lamina propria.

Histological analysis of totoaba liver confirms dose-dependent damage of this organ with SBM inclusion in the diets. Totoaba is considered a moderately lean fish and has their main energy reserves in the liver, which is reflected in a liver with 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 in the California yellowtail 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. It is not well known, if the presence of high levels of peripancreatic fat, could lead 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 (10:00 am), and the objective of the present study was to compare 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., 2001a). However, both products used in the present study should be free of trypsin inhibitors according to 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, despite the lack of statictic difference, lipase activity tends to increase with higher levels of 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 fibres, 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).

Based on the overall performance of the fish and through the histological analysis from the liver and distal intestine performed in the present study, we suggest the antinutritional factors in SBM are related to adverse effects found in this work even at a relative low inclusion level (i.e. 16 % SBM in combination with 6 % SPC), such as tissue disruption in the distal intestine with reduction of the digestive enzyme activity and nutrient absorption, late atrophy of the intestinal mucosa and growth impairment that consequently affects the fish welfare. 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 digestive capacity level) as well as the overall digestive enzyme activity and consequently impairing growth when included at the intermediate (44 % SBM) 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.

Chapter 3. Fishmeal replacement with poultry by-product meal and soybean meal in *Totoaba macdonaldi* diets and the effect of agavin as a prebiotic

3.1 Introduction

The use of fishmeal (FM) in diets for carnivorous fish species is one of the major concerns of the aquaculture industry. Due to the limited supply, increasing prices and the lack of sustainability makes the use of FM in fish diets an urgent mater to address, sparking the much needed research to find cost-effective alternative protein sources. Among the rendered animal protein ingredients, poultry by-products meal (PBM) is a good substitute to FM. PBM has high protein content (60-65 %) with adequate levels and balance of amino acids, is highly digestible, has a lower price than FM and can be a successful replacement due the largest quantities produce around the world (Bureau et al., 2006; Metts et al., 2011; NRC, 2011). In totoaba, Badillo et al., (2014) found that PBM can be included up to 45 % of the diet replacing up to 67 % of the FM protein in a diet with 52 % of crude protein resulting in suitable growth rates and high survival. Nonetheless, the authors mention that complete replacement of the FM resulted in lower growth and high mortality possibly associated with inadequate quantities and balance of essential fatty acids to meet the high requirements for these nutrient in marine fish.

The formulation of practical diets in several species with PBM to replace FM, have also included a fixed level of SBM in the diet without negative effects on growth performance. For example, a 26 % SBM in the diet for sunshine bass *Morone chrysops* ♀ × *M. saxatilis* ♂ (Rawles et al., 2011), 15 % in Malabar grouper *Epinephelus malabaricus* (Li et al., 2009), 11 % in cobia *Rachycentron canadum* (Zhou et al., 2011), 50 % in Florida pompano *Trachinotus carolinus* L. (Rossi and Davis, 2012), up to 45.5 % in golden pompano *Trachinotus ovatus* (Ma et al. 2014) and 24 % in Largemouth Bass *Micropterus salmoides* (Ren et al., 2018). However, none of these studies evaluated the health condition of the intestine of fish fed with these practical diets. The use of SBM has been recommended with caution, since several studies have reported of damage to the intestinal integrity of marine fish (Krogdahl et al., 2003; Heikkinen et al., 2006; Hedrera et al., 2013; Gu et al., 2016). In the totoaba, Fuentes-Quesada et al. (2018) described the changes to the distal intestine in fish fed diets with increasing levels of SBM and found, even in the lower level tested (22 %: 16 % SBM + 6 % SPC), alterations in the distal intestinal morphology typically associated with enteritis, follow by a reduced growth performance and feed utilization.

Research to improve growth performance and gut health in fish, in particular when high levels of plant ingredients are included in the diet, has promoted the use of several nutrients, supplements or compounds with a biological function that protects the intestine and reduces the presence of enteropathies. The use of prebiotics in diets with SBM inclusion levels ranging from 13.5 % to 40 % improve the growth performance, feed utilization and reduce SBM-induced enteritis (Burr et al., 2008; Resftie et al., 2010, Dimitriglou et al., 2010b; Bai et al., 2017). One promising prebiotic is the agavin, a highly branched fructan obtained from the agave plant grown in Mexico to produce tequila (López et al., 2003; Escamilla-Treviño, 2012). Agavin is a type of oligofructose (a heterogeneous blend of fructose polymers) that has been investigated for its prebiotic effects conferring benefits the hosts general health by providing specific changes in the composition, diversity and activity of the gut microbiota in mammals (Mancilla-Margalli and López, 2006; Gibson et al., 2010; Huazano-García et al., 2017; Huazano-García and López, 2013, 2015, 2018).

Research with new prebiotics in emerging species is necessary to determine if the prebiotic of interest has beneficial effects on the species being evaluated. In particular, agavin is a novel prebiotic that has not been studied in marine fish and can potentially reduce the negative effects of the incorporation of SBM in totoaba diets. Recent research evaluated the inclusion of two commercial products in totoaba diets, 2 % of a prebiotic based on yeast, GroBiotic®-A, and a probiotic based on *Bacillus* sp., Aquablend®, in diets with 21.8 % SBM inclusion fed for 109 days. Researchers did not find significant differences in survival and growth parameters at the end of the experiment compared to a control group fed a diet based on fishmeal and 29 % of SBM (González-Felix et al., 2018).

The use of prebiotics in the diets for marine fish when plant ingredients are included has been shown to maintain the overall growth and adequate digestive physiology, and can reduce the adverse effects when SBM is included. Nevertheless, there is a lack of information with respect to the use of prebiotics and the possible benefits in totoaba culture. Likewise, the high replacement achieved and lower price of PBM in fish diets, represents an advantage to reduce the cost of commercial diets for marine fish. However, its effect on the intestinal integrity and health of totoaba juveniles is unknown. Therefore, the objective of this work was to evaluate the use of a prebiotic, agavin, in low FM diets containing PBM and SBM as the main protein sources using growth performance, feed utilization, digestive capacity, gut and liver integrity in *Totoaba macdonaldi* juveniles as response variables.

3.2. Materials and methods

3.2.1 Diet formulation

Four experimental diets were formulated to be isoproteic (511 g kg^{-1} crude protein, CP) and isolipidic (119 g kg^{-1} crude lipid, CL) of diet dry matter based on recommended nutritional requirements for totoaba (Perez-Velazquez et al., 2016; Rueda-López et al., 2011), with a DHA/EPA ratio of 1 and DHA+EPA content >1.5 % of diet (NRC, 2011). For the first diet, fishmeal (FM) was the sole protein source (69 % CP, 6 % CL, Maz Industrial SA de CV, Mazatlán Sinaloa, México), this diet termed reference diet (FM). In the second diet (PMB), 67 % of the FM was replaced by poultry by-product meal (PBM, 68 % CP, 14 % CL, pet food grade, National Renderers Association, USA) maintaining a ratio of 2:1 (PBM:FM) according to Badillo et al., (2014) and termed as diet PBM. In the third diet (SBM), soybean meal (SBM, 48 % CP, 6 % CL, Alimentos COLPAC, Sonora, México) was also included at 240 g kg^{-1} diet DM, and the PBM and FM were reduced in the same proportion to keep the ratio 2:1. The fourth diet had the same formulation of the third diet (PMB+SBM) but included a prebiotic, 20 g kg^{-1} agavin (donated by Instituto de Biotecnología, UNAM, Cuernava, México) of the diet DM, termed as 2% AGA. All diets included 1 g kg^{-1} of krill oil (Biogrow, ProAqua, México) as an attractant and 10 g kg^{-1} of taurine (Insumos Nubiot, SA de CV, México) to satisfy the minimum recommend for marine fish diets when SBM is include in the diets (Table 7). Four essential amino acids (lysine, methionine, threonine, and arginine; EVONIK, Degussa, México) were supplemented based on recommended levels from totoaba's whole body amino acids profile (data from our laboratory). Diets were manufactured in a mixer (Robot-Coupe, model R10, USA), pelleted through a 5 mm die in a meat grinder (Tor-Rey, Model M32-5, Mexico) and dried at 60 °C in a forced air-dried oven for 24 h.

Table 7. Formulation and proximate composition of the experimental diets. Dietary formulation is presented as g kg^{-1} on as fed basis and proximate composition in g kg^{-1} on a dry matter basis.

	Experimental diets			
Ingredients (g kg^{-1} DM)	**FM**	**PBM**	**SBM**	**2% AGA**
Sardine meal (69 % CP)[a]	701.0	230.0	180.0	180.0
Poultry by-product meal (68 % CP)[b]	0.0	458.0	340.0	340.0
Soybean meal (48 % CP)[c]	0.0	0.0	240.0	240.0
Starch	78.5	114.2	37.9	17.9
Pregelatinized starch	80.0	80.0	80.0	80.0
Sardine oil[a]	77.0	38.0	43.0	43.0
Rovimix for carnivorous fish[d]	30.0	30.0	30.0	30.0
Stay-C[d]	10.0	10.0	10.0	10.0
Taurine[e]	10.0	10.0	10.0	10.0
Methionine[f]	2.2	4.7	5.5	5.5
Lysine[f]	0.0	16.0	15.6	15.6
Arginine[f]	4.7	3.0	1.8	1.8
Threonine[f]	1.5	1.0	1.1	1.1
Attractant (krill oil)[g]	1.0	1.0	1.0	1.0
Sodium benzoate	2.5	2.5	2.5	2.5
Choline chloride	1.5	1.5	1.5	1.5
BHT	0.1	0.1	0.1	0.1
Agavin[h]	0.0	0.0	0.0	20.0
Proximate composition (g kg^{-1} DM)				
Dry matter	965 ± 0.6	961 ± 0.4	969 ± 0.7	967 ± 0.4
Crude protein	513 ± 2.7	511 ± 8.9	513 ± 11.3	508 ± 0.7
Crude fat	110 ± 1.4	119 ± 0.8	122 ± 7.2	123 ± 0.8
Ash	157 ± 3.7	131 ± 1.3	124 ± 0.3	128 ± 1.0
NFE[i]	220 ± 7.0	239 ± 8.5	239 ± 8.0	241 ± 5.9

[a] Maz Industrial SA de CV, Mazatlán, Sinaloa, México.
[b] Pet food grade, National Renderers Association, USA.
[c] Alimentos COLPAC, Sonora, México.
[d] Rovimix; Stay-C, DSM, Guadalajara, México.
[e] Insumos NUBIOT SA de CV, México.
[f] Free aminoacids, EVONIK, Degussa, México.
[g] Biogrow, Proveedora de Insumos Acuícolas, SA de CV, Mazatlán, Sinaloa, México.
[h] Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México
[i] Nitrogen-free extract (NFE, %) = 100 - (% crude protein + % total lipid + % ash).

3.2.2 Experimental design, animals and facilities

Totoaba juveniles, were obtained from the Centro de Reproducción de Especies Marinas del Estado de Sonora (CREMES) in Kino bay, Mexico and transported by land to the experimental facilities in the Instituto de Investigaciones Oceanográficas of Universidad Autónoma de Baja California in Ensenada, B.C., México. The four experimental treatments were evaluated in triplicate. Five fish (60.8 ± 22.6 g) were randomly stocked into twelve 350-L cylindrical fiber-glass tanks in a closed recirculation system with a biofilter

(PolyGeyser®; Pneumatic Drop Bead Filter model PG7 International Filter Solutions, TX, USA) connected to a 750-L reservoir tank with a titanium heater of 6000 W, H60T (Pentair Aquatic Eco-Systems, FL, USA) and a daily water renewal of 5 %.

Water quality was monitored daily, with mean values for temperature of 23.6 ± 0.3°C, dissolved oxygen above 5.7 ± 0.2 mg L^{-1} with oxygen saturation up to 80 %, salinity equal to 35.0 ± 0.5 ‰ and a water flow of 2.2 L min^{-1}. Every three days the total ammonia nitrogen, nitrite-nitrogen and nitrate-nitrogen levels were measured (Api Pharmaceutic Aquarium Kit) to keep values < 0.75 mg L^{-1}, < 0.5 mg L^{-1} and < 40 mg L^{-1}, respectively and considered optimum for totoaba culture. Fish were kept under natural photoperiod between September and November of 2017 (31°86 N, 116°67 W). Each dietary treatment was randomly assigned into triplicate experimental units. Fish were hand-fed daily to apparent satiation three times a day at 08:00, 12:00 and 16:00 h during 44 days. Daily, all uneaten feed was removed within an hour of feeding and dry weighed and deducted from the quantity offered by hand.

3.2.3 Sampling

Growth response indexes and somatic indexes were calculated as previously described in section 2.2.3. Fish were harvest at the end of the experiment (i.e., day 44). In addition to the described indexes the following growth indexes were recorded:

Weight gain, g = final weight – initial weight (9)

Relative weight gain % = [(final weight – initial weight) / initial weight] x 100 (10)

Daily weight gain (DWG) = final weight gain / experimental days (11)

For histological analysis, samples were processed using the protocol described in section 2.2.3., but with a minor modification for the intestine samples, were the complete distal intestine of each fish (three per treatment) was dissected at the end of the experiment.

3.2.4 Analytical methods

The proximate analyses of the experimental diets and liver were performed previously described in section 2.2.4.

3.2.5 Intestine and liver histology

The intestine and liver samples dissected for histology analysis were processed according to the methodology described in the section 2.2.5 with the next minor modification: after dehydrated and clarified in benzene, the complete intestine was cut in six equal transversal sections to characterized and analyze the entire distal intestine (see Fig. 17).

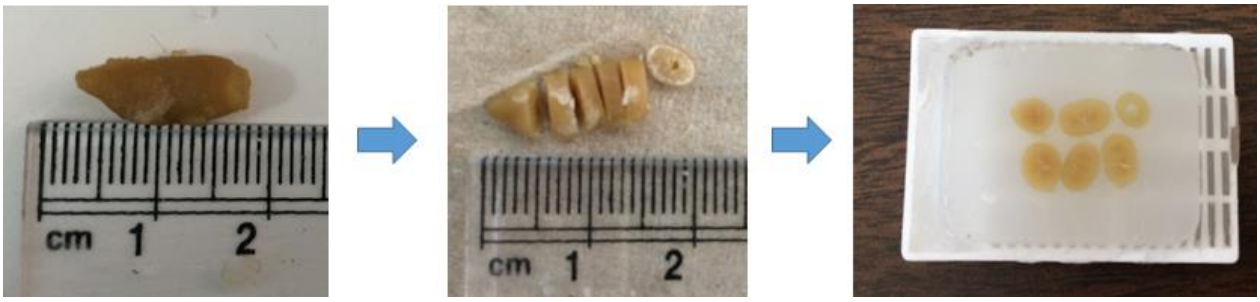

Figure 17. Illustration of the distal intestine cuts and the inclusion in paraffin of the sections for the histology analysis of *Totoaba macdonaldi*.

To characterize the morphological changes occurring in the distal intestine, the following measurements were made per cut section (i.e., 6 sections per fish); at least 30 measurements were made for each histometric variable used. The following histometric measurements were made; 1) the mucosal fold length (MF) (Fig. 18), 2) the enlargement of sub-epithelial mucosa (SM), 3) the wide of the lamina propia (LP), 4) the enterocyte height (EH) and 5) the brush border height (BBH) using 6 measurements per section. The MF length was made on the longest and complete MF per each section (Fig. 18). For the MF number, all the MFs were counted to quantify the number of MF per section according to methodology described in section 2.2.5.

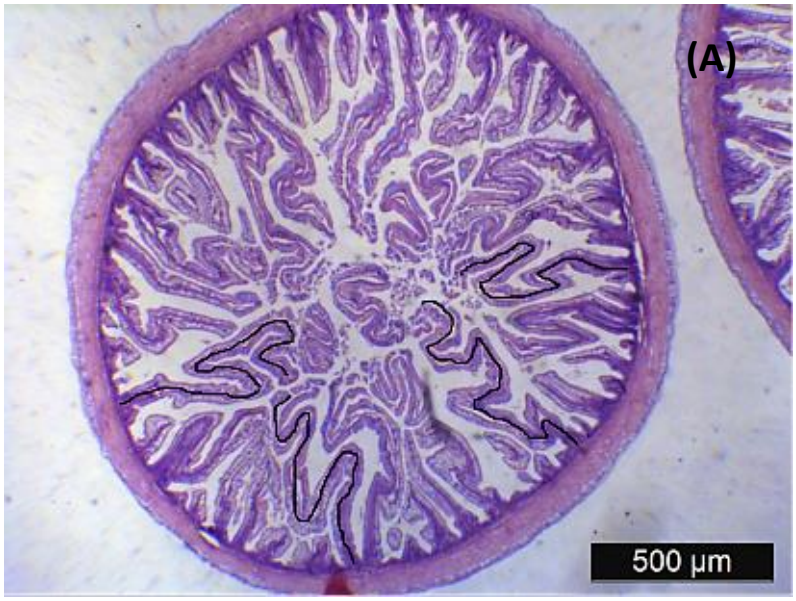

Figure 18. Illustration of the measurements (black lines) made of the mucosal folds in the distal intestine of *Totoaba macdonaldi.*

The percentage of MF with wider SM (% of MF with wider SM) was calculated counting the number of MF with enlargement of the SM (Fig. 19A) and divided by the total number of MF. The brush border height (BBH) was estimated using the height measurements described in figure 19B.

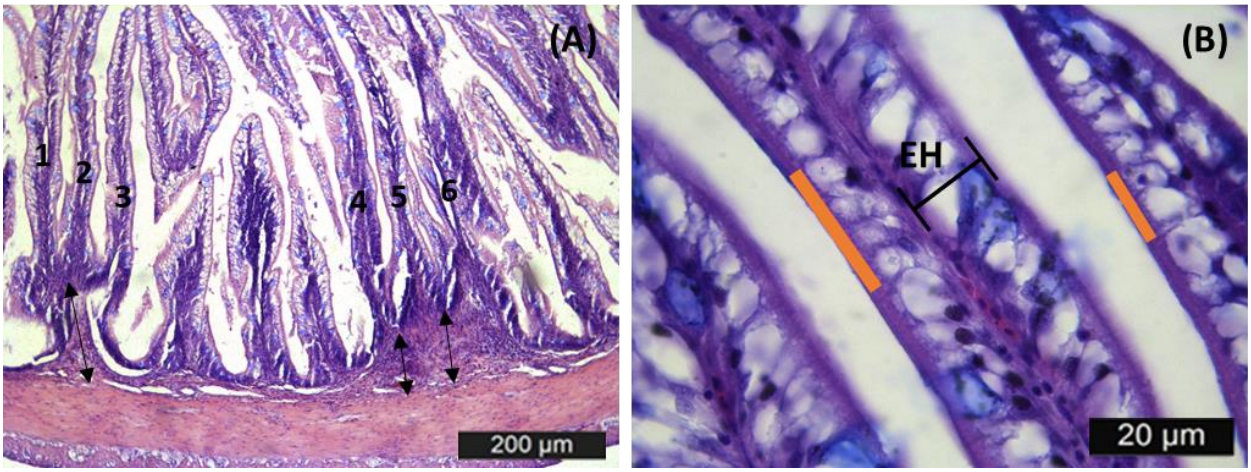

Figure 19. Illustration of the mucosal folds with wider sub-epithelial mucosa (A) and the measurement of brush border height (yellow-orange bar) in the distal intestine of *Totoaba macdonaldi.*

Based on the appearance and structure of the tissue from the reference diet, a semi-quantitative scoring system was used to evaluate the histomorphological changes in the distal intestine according to Urán et al. (2008a) and Penn et al. (2011) (see Table 11). The selected variables such as the appearance and length of the mucosal folds (MF), abundance of the goblet cells (GC), presence of the supranuclear vacuoles (SNV), the degree of enlargement of the lamina propria (LP), the degree of widening of the sub-epithelial mucosa (SM) and the infiltration of eosinophilic granulocytes (EG) into the SM and LP were scored from 1 to 5; where 1 is similar to reference diet samples and 5 is highest damage observed.

3.2.6 Digestive enzyme activity

Digestive enzyme activity assays were performed following the protocols described in the section 2.2.6.

3.2.7 Fishmeal protein dependency ratio (FPDR)

Since one of the goals of our research is to reduce the use of fishmeal in totaoba's diets, we utilized a previously published index relating the used of fishmeal protein relative to our alternative protein sources in the production of fish body-protein. The quantity of fishmeal used in each the dietary treatment was approach through a relative protein ratio. Fishmeal protein consumed in the feed and produced as body mass by farmed fish is termed the fishmeal protein dependency ratio (FPDR). To estimate this ratio, we followed the equation described by Sarker et al., (2013) as follows;

$$\mathrm{FPDR} = \frac{\text{kg fishmeal protein used}}{\text{kg fish protein produced}} \tag{12}$$

$$\mathrm{FPDR} = \frac{\text{FMfeed} \times \text{Protein FM} \times \text{FCR}}{\text{Protein in fish}} \tag{13}$$

FMfeed is the inclusion level of fishmeal in each experimental diet;

Protein FM is the average concentration of protein (69 %, dry matter basis) in the fishmeal used in each experimental diet (weighted by their inclusion level and expressed as a proportion);

FCR is the feed conversion ratio (kg of feed consumed per kg fish mass produced);

Protein in fish, is the concentration of protein in totoaba on a whole fish basis (i.e., 17.6 % crude protein, data from our laboratory for totoaba whole body).

3.2.8 Economic conversion ratio (ECR)

In addition, feed price highly depends on the quantity of protein ingredients in the diet formulation and the price of these ingredients in the diet. To estimate ECR the cost of the protein ingredients in each experimental diet required to produce 1 kg of biomass were considered. The economic conversion ratio (ECR) was calculated with the following equation from Hernández et al., (2007):

$$ECR = Protein\ ingredients\ cost\ (US\$) \times Feed\ conversion\ rate\ (FCR) \quad (14)$$

3.2.9 Statistical analyses

The assumptions of normality and homogeneity of variances were evaluated using the Shapiro-Wilks and Barlett test respectively (Zar, 2010). Prior to analysis, percentage data were arcsine transformed. Significant differences in performance indexes, somatic indexes, histological measures of the distal intestine, enzyme activities levels, economic conversion ratio and fishmeal dependency ratio were analyzed by one-way ANOVA, followed by post-hoc Fisher's least significant difference rank test. The values of individual parameters and mean score of the semi-quantitative scoring system were analyzed by no parametric Kruskal-Wallis test and the existing variation between all samples was determined and presented as the pooled standard error (PSE). For all data, statistical significance was set at $P<0.05$. Statistical analysis was performed using the software STATISTICA 8.0™ (StatSoft, Inc. USA).

3.3 Results

3.3.1 Growth performance, feed utilization, and somatic indexes

The effects of the experimental diets on totoaba growth performance are shown in Table 8. At the end of the experiment fish fed with SBM + 2 % of agavin presented a significantly higher final weight, TGC, WG, RWG, DWG, and PER than those fed the SBM diet without the prebiotic, and significantly higher in terms of final weight and DWG compare to fish fed the PBM diet. Fish fed the diet with agavin addition resulted in lower FCR than fish fed without the prebiotic but only significantly better compared to SBM alone. The alternative ingredients to fishmeal did not significanlty affec FI of the experimental diets and no significant differences were found in the CF among the treatments. No mortality was recorded during the 44-days feeding trial.

Table 8. Growth performance and feed utilization of *Totoaba macdonaldi* fed with the experimental diets during 44 days. Different letters represent significantly different values (P<0.05) within the same row.

	FM	PBM	SBM	2% AGA	P value
Initial weight (g)	60.7 ± 1.5	60.7 ± 2.5	61.0 ± 2.0	60.7 ± 1.5	0.995
Final weight (g)	200.2 ± 3.0^{ab}	196.7 ± 1.1^{b}	195.0 ± 3.4^{b}	207.1 ± 3.8^{a}	0.005
TGC[1]	1.85 ± 0.01^{ab}	1.82 ± 0.05^{ab}	1.79 ± 0.05^{b}	1.91 ± 0.02^{a}	0.023
WG[2] (g)	139.6 ± 1.6^{ab}	136.0 ± 2.9^{ab}	134.0 ± 3.7^{b}	146.4 ± 2.6^{a}	0.003
RWG[3] (%)	230.1 ± 3.9^{ab}	224.6 ± 13.9^{ab}	219.9 ± 11.1^{b}	241.4 ± 4.4^{a}	0.045
DWG[4] (g)	3.17 ± 0.04^{ab}	3.09 ± 0.01^{b}	3.05 ± 0.08^{b}	3.33 ± 0.06^{a}	0.003
FCR[5]	0.85 ± 0.01^{ab}	0.86 ± 0.04^{ab}	0.90 ± 0.02^{a}	0.83 ± 0.02^{b}	0.039
PER[6]	2.29 ± 0.02^{ab}	2.27 ± 0.11^{ab}	2.17 ± 0.05^{b}	2.37 ± 0.04^{a}	0.028
CF[7]	1.61 ± 0.05	1.50 ± 0.05	1.51 ± 0.03	1.63 ± 0.09	0.060
FI[8] (% day^{-1})	1.67 ± 0.01	1.65 ± 0.04	1.64 ± 0.03	1.71 ± 0.01	0.051

[1] TGC (Thermal Growth Coefficient) = [(final weight^{⅓} - initial weight^{⅓}) / (T°C x Days)] x 1000 (Jobling, 2003).
[2] Weight gain, g = final weight – initial weight
[3] RWG (Relative weight gain, %) = [(final weight – initial weight) / initial weight] x 100
[4] DWG (Daily weight gain) = final weight gain / experimental days
[5] FCR (Feed Conversion Ratio) = total feed consumed / wet weight gained.
[6] PER (Protein Efficiency Ratio) = weight gain / protein intake.
[7] CF (Condition Factor) = final body weight x (body length)3 x 100 (Hardy and Barrows, 2002).
[8] FI, Feed Intake = FI (%/day) = 100 x (total amount of the feed consumed per fish / ((initial body weight + final body weight) / 2) / days).

At the end of the feeding trial fish fed the experimental diets resulted in significantly different ISI, where fish fed 2% AGA diet present a higher ISI compared to fish fed the PBM diet (Table 9). Although no significant differences in HSI and VSI, fish fed the SBM diet resulted in the lowest HSI values, while VSI values were lowest in fish fed the PBM and SBM diets.

Table 9. Somatic indexes of *Totoaba macdonaldi* fed with the experimental diets during 44 days (n=6). Different letters represent significantly different values (P<0.05) within the same row.

	FM	PBM	SBM	2% AGA	P value
HSI %[1]	1.65 ± 0.45	1.54 ± 0.49	1.39 ± 0.30	1.63 ± 0.25	0.629
VSI %[2]	4.27 ± 1.01	3.68 ± 0.45	3.86 ± 0.21	4.36 ± 0.45	0.492
ISI %[3]	0.67 ± 0.11^{ab}	0.52 ± 0.05^{b}	0.57 ± 0.10^{ab}	0.72 ± 0.04^{a}	0.019

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

3.3.2 Distal intestine and liver morphology

Fish fed the SBM diet resulted in decreased SNV size in the complete MF (Fig. 20C and G). Although not significantly different from other treatments, fish fed with SBM diet resulted in a lower EH, an increase in the % of MF with wider SM (Table 10) and hyperplasia and hypertrophy of the GC (Fig. 20G). In addition, a higher migration of EG to the SM and a reduction in the BBH was observed (Table 10). These changes are tipcially associated with inflammation of the intestinal mucosa, but the supplementation of 2 % of agavin in the diet reduced the negative effects of SBM inclusion when assesing the DI of the fish. For example, by increasing the SNV (Fig. 20D and H) maintain the EH, reduceding the % of MF with wider SM (Table 10), the GC did not present changes, and the BBH is higher compared to the diet of SBM. Moreover, the values of theses parameters of fish fed the 2% AGA diet are intermediate and close to those observed for fish fed the FM and PBM diets (Fig. 20, Table 10 and 11). Interestingly, the fish fed with SBM diet resulted in increased number of MF (Table 10). The supplementation with agavin to the diet did not reduce migration of EG to the SM (Table 11).

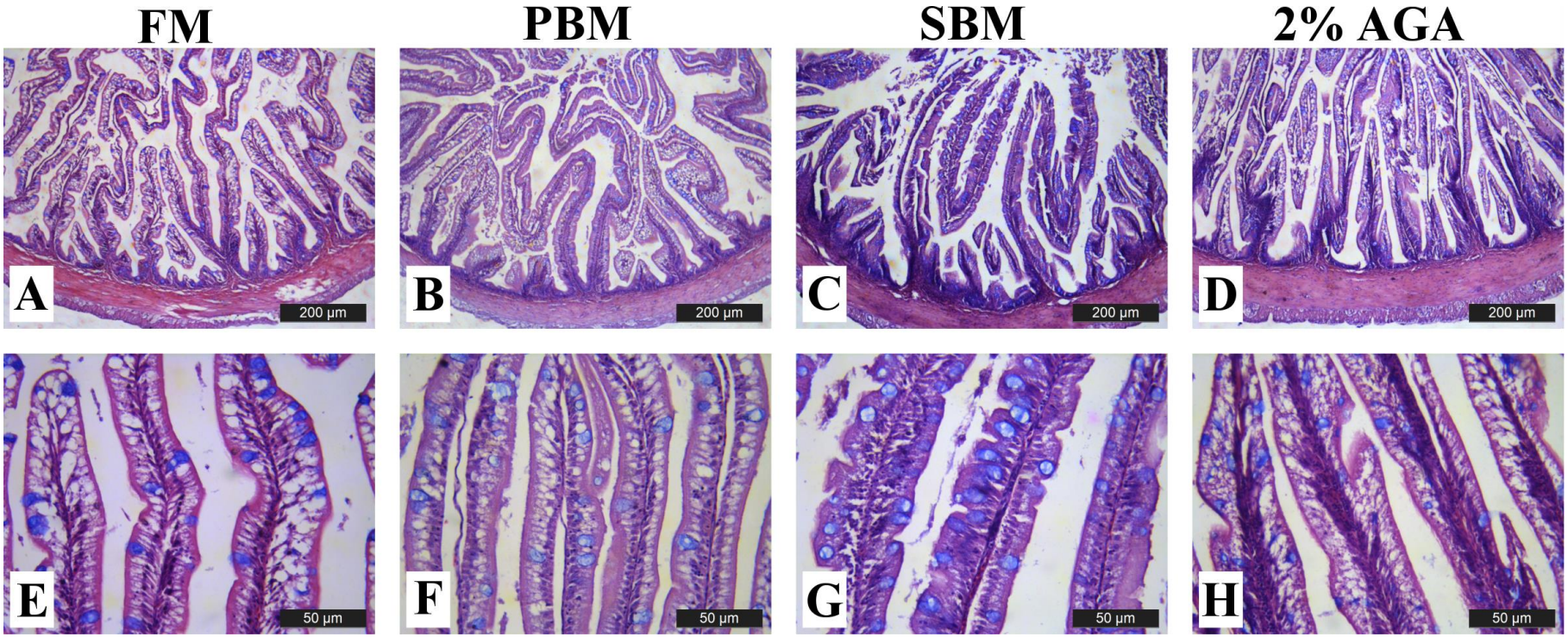

Figure 20. Light microscopy images of the morphological changes in distal intestine in *Totoaba macdonaldi* fed with 100 % FM (A and E), relation 2: 1 PBM: FM (B and F), relation 2: 1 PBM: FM and 24 % of SBM (C and G) and relation 2: 1 PBM: FM with 24 % of SBM and supplemented with 2 % of agavin (D and H) at 44 days. Figures from A to D bar = 200 µm, and from E to H bar = 50 µm.

Table 10. Morphometric measures of the distal intestine of *Totoaba macdonaldi* fed with the experimental diets during 44 days. Different letters represent significantly different values (P<0.05) within the same row.

	FM	PBM	SBM	2% AGA	P value
MF number[1]	43.8 ± 3.2	44.3 ± 4.0	47.1 ± 4.7	48.5 ± 0.7	0.361
MF length[2] (µm)	890.7 ± 135.6	762.2 ± 143.9	933.3 ± 170.8	763.6 ± 93.0	0.369
EH[3] (µm)	20.3 ± 2.5	20.0 ± 1.3	17.5 ± 1.8	20.3 ± 1.5	0.248
LP[4] (µm)	5.2 ± 0.4	4.5 ± 0.8	5.1 ± 0.8	4.4 ± 0.8	0.504
SM[5] (µm)	17.1 ± 1.8	14.8 ± 1.8	14.4 ± 1.9	17.6 ± 1.9	0.161
% MF with wider SM[6]	25.1 ± 2.1^{b}	26.0 ± 4.9^{b}	38.0 ± 11.1^{a}	28.0 ± 1.8^{ab}	0.034
BBH[7] (µm)	2.89 ± 0.11^{a}	2.65 ± 0.24^{a}	2.18 ± 0.05^{b}	2.64 ± 0.19^{a}	<0.001

[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]% MF with wider SM = percentage of mucosal folds with wider sub-epithelial mucosa; [7]BBH = brush border height.

Table 11. Individual and mean score the parameters evaluated to assess the degree of histological changes in the distal intestine of *Totoaba macdonaldi* fed with the experimental diets during 44 days. Different letters represent significantly different values (P<0.05) within the same row.

	FM	PBM	SBM	2% AGA	PSE	P value
Mucosal folds	1.3	1.3	2.3	1.3	0.19	0.198
Supranuclear vacuoles	2.3	2.7	4.0	3.0	0.23	0.140
Goblet cells	2.0	2.3	3.3	2.3	0.19	0.106
Lamina propia	1.3	2.0	3.0	2.3	0.21	0.159
Sub-epithelial mucosa	2.00	2.7	3.3	2.3	0.19	0.086
Eosinophilic granulocytes	1.3^{b}	1.3^{b}	3.3^{a}	3.0^{a}	0.30	0.024
Mean score	1.8^{c}	2.1^{bc}	3.2^{a}	2.4^{b}	0.17	<0.001

Fish fed the FM and PBM diets showed larges vacuoles in the hepatocytes (Fig. 21A and B) with a displacement of the nucleus from the central position and reduction of the sinusoid space (Fig. 21E and F). In contrast fish fed the SBM and 2% AGA diets presented a reduction of the cytoplasmic vacuoles (Fig. 21C and D) with a displacement of the nucleus to the central position and higher sinusoid space (Fig. 21G and H). Fish fed the SBM diet without agavin (2% AGA) presented few peripancreatic fat infiltrations (Fig. 21C).

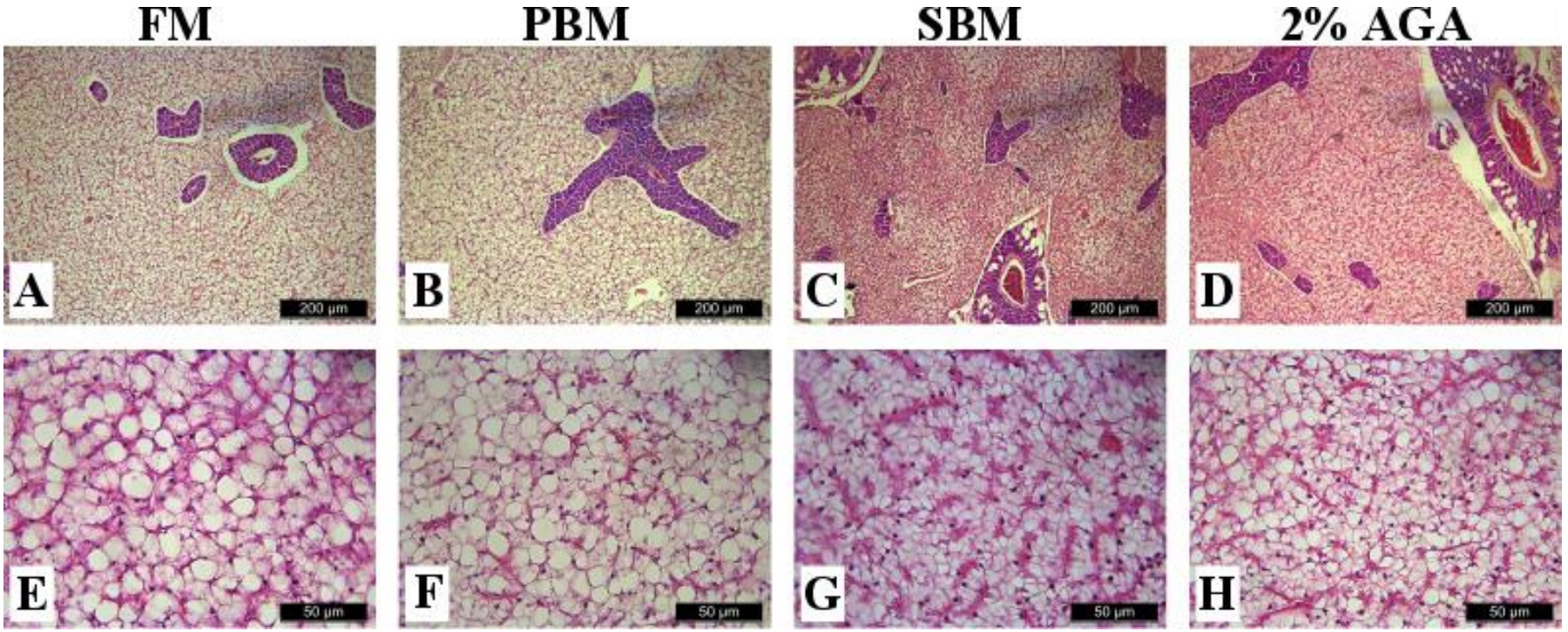

Figure 21. Light microscopy images depicting morphological changes in *Totoaba macdonaldi* liver fed with 100 % FM (A and E), relation 2: 1 PBM: FM (B and F), relation 2:1 PBM:FM and 24 % of SBM (C and G) and relation 2: 1 PBM: FM, 24 % of SBM and supplemented with 2 % of agavin (D and H) at 44 days. Figures from A to D bar = 200 µm, and from E to H bar = 50 µm.

3.3.4 Liver proximate composition

No significant differences in the proximate composition of the liver among treatment were found (Table 12).

Table 12. Liver proximate composition (% of wet weight) of *Totoaba macdonaldi* fed with the experimental diets during 44 days (n=6). Different letters represent significantly different values (P<0.05) within the same row.

	FM	PBM	SBM	2% AGA	P value
Moisture	49.1 ± 1.3	53.9 ± 6.2	49.2 ± 9.5	53.4 ± 4.2	0.664
Crude Protein	11.2 ± 1.6	11.0 ± 2.4	10.3 ± 2.5	12.0 ± 0.6	0.771
Lipids	24.2 ± 2.8	25.7 ± 4.4	22.7 ± 4.1	21.6 ± 3.9	0.307
Ash	0.5 ± 0.2	0.5 ± 0.3	0.5 ± 0.3	0.7 ± 0.2	0.616
NFE[1]	14.9 ± 3.0	9.0 ± 5.6	17.4 ± 9.5	12.2 ± 5.1	0.448

[1] Nitrogen Free Extract (NFE, %) = 100 - (% crude protein + % total lipid + % ash).

3.3.3 Digestive enzyme activity

No significant differences (P = 0.852) in pepsin activity were observed among dietary treatments (Fig. 22).

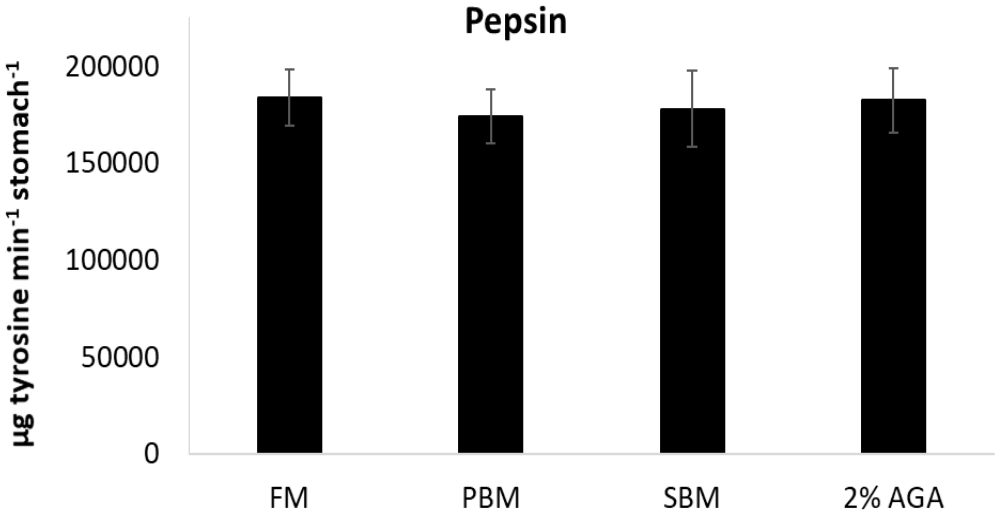

Figure 22. Acid protease (pepsin) activity with different SBM inclusion levels in diet in *T. macdonaldi* at 44 days fed with 100 % FM, relation 2: 1 PBM: FM, relation 2: 1 PBM: FM and 24 % of SBM and relation 2: 1 PBM: FM, 24 % of SBM and supplemented with 2 % of agavin.

In general terms, protease activity was significantly lower fish fed the PBM diet (Fig. 23). The activity of trypsin, chymotrypsin and total alkaline proteases in the intestine were significantly lower in fish fed the PBM diet compared to those fed the FM, SBM and 2% AGA diets, except for chymotrypsin in the 2% AGA treatment. In addition, significantly lower activity was found in the pyloric caeca for trypsin and L-aminopeptidase of fish fed with PBM diet. Lipase activity in both digestive organs was significantly higher

in fish fed with the 2% AGA diet compared to those fed the FM diet, but without a significant differences with fish fed the PBM and SBM diets. No significant differences were observed in amylase activity among the treatments.

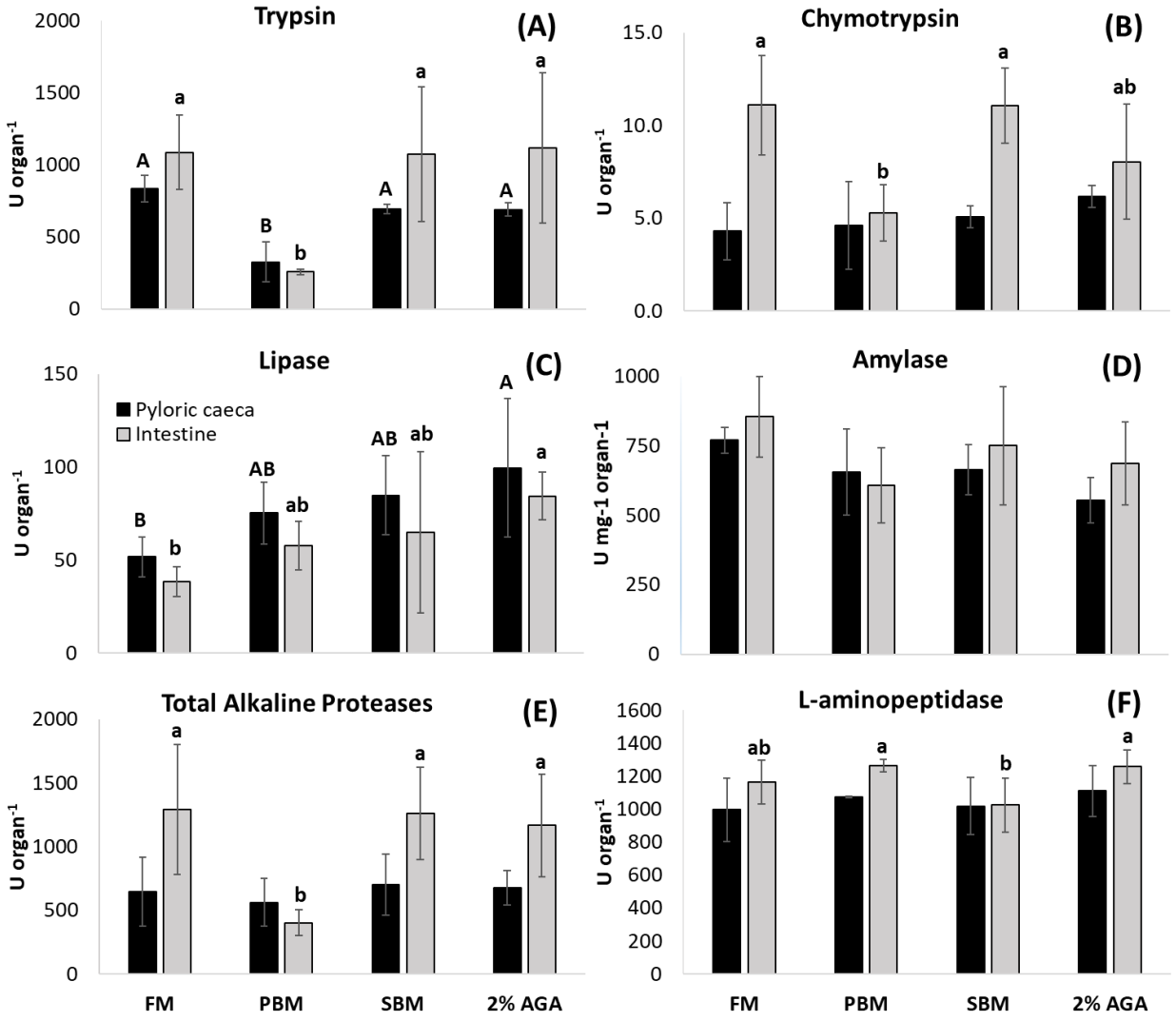

Figure 23. Total enzyme activity (U organ^{-1}) per pyloric caeca and intestine for trypsin (A), chymotrypsin (B), lipase (C), amylase (D), total alkaline proteases (E) and L-aminopeptidase (F) in *Totoaba. macdonaldi* at 44 days fed with 100 % FM, relation 2: 1 PBM: FM, relation 2: 1 PBM: FM and 24 % of SBM, and relation 2: 1 PBM: FM, 24 % of SBM and supplemented with 2 % of agavin. Different capital letters for pyloric caeca and lowercase letters for intestine represent significantly different values ($P<0.05$) within the same organ (n=3).

3.3.4 Fishmeal protein dependency ratio and economic conversion ratio

The fishmeal protein dependency ratio (FPDR) is typically used to assess the reduction in FM protein used to produce 1kg of farmed fish protein. The inclusion of PBM, SBM and 2 % of agavin significantly decrease the FPDR from 2.34 in the FM based diet to 0.78, 0.64 and 0.59, respectively (Fig. 24).

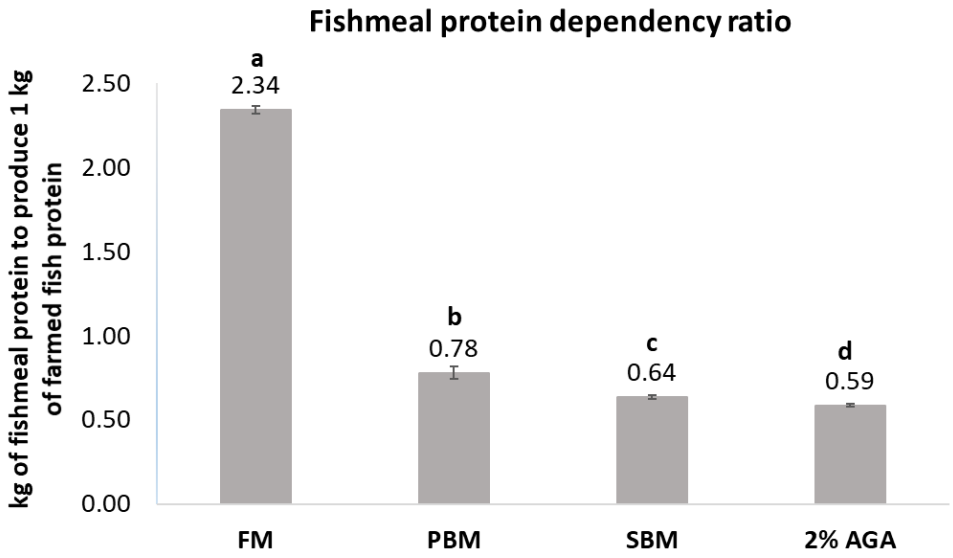

Figure 24. Fishmeal protein dependency ratio (FPDR) values for the experimental diets. Kilograms of fishmeal protein requires to produce 1 kg of farmed fish protein. Different lowercase letters represent significantly different values ($P<0.05$) among treatments.

The substitution of the FM by alternative ingredients reduce the protein ingredients cost of produce 1 kg of totoaba from $1.89 USD in the FM based diet to $0.88 USD in the PBM diet and $0.79 USD in the SBM diet. The diet supplemented with 2 % of agavin resulted in the better economic conversion ratio with $0.73 USD per kilogram of totoaba produced under culture conditions (Table 13).

Table 13. Economic conversion ratio (ECR) of experimental diets.

	FM	PBM	SBM	2% AGA
Feed conversion ratio (FCR)	0.85	0.86	0.90	0.83
Protein ingredients cost (USD kg^{-1})[z]	2.21	1.02	0.87	0.87
Economic conversion ratio (ECR)[y]	1.89	0.88	0.79	0.73

[z] Cost of fishmeal, poultry by-product meal and soybean meal

[y] ECR = FCR x Protein ingredients cost

3.3.4 Discussion

The use of prebiotics in fish diets is a new strategy trying to maintain the intestinal homeostasis of fish fed with alternative ingredients to replace fishmeal, especially those of plant origin, as well as to improve the growth performance and feed utilization, enhance the capacity to overcome stressful management and improve the response to pathogen challenges during the culture period. To our knowledge, the present study is the first to evaluate the use of agavin supplemented as a prebiotic in a marine fish diets with SBM inclusion. The main findings of this experiment were the agavin in low FM (18 %) diet containing 34 % of PBM and 24 % of SBM improve the growth performance, ameliorate the intestinal changes associated with enteritis, decrease the dependency for FM protein and reduce the cost of producing 1 kg of totoaba farmed.

Fish fed with SBM + 2 % of agavin in the diet not result in significant differences in growth performance and feed utilization with fish fed the 100 % FM diet. Agavin supplementation significantly increased the final weight, TGC, DWG, PER and lower FCR compared to fish fed the same diet (SBM) but without the prebiotic. In addition, fish fed the PBM diet resulted in lower final weight and DWG than fish fed the diet supplemented with agavin. This reduction in growth when using PBM is contrary to the study reported by Badillo et al., (2014) with the same species, where no difference was found with the control diet containing 100 % FM. A lower quality of the PBM or higher quality of the FM used in this experiment can explain the lower fish performance in PBM treatment. In this study, the reduction in fish growth cannot be associated with a decrease in feed intake in any of the diets since no significant difference was found among the treatments. The addition of krill oil as an attractant to avoid a lack of palatability associated with the inclusion of plant ingredients (NRC, 2011) and amino acid supplementation as part of the optimization in the formulation of fish diets can improve feed intake.

The benefits of agavin as a prebiotic in the growth performance and feed utilization found in this study agree with the results observed using another type of fructan, such as inulin in several species of fish. For example, in the Siberian sturgeon (*Acipenser baerii*) fed with 2 % inulin for 30 days (Mahious et al. 2006a), in the rainbow trout fed with commercial diets supplemented with 0.5 % and 1 % of inulin for 49 days (Ortíz et al., 2012), or in Tilapia (*Oreochromis niloticus*) with initial weight (IW) of 11 g fed with diets that included 52.3 % of SBM and 0.5 % of inulin for 61 days (Ibrahem et al., 2010), and the red drum (IW 7.1 g) fed with 30 % SBM and 1 % of inulin for 56 days (Zhou et al., 2010). Other studies did not find differences on growth and feed utilization when evaluating inulin in fish diets. For example, in the hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) (IW 34 g) fed with 35.5 % SBM and 1 % inulin for 56 days (Burr et al.,

2010), common carp (*Cyprinus carpio*) (IW 0.6 g) fed with 13.5 % SBM, supplemented with 0.5 % and 1 % inulin for 35 days (Eshaghzadeh et al., 2015), and sharpsnout sea bream (*Diplodus Puntazzo*) (IW 100 g) fed diets with 40 % of SBM and 0.8 % of inulin for 150 days (Piccolo et al., 2013). Other fructans have also been evaluated with similar results. For example, in gilthead sea beam (IW 32 g) fed with diets containing 25 % of SBM, and 0.1 %, 0.25 % and 0.5 % of scFOS for 56 days (Guerreiro et al., 2015c), European sea bass of 60 g with inclusion of 25 % of SBM and 1 % scFOS for 35 days (Guerreiro et al., 2015d). Similarly, in diets with 100 % FM, no growth improvements were found in the Atlantic salmon (IW 200 g) fed diets with 1 % FOS for 111 days (Grisdale-Helland et al., 2008), and in some study negative effects have been found, such as in the beluga juveniles (*Huso huso*) (IW 16 g) fed with diets containing 1 %, 2 % and 3 % inulin for 56 days reduced growth and the feed efficiency as inulin content in the diet increased (Reza et al., 2009).

Since supplementing prebiotics in diets containing SBM did not result in significantly lower growth rates or feed efficiency compared to diets with FM, demonstrates the benefits of using these functional nutrients to aid in digestion and maintain adequate intestinal health status to improve absorption of nutrients from vegetable ingredients resulting in adequate growth rates for each specie. In a recent study with totoaba juveniles (IW 215 g), González-Felix et al., (2018) included 2 % of a commercial prebiotic based on yeast (GroBiotic®-A) and the commercial probiotic containing *Bacillus,* Aquablend®, in diets with 21 % SBM and fed for 109 days. The authors found no significant differences in survival and growth at the end of the experiment among treatments and concluded that the inclusion of SBM in the diets was not the cause of the lack of positive response when using pre and probiotic since the diet without supplementation and SBM inclusion resulted in similar response, and recommend using a stress challenge in the study that can elucidate the benefits of using prebiotics and probiotics. In contrast, Fuentes-Quesada et al. (2018) evaluated the inclusion of SBM in the diets for totoaba with an initial weight of 71 g and found significant negative effects on the growth, digestive capacity and intestinal health when including SBM in the diet (i.e., > 22 %). In the present study, using 24 % SBM in the diet (fish initial weight 60 g), the growth rate obtained among treatments was quite high ranging from 3.1 to 3.3 g per day, while in the study of Felix-González et al. (2018) was from 1.6 to 1.7 g for animals of 215 g. This lower growth rate could be attribute in part to the inclusion of SBM, however the lack of negative histological changes in the intestine suggests that there are other causes for the differences found between studies, such as the initial size of the organisms (60 vs 215 g), duration of the trial (44 vs 109 days), diet nutrient level (CP 48.5 % vs 43 %), the source of the SBM or the environmental conditions during the bioassays (23.6 vs 27.7 °C). Nonetheless, the better growth performance and feed utilization from the present study can be associated to the fructooligosaccharides present in the agavin, which can modulate the intestinal bacterial communities to promote the proliferation of specific beneficial bacterial groups such as the lactic acid bacteria (LAB, e.g.,

Lactobacillus) and *Bifidobacterium* (Patel and Goyal, 2011). These bacteria have the capacity to hydrolyze the prebiotic β-bonds between the monosaccharides in the oligosaccharides resulting in end-products of the fermentation such as the short chain fatty acids (SCFAs) (Mussatto and Mancilha, 2007). As part of the production of SCFAs during fermentation, a decrease of the intestinal pH is observed. This acidification of the intestinal environment may help in the reduction of certain minerals, resulting in increased solubility and facilitate the uptake of many metal ions by the intestine (Merrifield et al., 2010). In a study with rainbow trout fed with inulin and FOS, Ortíz et al., (2012) found the Ca^{+2} content increased in the whole body of the fish, suggesting that the intestinal absorption of Ca^{+2} was improved by the prebiotics and latter incorporated into bone tissue.

Agavin is a known fructan and the main products of the fermentation of fructans in the intestine are the linear SCFAs such as acetate (C2:0), propionate (C3:0) and butyrate (C4:0) (Gibson et al., 1999; Franco-Robles and López, 2015). These SCFAs in mammals are known indirect energy substrates and metabolic regulators (Delzenne et al., 2003, 2008). In addition, the acetate transported to the liver is the primary substrate for cholesterol synthesis and lipogenesis, and can be utilized in the muscle (Laparra and Sanz, 2010). Propionate is known to inhibit lipogenesis in liver by direct competition with acetate and is a substrate for gluconeogenesis (Delzenne et al., 2008), and the butyrate is metabolized by the enterocyte as energy source and plays a role in the maintenance of colonic homeostasis (Hamer et al., 2008). Although, little is known of the effects of SCFAs in fish, a recent interest has been developed in these SCFA because they have been proposed as modulators of the metabolism of lipids and carbohydrates in fish and the energy status of the host (Guerreiro et al., 2015d; Ringø et al., 2016).

In addition, studies in humans have found that the modulation of the microbiota by prebiotics promotes the colonization of benefical commensal bacteria, up-regulate the expression of an intestinal monosaccharide transporter and key enzymes (acetyl-CoA carboxylase and fatty acid synthase) of *de novo* fatty acid biosynthetic pathways, involved in the metabolism of carbohydrates and lipids that can result in increase body weight gain and fat storage (Laparra and Sanz, 2010). Moreover, the use of complex polysaccharides by the microbiota has been shown to contribute up to 10 % of the energy supplied by the diet (Flint et al., 2008) and the active metabolism of the microbiota by the presence of fermentable carbohydrates can contribute with the supply of amino acids required by the organism. For example at least, between 1 % to 20 % of the circulating lysine and threonine in the blood have been associated with the intestinal microbiota (Metges, 2000; Hooper et al., 2002). In addition, *Bifidobacteria* present in the intestine have the ability to encode nucleotide biosynthesis (pyrimidine and purine) and B vitamins such as folic acid, thiamin, and niacin (Ventura et al., 2009). Therefore, the additional production of

intermediate metabolites and the increase of SCFAs by commensal bacteria used in the metabolism of enterocytes, liver, and muscle, exert beneficial effects and can be related with the efficiently utilization of the dietary protein, the improved growth and reduce the feed conversion in fish supplemented with the prebiotic evaluated in our study.

Some authors report that the different manufacture process of fructans, the degree of polymerization, dosage levels, species, feeding habit, life stage, culture system and fermented metabolites by the gut microbiota, might explain the contradictory results reported in several species when evaluating fructans as a prebiotics and makes ambiguous the possible benefits on growth performance, feed utilization and intestine histology of such a products (Merrifield et al., 2010; Guerrero et al., 2017). In the present study, the positive effects can be associated with the molecular structure of the agave fructans. The agavin is a complex mixture of highly branched molecules with β(2-1) and β(2-6) linkages (Mancilla-Margalli et al., 2006). Using *in vitro* assays with bifidobacteria and lactobacilli, Urías-Silvas and López, (2009) evaluated the prebiotic potential of fructans extracted from five different species of *Agave* spp., *Dasylirion* sp. and commercial inulins, and found that branched fructans from agavins produce higher quantities of SCFAs than linear fructans (inulins). In another study with mice, the supplementation with an inulin-type fructan extracted from *Agave angustifolia* with an average degree of polymerization of 32 and a complex molecular structure highly branched produced greater quantities of SCFAs than those fed with Raftiline ST, an inulin-type fructan with average degree of polymerization of 25 and completely linear in structure (Fig. 25, Huazano-García and López, 2013). The authors suggested the presence of branches in the agave fructans could make the molecule more accessible to the enzymes (e.i., fucosyltransferases) of the bacteria, rendering the agave fructans easily fermentable, independently of the high degree of polymerization. A study in turbot (*Psetta maxima*) larvae of 46 mg, fed with FM-based diets added with 2 % of Raftilose P95 which contains mainly oligofructose produced by partial enzymatic hydrolysis of chicory plant, increased the final weight and SGR of the larvae during 26 days compared with diet supplemented with 2 % of Raftiline ST, a standard form of chicory inulin and the control diet without prebiotic. The main difference of these inulin-type prebiotics is the degree of polymerization. For example, in Raftiline ST degree of polymerization ranges from 2 to 60 and in Raftilose P95 from 2 to 8 (Mahious et al. 2006b).

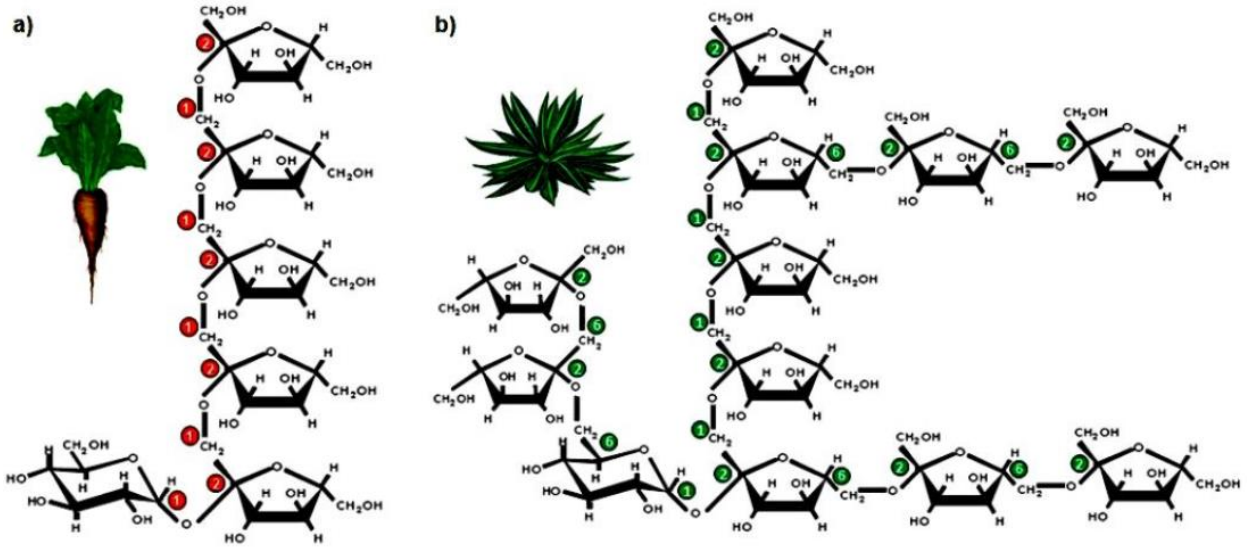

Figure 25. Schematic representation of the main structural differences between a) chicory fructans with a linear structure called inulins and b) agave fructans with a branched structure called agavins. The figure is taken from Huazano-García and López (2013).

In terms of the assessment of the intestinal structure, the supplementation of 2 % agavin in the diet containing 24 % SBM ameliorate the alteration of the intestinal epithelium compare to fish fed with SBM diet without prebiotic. The latter changes observed in the intestinal epithelium are typically associated with enteritis condition evaluated through a semi-quantitate scoring system (REF). The assessment of the intestinal integrity of the fish fed with the prebiotic resulted in a lower mean score (2.4) compared to fish fed the SBM with a mean score (3.2), but higher than PBM and FM with a mean score value of 2.1 and 1.8, respectively (see Table 11). The histometric measurements revealed that agavin supplementation reduced the percentage of mucosal folds with wider sub-epithelial mucosa (% MF with wider SM), and reduced the decrease of the brush border height (BBH) compared to fish fed the SBM diet (Table 12). Histological alterations of the intestine of fish fed the SBM diet without agavin are in accordance with previous study in totoaba assessing the effect of increasing SBM in the diet (Fuentes-Quesada et al., 2018).

To date several studies have been performed evaluating the effect of different fructans as prebiotics in fish diets using intestine morphology as a response variable. For example, in European sea bass juveniles (Guerreiro et al., 2015a), white sea bream (Guerreiro et al., 2016a) and gilthead sea bream (Guerreiro et al., 2016c) fed diets containing 25 % SBM supplemented with increasing levels of scFOS ranging from 0.1 % to 1 %, did not observed any significantly changes in the histomorphology of the intestine and demonstrated that the prebiotic helped maintain the intestinal integrity with moderate SBM inclusion.

Similar results were found by Refstie et al., (2006) and Bakke-McKellep et al., (2007) in Atlantic salmon (IW 172g) fed a diet using FM as the main protein source but with 7.5 % inulin. In contrast, several studies using inulin as a prebiotic have failed to demonstrate any positive effect on the intestinal integrity. For example, in Artic charr (IW 218 g) fed a casein-based diet with 15 % inulin for 21 days (Olsen et al., 2001), in gilthead sea bream (IW 50g) fed a commercial diet with 1 % of inulin for 28 days (Cerezuela et al., 2013) and in the sharpsnout sea bream (IW 100g) fed a 40 % SBM diet with 0.8 % of inulin inclusion for 114 days (Ferrara et al., 2015), the authors found no positive effects of the prebiotic. The authors concluded the lack of effect to be associated to a high level of prebiotic (Olsen et al., 2001), a reduction of gut microbiota richness (Cerezuela et al., 2013) and the high sensitivity of the sharpsnout sea bream to high levels of SBM (Hernández et al., 2007; Ferrara et al., 2015).

Changes in the intestinal integrity can affect intestinal health, feed digestion and nutrient absorption with ultimate consequences in animal growth. In particular, in the brush border structure of the intestine which contributes with more than 90 % of the total intestinal surface area (Sauvanet et al., 2015). In the present study, the histology analyses revealed that the fish fed with 24 % of SBM supplemented with the agavin resulted in higher brush border height (BBH) compared to fish fed without the prebiotic. This suggests that supplementation with agavin maintains adequate nutrients absorption in the enterocyte, observed in the presence of higher number of supranuclear vacuoles compared with the fish fed SBM diet. In other studies, with prebiotics, light microscopy demonstrated that supplementation increased mucosal folds height, tunica mascularis thickness, number of goblet cells, height and density of microvilli in intestinal brush border from fish fed with inclusion of SBM at 30 % of the diet for red drum (Zhou et al., 2010), 10 % SBM for the European sea bass (Torrecillas et al., 2013), 18 % SBM for rainbow trout (Yarahmadi et al., 2014) and 40 % SBM for turbot (Bai et al., 2017).

The mitigation of the adverse effects of SBM on the intestinal epithelium can be related in part to the production of SCFAs from the agavin by the modulated microbiota. Studies in mice, using fructans with different chains length such as inulin, FOS, combination of inulin-FOS and agavin reported a significantly increase of SCFAs with a concomitant drop in the luminal pH (Klessen et al., 2001; Licht et al., 2006; Huazano-García and López et al., 2013). Kihara and Sakata (1997) in tilapia (IW 50 g) evaluated the fermentation of five dietary carbohydrates and found differences on the intestinal morphology and in the SCFAs produced after 14 days of feeding. Among the SCFAs, butyrate is known as the main energy source of the enterocyte and is associated with maintenance of the barrier function (Maslowski and Mackay, 2011). Butyrate can decrease epithelial barrier permeability by supporting epithelial cell growth and inducing the expression of tight junction proteins, as well as suppressing pro-inflammatory cytokines in

favour of antiinflammatory and regulatory cytokines (Van Nuenen et al., 2005). In addition, Berni-Canani et al. (2011) reported the antioxidant effects of SCFAs on the mucosa, which inhibits apoptosis and regulates cell growth and differentiation in the intestine. Moreover, Gibson et al. (2003) suggested the reduction of pH by the production of SCFAs stimulates the growth of Bifidobacteria and *lactobacilli,* which in turn secrete bacteriocins that promote unfavorable conditions for potentially pathogenic bacteria and boost the overall intestinal health. However, in fish studies evaluating the effect of prebiotics on SCFAs production and intestine structure are scare. More research is warranted to better understand the physiological mechanisms that underlie the results of the present study.

Liver histological analysis of fish fed the FM and PBM diets revealed large lipid vacuoles, which is common for totoaba fed lipid rich diets, since it is considered a lean species and the liver is the main energy storage of excess lipid (Perez-Velazquez et al., 2017). Fish fed the diet with SBM and supplemented with agavin (2% AGA), a reduction in the size of lipid vacuoles with an increase of the sinusoid space was observed. In fish fed the SBM diet a consistent decrease in HSI index was observed. Similar results were reported in previous studies with totoaba fed SBM diets (Fuentes-Quesada et al., 2018), sharpsnout seabream (Hernández et al., 2007) and turbot (Gu et al., 2016). The reduction in lipid deposition in the liver can be related to the phytoestrogens present in SBM diets that modified lipid metabolism and have hypocholesterolemic effects in fish (Kaushik et al., 1995; Caiozzi et al., 2012). Additionally, changes in intestinal health and structure that reduce nutrient absorption forces the fish to use liver reserves that can result in a lower HSI. On the other hand, in fish supplemented with agavin, the HSI was 15 % higher than fish fed with SBM diet. However, the typical characteristics of a fatty liver with large lipid vacuoles and little sinusoid space were not observed in the present study. Although not measure in our study, SCFAs have been reported to modulate lipid metabolism in the liver and propionate in particular is a competitive inhibitor of acetate, which is a precursor to the lipogenesis and cholesterogenesis pathways, thus a high concentrations of propionate in the liver has been proposed as the mechanism to explain the decrease lipid content in the liver (Delzenne et al., 2008). Further research is warranted evaluating this effect in totoaba.

Huazano-García and López (2013) found that acetate was the main SCFAs produced when mice were fed diets with agavin supplementation. However, they also observed an increment in the production of propionate and butyrate in the distal colon of mice receiving diets supplemented with agavin. In fish, *in vitro* incubation of rainbow trout intestines, produce SCFAS by the microbiota (Kihara and Sakata, 2001). Similarly, in the common carp (IW 100 g) fed various oligosaccharides resulted in the production of butyrate and propionate in high concentrations (Kihara and Sakata, 2002). Burr et al. (2010) in another *in*

vitro study with hybrid striped bass (IW 200 g), reported that after 24 and 48 h incubation with a probiotic Grobiotic®-A, MOS, inulin and GOS produce higher concentration of acetate and butyrate and lower concentrations of propionate. Similarly, in the Siberian sturgeon (*Acipense baerii*) fed with arabinoxylooligosaccharides (AXOS), the main SCFAs produced in the distal intestine were acetate and butyrate, while propionate concentration was very low, similar to the control diet (Geraylou et al., 2012). In European sea bass the supplementation with XOS decreased lipogenesis and in fish fed with FM-based diet XOS and scFOS increased glycolytic activity (Guerreiro et al., 2015a). The possible modulation of the glucose and lipid metabolism by the production of the main SCFAs, acetate and propionate and their relative ratios needs further investigations. The measurements of SCFAs patterns is necessary to determinate this hypothesis, since the pattern of SCFAs proportion varies according to the species of interest and the prebiotic.

Regarding the ISI, fish fed with SBM and PBM diets presented lower values of ISI. The reduction of the ISI can be associated with changes on the intestinal integrity causes by the SBM inclusion in totoaba diets (Fuentes-Quesada et al., 2018). Similar result was reported by Refstie et al., (2006) in the distal intestine of Atlantic salmon fed FM-based diet containing 25 % of SBM. The reduction of the ISI in the present study in fish fed with PBM is unclear. In contrast, fish fed with 2% AGA diet presented the higher ISI and similar to fish fed with FM diet, that demonstrate the agavin helps to maintain the intestinal structure.

Growth improvement and nutritional efficiency in fish fed the diet supplemented with prebiotics has additionally been associated with production of the exogenous enzymes, that are not present or in very low quantities in the host, by the microbiota in the gut and help in the digestion of certain nutrients (Ray et al., 2012; Merrifield and Rodiles, 2015). In juvenile fish, an adequate feed digestion is essential for nutrient absorption by the enterocytes to maintain fast growing rates. The reduced digestive enzyme activity of total alkaline proteases, trypsin, and chymotrypsin in the fish fed the PBM diet may be responsible to the lower growth observed in this treatment. Fish fed with 2% AGA did not present significant differences in the digestive enzyme activity between the FM and the SBM treatments. Similar results have been reported in Atlantic salmon (IW 172 g) fed with FM-based diet with 7.5 % inulin, resulting in no significant differences in trypsin, amylase, alkaline phosphatase and leucine aminopeptidase activity (Refstie et al., 2006). Likewise, in the red drum (IW 11 g) and hybrid striped bass (*Morone chrysops* x *M. saxatilis*) (IW 5 g) fed with diets containing 37.5 % SBM and supplemented with four prebiotics (1 % fructo-oligosaccharide, 1 % Bio-MOS, 1 % transgalacto-oligosaccharide and, 1 % and 2 % of GroBiotic-A), found no significant differences in activity in pepsin, trypsin, chymotrypsin, aminopeptidase, α-amylase, lipase, and both acid and alkaline phosphatase activities compared to the control group fed a SBM diet for 56

days (Anguiano et al., 2013). Guerreiro et al. (2016c) obtained the same results feeding gilthead sea bream of 32 g with 25 % SBM inclusion and supplemented with scFOS at 0 %, 0.1 %, 0.25 %, and 0.5 %. The authors report no changes in digestive enzyme activity of total alkaline protease, lipase, and α-amylase at 56 days.

In the present study, lipase activity of fish fed with 1 % inulin was higher than those fed with 100 % FM, but without significant changes with fish fed the SBM and PBM diets. Similar results were reported by Hoseinifar et al. (2016b) for the common carp (IW 0.5 g) who found a higher activity in amylase and lipase with 1 % scFOS supplementaion, but without changes in proteases. Additionally, fry of the Caspian roach (*Rutilus rutilus*) (IW 0.7g) fed diets with 2 % and 3 % FOS presented higher activity in total alkaline proteases, amylase, and lipase (Soleimani et al., 2012). Both studies used 13 % SBM in the diet and fed for 7 weeks. The authors mention that the highest digestive enzyme activity found was probably due to the activity of the exogenous enzymes produced by the microbiota modulated by the prebiotic. However, it is not yet possible to easily elucidate the quantity of exogenous enzymes derived from the microbiota of the total activity found in the fish intestine. The quantity of exogenous enzymes produced by the microbiota can be stimulated by the oligosaccharides of the SBM (Mussatto and Mancilha, 2007), which might explain why the fish fed with the SBM diet, did not result in significant differences in digestive enzyme activity compare with fish fed 2% AGA diet, but had worse fish performance.

The formulation of practical diets including PBM and SBM reduce the dependency for FM protein in fish diets (Fig 24). The use of the FPDR ratio assessing the dependence on FM, should be correlated with fish performance parameters to evaluate wheatear a diet containing less FM allow an optimal growth and feed utilization. The PBM diet had a relation of 2:1 PBM:FM decreasing three-fold the FPDR from 2.34 to 0.78, and the incorporation of SBM reduce to value to 0.64, but the growth performance of fish feed either diet was lower. Interestingly, fish fed the diet supplemented with 2 % of agavin resulted in better growth and feed utilization (i.e., lower FCR) that allow to produce 1kg of farmed totoaba protein with 0.59 kg of FM protein compared with the SBM diet (0.64). Additionally, agavin supplementation reduced the cost to produce 1 kg of totoaba to $0.73 USD compared with the higher cost using the PBM ($0.88 USD) and SBM ($0.79 USD) diets (Table 13). The use agavin as a prebiotic can overcome many problems that are associated when SBM or plant protein ingredients and should be included in practical diets for carnivorous fish. Reducing the dependency for FM in totoaba diets is a step to farmed fish as a net producer of protein and to increase the sustainability of the culture of this species.

In conclusion, supplementation with agavin as a prebiotic improves growth, nutritional efficiency, decreases the FM protein dependency in diet resulting in with lower production costs, and counteracts

the negative effects of SBM on intestinal health by maintaining the height of the brush border, reducing the percentage of mucosal folds with wider submucosal epithelium and increasing the intestinal somatic index. Although, agavin did not ameliorate the infiltration of eosinophilic granulocytes in the submucosal epithelium and should be further investigated. The use of agavin in diets for totoaba seems to be an adequate prebiotic with great potential, more investigations are needed to elucidate the mechanisms by which this improvement in growth and intestinal health is brought about.

Chapter 4. The prebiotic effect of agavin inclusion levels in practical diets for *Totoaba macdonaldi* juveniles

4.1 Introduction

The inclusion of alternative ingredients in diets for marine fish is one of the priorities to reduce dependency on marine resources such as fishmeal. The use of vegetable proteins such as soybean meal (SBM) is considered a viable option due to its high availability and a constant nutritional profile. However, it has been reported that medium to high SBM levels (i.e., > 22 %) can cause intestinal alterations and reduces growth in totoaba (Fuentes-Quesada et al., 2018).

The use of prebiotics in animal nutrition is a nutritional strategy to improve or maintain the nutritional efficiency of feeds, organism immune response, intestinal health and even growth in fish fed practical diets that contain alternative ingredients such as SBM (Refstie et al., 2006, Bakke-McKellep et al., 2007, Buentello et al., 2010; Zhou et al., 2010; Piccolo et al., 2011; Torrecillas et al., 2013; Guerreiro et al., 2015c, 2015d, 2016, 2018a). However, it has been found that the inclusion of prebiotics can also decreased growth, feed efficiency and can damage the intestinal structure (Olsen et al., 2001; Reza et al., 2009; Cerezuela et al., 2013; Ferrara et al., 2015). The contradictory results of using prebiotics in fish diets have been explained by the source, type, level of inclusion, species of fish and age of the fish evaluated (Gatlin et al., 2015, Guerreiro et al., 2017). Therefore, the benefits of a particular prebiotic for each species assessed cannot be generalized and specific species investigations must be carried out to determine the effects of each prebiotic.

Agavin is a new prebiotic primarily evaluated in terrestrial animal and recently tested in marine fish. For example, in totoaba, the inclusion of agavin at 2 % in a diet containing low in fish meal (< 18 %) and 24 % SBM improved the growth performance and integrity of the intestine compared to the same diet without the prebiotic (chapter 3). These promising results allow the formulation of cost-effective diets that depend less on marine ingredients and reduce the production cost of this promising species. On the other hand, based on previous research with prebiotics it is proposed the hypothesis of whether increasing levels of inclusion of agavin in the diet would improve the results previously observed and possibly define an optimum level. Therefore, this study was design to we evaluated the effect of increasing levels of agavin in the diet on the growth performance, feed efficiency, digestive capacity, distal intestine histology in juveniles of *Totoaba macdonaldi* fed low fishmeal diets containing 24 % of SBM.

4.2 Materials and methods

4.2.1 Diet formulation

Four experimental diets were formulated to be isoproteic (509 g kg^{-1} crude protein (CP)) of diet dry matter (DM) and isolipidic (120 g kg^{-1} crude lipid (CL)) of diet DM based on recommended nutritional requirements for totoaba (Perez-Velazquez et al., 2016; Rueda-López et al., 2011), with a DHA/EPA ratio of 1 and DHA+EPA >1.3 % of diet (NRC, 2011). The first diet was formulated to contain poultry by-product meal (PBM, 68 % CP, 14 % CL, pet food grade, National Renderers Association, USA) and fishmeal (FM, 69 % CP, 6 % CL, Maz Industrial SA de CV, Mazatlán Sinaloa, México) as the main protein sources in a 2:1 ratio according to Badillo et al., (2014), and was a positive control (i.e., without SBM in diet) and termed control diet (CD). For the following three experimental diets, soybean meal (SBM, 48 % CP, 6 % CL, Alimentos COLPAC, Sonora, México) was included at 240 g kg^{-1} diet, for this the PBM and FM were reduced in the same proportion and holding the 2:1 relation. The prebiotic agavin (donated by Instituto de Biotecnología, UNAM, Cuernava, México) was added to the diets in increasing levels of 10, 20 and 30 g kg^{-1} DM of the diet. All diets included 1 g kg^{-1} of krill oil (Biogrow, ProAqua, México) as an attractant and 10 g kg^{-1} of taurine (Insumos Nubiot, SA de CV, México) (Table 14). Four essential amino acids (lysine, methionine, threonine, and arginine; EVONIK, Degussa, México) were supplemented to meet estimated amino acid requirements based on the totoaba whole body amino acids profile (data from our laboratory) Diets were manufactured in a mixer (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.

4.2.2 Experimental design, animals and facilities

Totoaba juveniles, obtained from the Centro de Reproducción de Especies Marinas del Estado de Sonora (CREMES) in Kino bay, Mexico, were transported to the research facilities at the Marine Fish Culture Laboratory, of the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), in Ensenada, B.C., México. The four experimental diets were evaluated in triplicate. One hundred and twenty fish (initial body weight 28.8 ± 1.3 g) were randomly distributed and stocked into twelve 200-L cylindrical fiberglass gray tanks (i.e., ten fish per tank) in a closed recirculating seawater system equipped with biofilter (Model BBF XF, Patent 5232586, AST, USA), heat pump (Delta Star DS-5 ½ hp, Aqualogic, USA) and UV sterilized (Model QL 25, Lifegard Aquatics, USA).

Table 14. Formulation of the experimental diets containing increasing levels of agavin. Dietary formulation is presented as g kg^{-1} on as fed basis and proximate composition in g kg^{-1} on a dry matter basis.

	Experimental diets			
Ingredients (g kg^{-1} DM)	**Control diet**	**1% AGA**	**2% AGA**	**3% AGA**
Sardine meal (69 % CP)[a]	230.0	180.0	180.0	180.0
Poultry by-product meal (68 % CP)[b]	460.0	357.5	357.5	357.5
Soybean meal (48 % CP)[c]	0.0	240.0	240.0	240.0
Corn starch	126.6	20.0	10.0	0.0
Pregelatinized corn starch	80.0	80.0	80.0	80.0
Sardine oil[a]	31.0	43.0	43.0	43.0
Rovimix for carnivorous fish[d]	30.0	30.0	30.0	30.0
Stay-C[d]	10.0	10.0	10.0	10.0
Taurine[e]	10.0	10.0	10.0	10.0
Methionine[f]	4.1	4.7	4.7	4.7
Lysine[f]	11.0	9.7	9.7	9.7
Arginine[f]	1.8	0.0	0.0	0.0
Threonine[f]	0.4	0.0	0.0	0.0
Attractant (krill oil)[g]	1.0	1.0	1.0	1.0
Sodium benzoate	2.5	2.5	2.5	2.5
Choline chloride	1.5	1.5	1.5	1.5
BHT	0.1	0.1	0.1	0.1
Agavin[h]	0.0	10.0	20.0	30.0
Proximate composition (g kg^{-1} DM)				
Dry matter	983.7 ± 0.3	965.8 ± 0.3	995.5 ± 0.2	995.0 ± 0.4
Crude protein	508.9 ± 5.5	510.5 ± 3.6	509.3 ± 1.6	506.5 ± 9.5
Crude fat	114.9 ± 0.7	121.2 ± 0.3	122.0 ± 3.4	120.3 ± 2.3
Ash	133.3 ± 1.9	128.0 ± 0.3	127.3 ± 0.7	126.5 ± 1.0
NFE[h]	241.7 ± 5.5	239.6 ± 3.9	241.4 ± 4.2	250.7 ± 8.0

[a] Maz Industrial SA de CV, Mazatlán, Sinaloa, México.
[b] Pet food grade, National Renderers Association, USA.
[c] Alimentos COLPAC, Sonora, México.
[d] Rovimix; Stay-C, DSM, Guadalajara, México.
[e] Insumos NUBIOT SA de CV, México.
[f] Free aminoacids, EVONIK, Degussa, México.
[g] Biogrow, Proveedora de Insumos Acuícolas, SA de CV, Mazatlán, Sinaloa, México.
[h] Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México.
[i] Nitrogen-free extract (NFE, %) = 100 - (% crude protein + % total lipid + % ash).

Water quality was monitored daily, with mean values for temperature equal to 24.1 ± 1.0 °C, dissolved oxygen equal to 6.2 ± 0.4 mg L^{-1} with an oxygen saturation up to 83 %, salinity around 34.8 ± 1.0 ‰, pH = 7.8 ± 0.1 with a water flow of 2.1 L min^{-1}. Every three days the total ammonia nitrogen, nitrite-nitrogen and nitrate-nitrogen levels were measured (Api Pharmaceutic Aquarium Kit) to keep values < 0.75 mg L^{-1}, < 0.75 mg L^{-1} and < 80 mg L^{-1}, respectively. Fish were kept under a 12 h light: 12 h dark photoperiod schedule. Each dietary treatment was randomly assigned into triplicate experimental units. Fish were

hand-fed daily to apparent satiation at 08:00, 12:00 and 16:00 h during 56 days. Daily, all uneaten feed was removed within an hour of feeding, dried and weighed to account for uneaten feed in our estimates of daily feed intake.

4.2.3 Sampling

The growth response indexes and somatic indexes were calculated at the end of the experiment as previously described in section 3.2.3. For histological samples the sampling protocol described in section 3.2.3. was followed. At the end of the growth experiment, and during fifteen days, feces from each dietary treatment were collected with a glass siphon, gently washed with distilled water to remove the salts from the sea water, dried at 60 °C and stored at -20 °C until analyses for digestibility.

4.2.4 Analytical methods

The proximate analyses of the experimental diets were performed as previously described in section 2.2.4.

4.2.5 Distal intestine histology

The intestine and liver samples for histology follow the methodology described in the section 3.2.5 with a minor modification. Briefly, for this experiment distal intestine was cut in 4 sections related for the small size of fish instead in 6 sections mention in the section 3.2.5.

4.2.6 Digestive enzyme activity

The enzymatic assays were performed as previously described in section 2.2.6.

4.2.7 *In vivo* apparent digestibility coefficient (ADC)

The apparent digestibility coefficient (ADC) of dry matter, crude protein and lipids, were determined using the insoluble ash method in hydrochloric acid as an inert marker as described by (Montaño-Vargas et al., 2002). The following formulas were used:

ACD of dry matter, protein and lipid: 1 – [(% insoluble diet ash / % insoluble feces ash) x (% dry matter in feces / % dry matter in diet)] (15)

The insoluble ash (IA) was calculated as follows:

IA (%) = insoluble ash (g) x 100 / sample (g) (16)

4.2.8 Fishmeal protein dependency ratio (FPDR)

The fishmeal protein dependency ratio was estimated using the formula described in section 3.2.7.

4.2.9 Economic conversion ratio (ECR)

The economic conversion ratio was estimated using the formula described in section 3.2.8.

4.2.10 Statistical analyses

The assumptions of normality and homogeneity of variances were evaluated by the Shapiro-Wilks and Barlett test respectively (Zar, 2010). Prior to analysis, percentage data were arcsine transformed. Significant differences in performance indexes, somatic indexes, histological measures of the distal intestine, enzyme activities levels and fishmeal protein dependency ratio were analyzed by one-way ANOVA, followed by post-hoc Fisher's least significant difference rank test. The values of individual parameters and mean score of the semi-quantitative scoring system were analyzed by the non-parametric Kruskal-Wallis test and the existing variation presented between all samples was determined with the

pooled standard error (PSE). For all cases, statistical significance was set at P<0.05. Statistical analysis was performed using the software STATISTICA 8.0™ (StatSoft, Inc. USA).

4.3 Results

4.3.1 Growth performance, feed utilization, and somatic indexes

Supplementation with agavin improved growth performance compared with the control diet (Table 15). Fish fed with 1 % of agavin diet resulted in final growth, WG, RWG and, DWG significantly higher compared to the control diet. Increasing the level of agavin in the diet above 1 % did not result in significant improvements in growth performance or feed utilization among agavin supplemented diets. Diet with 2 % of agavin did not present significant differences in growth to the control diet. The parameters of TGC, CF, FCR, PER and FI were not significantly different among experimental treatments.

Table 15. Growth performance of *Totoaba macdonaldi* fed the experimental diets supplemented with 1 %, 2 % and 3 % of agavin for 56 days. Different letters represent significantly differences values (P<0.05) within the same row.

	Control Diet	**1% AGA**	**2% AGA**	**3% AGA**	**P *value***
Initial weight (g)	29.2 ± 1.3	28.9 ± 0.9	28.7 ± 1.2	28.5 ± 2.4	0.946
Final weight (g)	133.8 ± 1.3^{b}	146.2 ± 5.2^{a}	141.6 ± 4.2^{ab}	145.9 ± 7.6^{a}	0.023
TGC[1]	1.52 ± 0.02^{b}	1.64 ± 0.03 ^{ab}	1.60 ± 0.07 ^{ab}	1.65 ± 0.10^{a}	0.039
WG[2] (g)	104.6 ± 0.4^{b}	117.3 ± 4.6^{a}	113.0 ± 5.4^{ab}	117.4 ± 8.5^{ab}	0.032
RWG[3] (%)	358.7 ± 17.3^{b}	405.3 ± 12.8^{a}	395.0 ± 35.8^{ab}	414.7 ± 53.6^{a}	0.041
DWG[4] (g)	1.87 ± 0.01^{b}	2.09 ± 0.08^{a}	2.02 ± 0.10^{ab}	2.10 ± 0.15^{a}	0.032
FCR[5]	0.79 ± 0.01	0.78 ± 0.03	0.79 ± 0.03	0.78 ± 0.01	0.850
PER[6]	2.48 ± 0.02	2.52 ± 0.09	2.48 ± 0.10	2.54 ± 0.03	0.832
CF[7]	1.47 ± 0.05	1.46 ± 0.02	1.50 ± 0.03	1.43 ± 0.04	0.205
FI[8] (% day^{-1})	1.22 ± 0.06	1.25 ± 0.04	1.20 ± 0.06	1.27 ± 0.03	0.259

[1] TGC (Thermal Growth Coefficient) = [(final weight^{⅓} - initial weight^{⅓}) / (T°C x Days)] x 1000 (Jobling, 2003).
[2] Weight gain, g = final weight – initial weight
[3] Relative weight gain % = [(final weight – initial weight) / initial weight] x 100
[4] DWG (Daily weight gain) = final weight gain / experimental days
[5] FCR (Feed Conversion Ratio) = total feed consumed / wet weight gained.
[6] PER (Protein Efficiency Ratio) = weight gain / protein intake.
[7] CF (Condition Factor) = final body weight x (body length)3 x 100 (Hardy and Barrows, 2002).
[8] FI, Feed Intake = FI (%/day) = 100 x (total amount of the feed consumed per fish / ((initial body weight + final body weight) / 2) / days).

At the end of the experiment, somatic indexes estimated on fish from each treatment; HSI, VSI and ISI did not result in significant differences among dietary treatments (Table 16).

Table 16. Somatic indexes of *Totoaba macdonaldi* fed the experimental diets for 56 days. Different letters represent significantly differences values (P<0.05) within the same row.

	Control Diet	1% AGA	2% AGA	3% AGA	P value
HSI %[1]	1.49 ± 0.25	1.37 ± 0.22	1.45 ± 0.17	1.24 ± 0.16	0.199
VSI %[2]	4.20 ± 0.42	4.45 ± 0.88	4.31 ± 0.41	4.02 ± 0.37	0.451
ISI %[3]	0.65 ± 0.04	0.69 ± 0.10	0.67 ± 0.04	0.66 ± 0.08	0.651

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

4.3.2 Distal intestine morphology

The distal intestine of the fish supplemented with 2 % of agavin presented a significantly higher number of MF than fish fed the control diet (Table 17). Fish fed with 1 % of agavin diet resulted in significantly lower number of MF compared to the fish fed 2 % and 3 % agavin diets. On the other hand, the BBH of the fish fed with 1 % and 2 % of agavin in diet did not show significant difference with the control diet, while 3 % of agavin presented a significant reduction in the BBH (Table 17). Increasing the levels of agavin in totoaba diets did not result in changes in the MF length, EH, SM and % MF with wider SM compared with fish fed with the control diet.

Table 17. Morphometric measures of the distal intestine of *Totoaba macdonaldi* fed the experimental diets for 56 days. Different letters represent significantly differences values (P<0.05) within the same row.

	Control Diet	1% AGA	2% AGA	3% AGA	P value
MF number[1]	43.8 ± 1.6^{bc}	41.5 ± 3.3^{c}	49.8 ± 1.8^{a}	47.8 ± 1.7^{ab}	0.007
MF length[2] (µm)	587.7 ± 77.7	665.7 ± 27.2	642.1 ± 33.4	609.6 ± 148.9	0.740
EH[3] (µm)	18.7 ± 1.1	20.2 ± 0.7	19.9 ± 2.2	18.4 ± 0.8	0.343
SM[4] (µm)	11.5 ± 1.2	11.5 ± 1.8	11.6 ± 2.5	11.1 ± 0.9	0.986
% MF with wider SM[5]	20.3 ± 3.7	22.3 ± 1.6	16.9 ± 3.9	20.2 ± 1.8	0.399
BBH[6] (µm)	2.70 ± 0.02^{a}	2.57 ± 0.15^{ab}	2.54 ± 0.18^{ab}	2.40 ± 0.08^{b}	0.016

[1]MF number = mucosal fold number; [2]MF length = mucosal fold length; [3]EH = enterocyte height; [4]SM = sub-epithelial mucosa; [5]% MF with wider SM = percentage of mucosal folds with wider sub-epithelial mucosa; [6]BBH = brush border height.

Regardless of the level of agavin, fish fed with soybean meal did not present significant changes compared to the control diet in the individual parameters and mean score to assess the degree of histological changes in the distal intestine associated with enteritis processes (Table 18). In the four experimental diets, the presence of SNV was observed, with GC scattered on the PM, without enlargement of the LP and SM, and a low to moderate infiltration of EG in the diets supplemented with agavin (Fig. 26, Table 18).

Table 18. Individual and mean score the parameters evaluated to assess the degree of histological changes in the distal intestine of *Totoaba macdonaldi* fed the experimental diets for 56 days. Different letters represent significantly differences values (P<0.05) within the same row.

	Control Diet	**1% AGA**	**2% AGA**	**3% AGA**	**PSE**	**P value**
Mucosal folds	1.3	1.3	1.7	1.3	0.19	0.794
Supranuclear vacuoles	2.7	2.0	2.7	1.3	0.28	0.287
Goblet cells	2.0	2.3	2.3	2.0	0.11	0.493
Lamina propia	2.7	2.0	2.3	2.3	0.19	0.721
Sub-epithelial mucosa	2.3	1.7	2.0	2.3	0.15	0.493
Eosinophilic granulocytes	1.7	2.7	2.3	2.7	0.22	0.721
Mean score	2.1	2.0	2.2	2.0	0.10	0.794

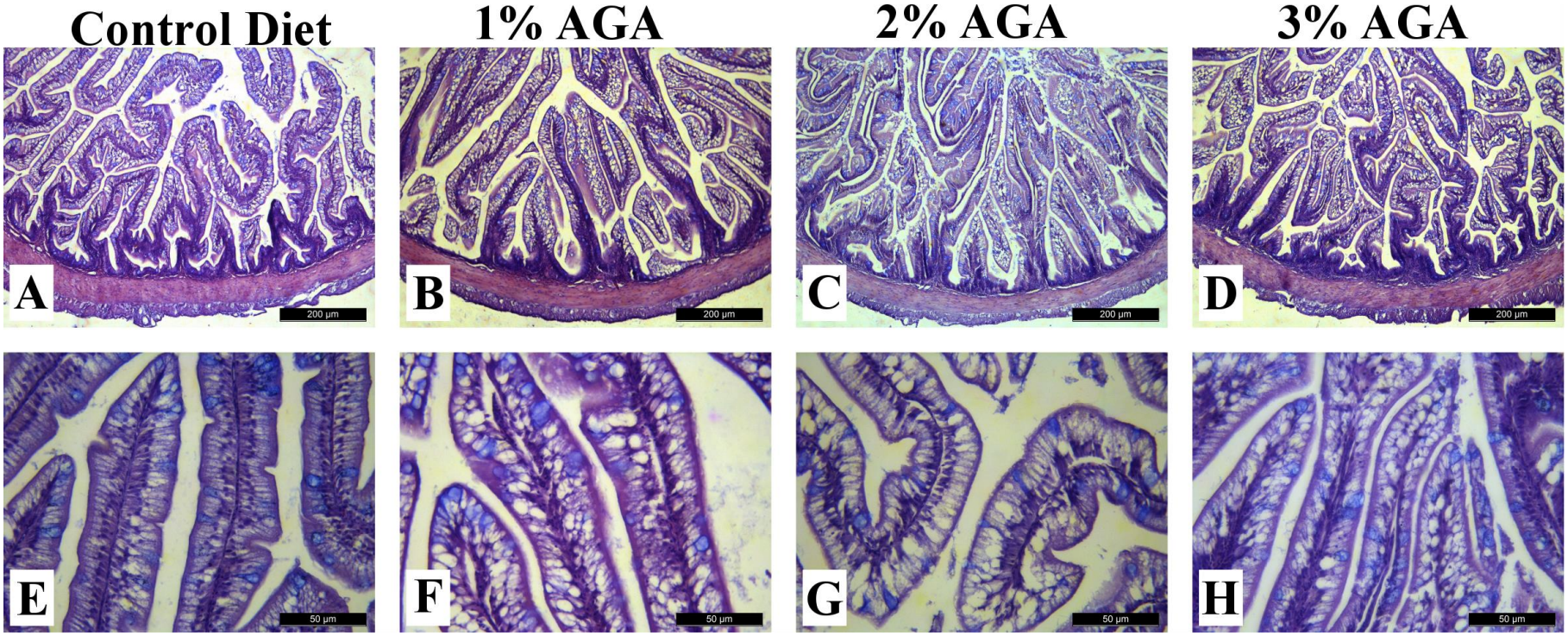

Figure 26. Light microscopy images of the morphological changes in distal intestine in *Totoaba macdonaldi* fed the experimental diets for 56 days. Figures from A to D bar = 200 µm, and from E to H bar = 50 µm.

4.3.3 Digestive enzyme activity

Pepsin activity was significantly higher in the fish fed with 2 % of agavin compared to fish fed with 1 % and 3 % of agavin, but not significant difference with the fish fed the control diet (Fig. 27).

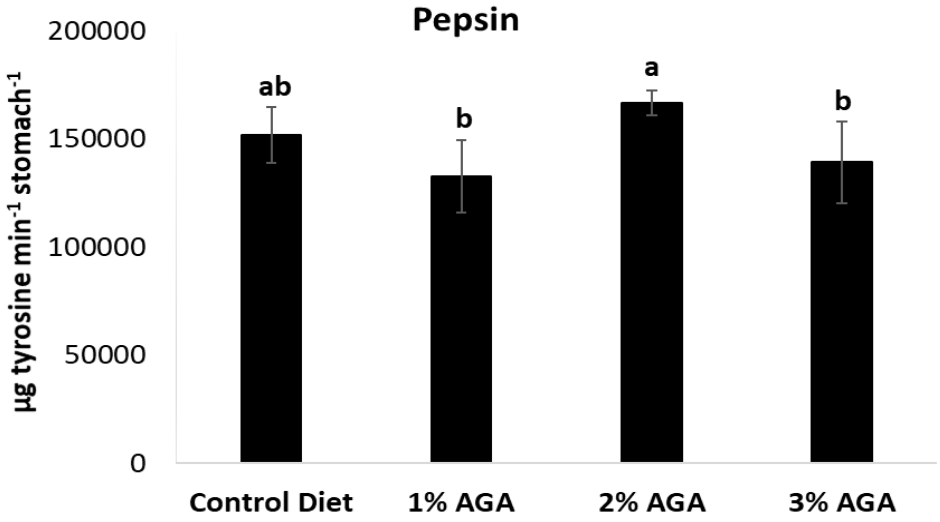

Figure 27. Acid protease (pepsin) activity in *T. macdonaldi* fed with different SBM inclusion levels in the diet for 56 days.

Digestive enzyme activity measurement on the intestine revealed significant differences among treatments. For example, trypsin activity was significantly higher in fish fed 2% AGA diet compared to fish fed 1% AGA diet, but no significant differences with fish fed the control and the 3% AGA diet (Fig. 28). Similarly, in fish fed the 2% AGA diet, total alkaline proteases were significantly higher than the other three treatments. No significant differences were found in chymotrypsin, lipase, amylase and L-aminopeptidase activity.

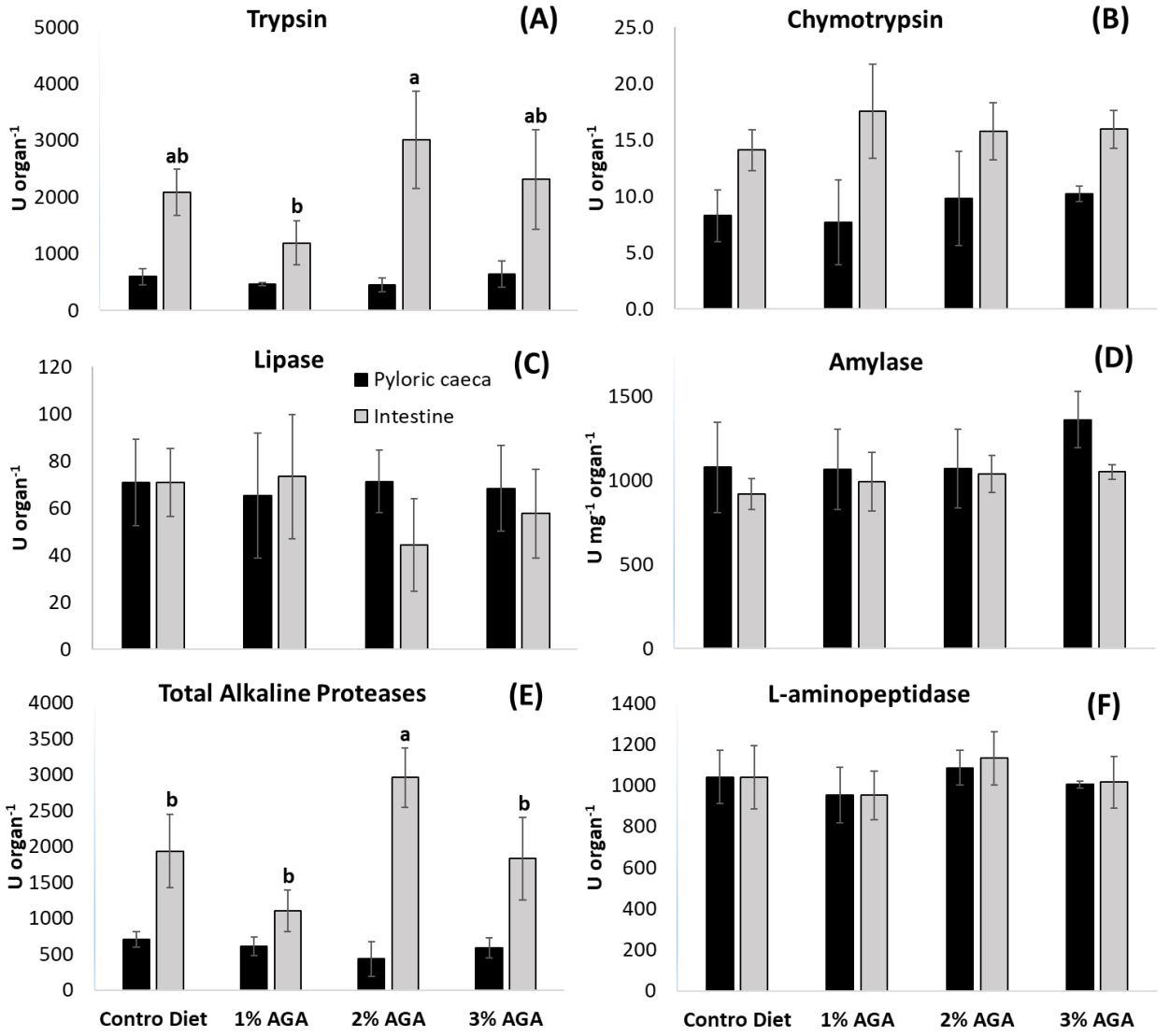

Figure 28. Total enzyme activity (U organ^{-1}) of the pyloric caeca (dark bars) and intestine (light bars) for trypsin (A), chymotrypsin (B), lipase (C), amylase (D), total alkaline proteases (E) and L-aminopeptidase (F) in *T. macdonaldi* after feeding with the experimental diets for 56 days.

4.3.4 *In vivo* apparent digestibility coefficient (ADC)

No significant differences were found in the ADC of dry matter, crude protein and lipid of the diets among the experimental treatments (Table 19).

Table 19. Apparent digestibility coefficients (% ADC) of the experimental diets.

	Control diet	1% AGA	2% AGA	3% AGA	P value
Dry matter ADC	50.8 ± 6.2	51.9 ± 4.7	47.2 ± 7.2	54.2 ± 6.3	0.593
Protein ADC	88.6 ± 1.6	89.1 ± 1.1	88.5 ± 1.0	89.5 ± 1.3	0.738
Lipid ADC	90.3 ± 1.9	92.8 ± 1.4	91.4 ± 1.7	93.4 ± 0.4	0.107

4.3.5 Fishmeal protein dependency ratio and economic conversion ratio

The inclusion of SBM with agavin in diet formulation significantly decreased the fishmeal protein required to produce 1 kg of farmed fish protein from 0.72 to 0.55. (Fig. 29).

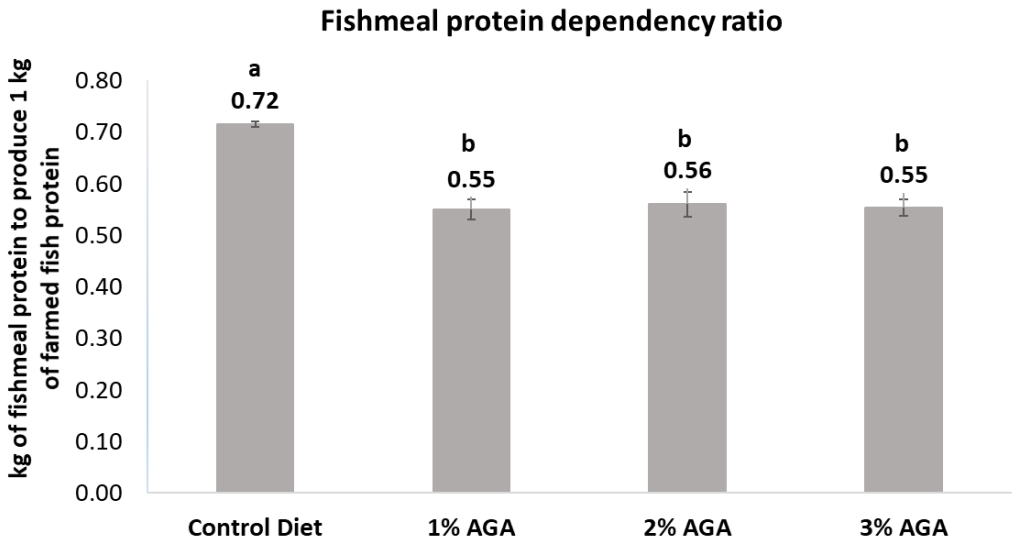

Figure 29. Fishmeal protein dependency ratio (FPDR) values for the experimental diets. Kilograms of fishmeal protein requires to produce 1 kg of farmed fish protein. Different lowercase letters represent significantly different values ($P<0.05$) among treatments.

Independently of the agavin level in the diet, supplementation of agavin reduced the cost of production from $0.81 USD in the control diet to $0.69 USD (Table 20).

Table 20. Economic conversion ratio (ECR) of experimental diets.

	Control Diet	1% AGA	2% AGA	3% AGA
Feed conversion ratio	0.79	0.78	0.79	0.78
Protein ingredients cost (USD kg^{-1})[z]	1.03	0.88	0.88	0.88
Economic conversion ratio (ECR)[y]	0.81	0.69	0.70	0.69

[z] Cost of fishmeal, poultry by-product meal and soybean meal

[y] ECR = FCR x Protein ingredients cost

4.4 Discussion

Increasing the levels of the prebiotic agavin resulted in increased growth that reached a certain plateau in growth performance and feed utilization that can be used to estimate a minimum dietary level. In this

study, fish fed with 1 % and 3 % of agavin presented higher FBW, RWG, DWG than those fed with control diet (i.e., 0 % agavin) and no significant difference with those fed the 2% AGA diet. Similarly, in other fish species fed with increasing levels of fructans prebiotics found positive effects until a certain level of inclusion. For example, in the blunt snout bream *Megalobrama amblycephala* fingerlings with initial weight (IW) of 1.4 g fed with a blend of plant ingredients (29 % SBM) in a low FM diet (5 %) supplemented with six levels of FOS (0 % to 0.8 %) present better results of growth performance, survival and feed utilization with 0.4 % level at 56 days (Wu et al., 2013). Soleimani et al. (2012) in Caspian roach *Rutilus rutilus* fry (IW 0.7 g) fed with diets containing 13.3 % SBM supplemented with four levels of FOS (0 %, 1 %, 2 % and 3 %) reported that fish fed with the 2 % and 3 % FOS inclusion had higher final weight, SGR and FCR at 49 days. The addition of 0 %, 1 % and 2 % FOS in a commercial diet for the stellate sturgeon *Acipenser stellatus* juvenile (IW 30 g), fish fed with the 1 % inclusion after 77 days resulted in higher growth performance and PER compared to fish fed with 0 % or 2 % inclusion level (Akrami et al., 2013). In another study, evaluating xylooligosaccharide (XOS) supplementation in four inclusion levels (0 %, 0.05 %, 0.1 % and 0.2 %) for the allogynogenetic crucian carp *Carassius auratus gibelio* (IW 17 g) fed with casein-based diets resulted in higher final weight at the 0.2 % level (Xu et al., 2009). In the European sea bass *Dicentrarchus labrax* (IW 0.2 g) supplemented with six levels (0 % to 0.4 %) of mannan oligosaccharide (MOS) and fed with diets containing 19 % SBM, resulted in higher FBW, SGR and PER at the 0.1 % level (Salem et al., 2016).

In contrast, several studies have found no significant improvements in growth performance or feed utilization with increasing levels of the prebiotic in species such as the common carp (Eshaghzadeh et al., 2014), rainbow trout (Akrami et al., 2009), beluga *Huso huso* (Reza et al., 2009; Hoseinifar et al., 2011), European sea bass (Torecillas et al., 2011b), Asian seabass *Lates calcarifer* (Ali et al., 2017) and gilthead sea bream (Dimitroglou et al., 2010b; Guerreiro et al., 2015c). In addition, some studies have reported growth impairment at higher prebiotic supplementation levels. For example, in two independent experiments with beluga juveniles (IW ≈18 g) fed with FM-based diets and supplemented with increasing levels (i.e., 0 %, 1 %, 2 % and 3 %) of inulin and oligofructose, the authors reported that fish fed with 3 % prebiotic showed a reduced growth performance and feed utilization (Reza et al., 2009; Hoseinifar et al., 2011). Some authors have reported that higher levels of the prebiotics cause deleterious effects on the intestine. For example, in a study evaluating fructans in the Arctic charr (IW 100 g) fed with casein-based diets supplemented with 15 % inulin, the authors reported the loss of brush border tissue and less straight microvilli, as well as damage to the enterocytes linked to the accumulation of intracellular lamellar bodies in the distal intestine and suggested that high levels of the prebiotic cannot be well fermented by the intestinal microbiota (Olsen et al., 2001). However, high levels have produced no negative effects in some

species evaluated. For example, Refstie et al. (2006) and Bakke-McKellep et al. (2007) fed FM-based diets with 7.5 % inulin for 3 weeks to Atlantic salmon and found no changes in the intestinal integrity compared to fish fed the control diet without prebiotic. Nevertheless, low levels of inulin supplementation to the diet have caused adverse effects in the intestinal structure. For example, in the gilthead sea bream (IW 50 g) fed with a commercial diet supplemented with 0.8 % inulin and fed for 28 days, Cerezuela et al. (2013) did not find changes in the intestinal adsorptive area, but did find brush border disruption, increase of intraepithelial leucocytes, signs of intestinal inflammation and a decreased microbiota richness in fish fed the inulin diet. Ferrara et al. (2015) working with the sharpsnout seabream (IW 99 g) fed diets containing 40 % of SBM supplemented with 1 % inulin reported a worsening of the morphometric characteristics of the mucosal fold and enterocytes in the proximal and distal intestines compared to the control diet containing only SBM. Hoseinifar et al. (2011) highlighted the importance of assessing the optimum prebiotic level. In this respect, the present study sought to optimize the minimum level of agavin inclusion in the diet, which resulted in the maximum growth and feed efficiency. Fish fed with agavin in their diets grew significantly higher than fish fed the control diet and without significant differences among the three levels of agavin. Moreover, feed utilization and gut histology of fish fed with agavin did not result in negative effects compared to fish fed with the control diet. For the reasons mentioned above, the use of 1 % of agavin is suggested as the optimal minimum level in diets with 24 % of SBM inclusion in practical diets for totoaba juvenile.

Enhance growth performance observed in the present study when using our prebiotic might be attributed to the fact that agavin helped maintain intestinal function (e.g., gut integrity and digestive capacity) in fish fed with our alternative ingredients. This was confirmed by not finding significant differences in the score of the semi-quantitative scoring system between the agavin diets and control diet (see Table 18). The scoring system is used to evaluate histomorphological changes in the distal intestine associated with enteritis when SBM is included in the diet above certain levels (Urán et al., 2008a; Penn et al., 2011). Moreover, the morphometric measures of MF length, EH, SM and % MF with wider SM did not revealed differences among dietary treatments (see Table 17). The brush border height (BBH) of totoaba fed with diets containing 24 % SBM remained without changes when the diet was supplemented with 2 % of agavin compared with the control diet and was reflected in better growth performance (see Chapter 3). In a similar study with the same species fed with diets containing 21.8 % SBM for 119 days, the addition of 2 % GroBiotic®-A did not find differences in mucosal folds length in the proximal or distal intestine among treatments, however not improvement in growth performance was observed (González-Felix et al., 2018).

In the present study fish fed with 3% AGA diet resulted in a reduction of 12 % in the BBH compared to fish fed the control diet (2.40 vs 2.70 µm). On the other hand, fish fed the diet supplemented with 1 % of agavin presented a significantly lower number of mucosal folds compared with fish fed the 2% AGA diet (see Table 17). Although, these intestinal alterations could potential reduce growth performance or feed utilization (Ferrara et al., 2015; Bai et al, 2017; Fuentes-Quesada et al., 2018), it was noticed that fish fed with the diet containing 3 % of agavin resulted in 9 % more number of mucosal folds (47.8 vs 43.8) than fish fed with control diet and, fish fed with the diet containing 1 % of agavin the mucosal fold length was 12 % longer than fish fed with control diet. These intestinal changes or "adaptations" to the diet can potentially compensate the low values of the histomorphological measurements observed, since increasing the mucosal surface area, would improve the digestion process and absorption of dietary nutrients. In addition, light photomicrographs (see Fig. 26) revealed the presence of supranuclear vacuoles that can indicate an adequate digestion and absorption of nutrients by the enterocyte, which resulted in higher growth of fish fed diet with agavin. Evaluating fructans, Guerreiro et al. (2016c) fed gilthead sea bream (32 g) with diets containing 25 % SBM and supplemented with scFOS levels of 0 %, 0.1 %, 0.25 % and 0.5 % for 56 days and reported that the intestine integrity was unaffected and demonstrated that the prebiotic help maintain the intestinal structure with moderate SBM inclusion. In other prebiotics, the inclusion of MOS at levels of 0.15 % and 0.3 % in a commercial diet significantly increased the mucosal fold length in juvenile of rainbow trout (IW 38 g) compared to the higher inclusion level of 0.45 % and the control diet (Yilmaz et al., 2007). In the gilthead sea bream, Dimitroglou et al. (2010b) evaluated MOS levels of 0 %, 0.2 % and 0.4 % in FM-based diets and 0 % and 0.4 % in diets formulated with 31 % SBM and found improvements in the anterior and distal intestine related with higher absorptive surface and microvilli density and length in fish fed diets supplemented with the prebiotic compared to the control diets.

The capacity to digest nutrients from a specific diet is largely determined by the fish digestive enzyme activity (Furne et al. 2005). In this study, fish fed with 2 % of agavin resulted in higher activities for pepsin (acid protease), trypsin and total alkaline proteases but was not reflected in growth improvements in fish fed the 2% AGA diet compared with the other agavin levels and the control diet. The activity of chymotrypsin, lipase, amylase and L-aminopeptidase a brush border enzyme were unaffected by the level of prebiotic compared with the control diet. In Atlantic salmon fed with FM-based diets and 7.5 % inulin, Refstie et al., (2006) found no differences in trypsin, amylase and L-aminopeptidase activities. In another study, Anguiano et al., (2013) fed red drum and hybrid striped bass diets with 37.5 % SBM supplemented with 1 % of Grobiotic ®, FOS, TOS and MOS for 56 days and did not find differences in activity for trypsin, chymotrypsin, aminopeptidase, α-amylase, lipase, and both acid and alkaline phosphatase. In contrast, the use of increasing dietary levels of FOS, MOS and XOS improve the intestinal proteases, lipases and

amylases activities in the blunt snout bream (Wu et al., 2013), Caspian roach (Soleimani et al., 2012) and crucian carp (Xu et al., 2009), respectively, and was correlated with higher growth. It is possible that the feeding habit and digestive physiology between the blunt snout bream, Caspian roach and crucian carp (i.e., herbivorous, Cyprinidae family) with the Atlantic salmon, red drum and totoaba (i.e., carnivorous) can explain the differences in digestive enzyme activity observed among studies. Additionally, in herbivorous fish the intestinal microbiota has been associated with the production of exogenous enzymes in the host that helps the digestion of the nutrients (Merrifield and Rhodiles, 2015). Interestingly, the use of different type of fructans, inulin and scFOS, in common carp (*Cyprinus carpio*) with similar experimental conditions (i.e., fish of 0.6 g) fed with FM-based diets containing 13 % SBM and supplemented with the prebiotics at levels of 0 %, 0.5 % and 1 % for 35 days resulted in no significant differences in proteases, lipases and amylase activities when inulin was the prebiotic (Eshaghzadeh et al., 2015) and significantly higher amylase and lipase activities with 1 % scFOS inclusion in the diets (Hoseinifar et al., 2016b).

These ambiguous results among the different published studies evaluating prebiotic effects on growth performance, feed utilization, digestive capacity and intestine histology can be partially explained by differences in type, source and level of inclusion of the prebiotic, fish feeding habit (i.e., herbivorous, omnivorous, carnivorous) and fish weight and age (i.e., larvae, juvenile), rearing conditions (i.e., temperature, salinity), diet formulation (i.e., fishmeal, casein-based, alternative protein-based or commercial diet), processing technology (i.e., extruded, pelleted), sensitivity to vegetal proteins and length of the bioassays. All these parameters can affect the results obtained in each study and makes difficult comparing among them to draw general conclusions. Therefore, the positive results reported for some species cannot be expected in other fish species or even in the same species. For each species, it is necessary to do species-specific research to generate accurate information regarding the effects of each prebiotic and the optimal level to use.

The lack differences in nutrient digestibility among diets supplemented with agavin and the control diet might be related to the lack of differences in digestive enzyme activity and intestinal integrity found in this study. The supplementation of agavin in the diets formulated with SBM resulted in similar ADC values compared to the control diet (i.e., PBM and FM). Similarly, Burr et al. (2008), working with the red drum (IW 500 g) demonstrated that prebiotic supplementation (1 % of GroBiotic ®-A, MOS, GOS and inulin) could enhance nutrient digestibility in diets formulated with 35.5 % SBM. In general terms, Anguiano et al., (2013) concluded the benefits of dietary nutrient utilization was more related to positive changes in intestine integrity and health than with effects on digestive enzyme activity when dietary prebiotics were supplemented in the diet.

The supplementation of agavin in the diets for totoaba reduced the FPDR values compared to the control diet, and did not result in significant difference between the three levels evaluated. The use of agavin allows reducing the FPDR by 23.6 % without affecting growth performance, feed utilization and intestinal health. This means agavin supplementation in diets containing 24 % SBM produce 1 kg of farmed totoaba protein with 0.55 kg FM protein compared with the control diet that required 0.72 kg FM protein. Additionally, the use of agavin reduce the economic conversion ratio from $0.81 USD to $0.69 USD. This represents a saving of 13.5 % in the cost of protein ingredients to produce 1 kg of totoaba. Future investigations can be performed to evaluate whether 1 % of agavin allows the inclusion of higher levels of SBM in the diet or other alternative vegetable proteins with lower cost. In addition, research is warranted evaluating agavin in low FM diets formulate with PBM or other rendered meals.

In conclusion, fish fed with agavin supplementation in their diets, with inclusion levels from 1 % to 3 % in low fishmeal diets containing poultry by-product meal and soybean meal, resulted in higher growth performance and but with similar feed utilization and nutrient digestibility compared with the control diet without the prebiotic. Additionally, the inclusion of agavin in diets formulated with SBM prevented any histological alterations in the distal intestine associated with enteritis caused by the SBM. The use of agavin contributes to reduce the fishmeal protein dependency and allows producing 1 kg of farmed totoaba protein with a lower cost than the control diet, improving the sustainability of farming this species.

Chapter 5. The effects of dietary glutamine on growth performance, feed utilization, gut integrity and digestive capacity in *Totoaba macdonaldi* juveniles fed with low fishmeal diets

5.1 Introduction

The limited supply of fishmeal (FM) associated with a constant increase in demand as the aquaculture industry grows, make diets for fish and shrimp, that depend on this natural resource, more expensive every year especially for carnivorous marine fish. Thus, feed producers have set to replace fishmeal using alternative proteins of animal and vegetable origin. Poultry by-product meal (PBM) and soybean meal (SBM) are some of the most promising ingredients due to their high availability worldwide at a lower cost compared to FM with constant amino acid profile and acceptable digestibility. Nevertheless, it has been reported that inclusion of medium or high SBM levels in fish diets can cause negative effects on growth and intestinal health in farmed marine species (Krogdahl et al., 2003; Urán et al., 2008b; Gu et al., 2016; Hedrera et al., 2013). For example, in totoaba the increase of SBM inclusion in the diet caused histopathological changes in the distal intestine, commonly associated with soybean meal-induced enteritis (SBMIE), which have been correlated with reduced growth performance, lower feed utilization, and considerable intestinal damage (Fuentes-Quesada et al., 2018).

The continued research to improve growth performance and gut health of fish fed high inclusion levels of plant ingredients in their diet has promoted the evaluation and utilization of certain nutrients, supplements or compounds with specific biological function that can aid in protecting the intestine and reduce the development of enteropathies. Among these compounds, the amino acid, glutamine, is considered a functional nutrient used to improve intestinal health (Rhoads and Wu, 2009; Lan et al., 2015; Wang et al., 2015). In humans, glutamine has been shown to modulate the intestinal mucosa homeostasis, helping to reduce inflammatory bowel disease (Lan et al., 2015), and to prevent intestinal atrophy and even enhanced growth in animals (e.g., weanling pigs) with intestinal damage and dysfunction (Wu et al., 1996). In vertebrates, it is the largest source of energy for the cells in rapid proliferation of the immune system, as well as enterocytes (Burrin and Stoll, 2009; Li et al., 2009; Pohlenz et al., 2012a), prevents the apoptosis of intestinal cells and is necessary for the stabilization and formation of a tight junction in the enterocytes (Rhoads and Wu, 2008; Xie et al., 2016; Liu et al., 2018). The incorporation of glutamine in fish diets with SBM or purified antinutritional factors (ANFs) has been shown to have a protective effect on the

intestine integrity and functionality with a reduction of the inflammatory process (Cheng et al., 2011, 2012; Jiang, Hu et al., 2015; Gu et al., 2017; Liu et al., 2018).

Supplementation of glutamine is now considered a nutritional strategy to minimize the negative effects of the utilization of plant-based diets, since it is a nutrient that is used directly by the enterocytes to help reduce enteropathies. Glutamine has also been shown to alter the intestinal microbiota in fish, but further investigations are needed to elucidate the possible effects on fish (Gu et al., 2017; Liu et al., 2018). In addition, prebiotics such as inulin and agavin have been used as dietary functional nutrients to selectively modulate the microbiota to exert beneficial effects on intestinal environment with concomitant positive effects on the enterocytes. Research with prebiotics in gilthead sea bream (Dimitroglou et al, 2010b), sharpsnout sea bream (Piccolo et al., 2011), European sea bass (Guerreiro et al., 2015a), white sea bream (Guerreiro et al., 2017), turbot (Bai et al., 2017) and totoaba (see Chapter 3) reported positive effects of fish when supplemented in their diets. The simultaneous supplementation of glutamine and a prebiotic with possible synergetic effects has not been evaluated in finfish. Therefore, the objective of this study was to evaluate the effects of dietary glutamine, agavin or a combination of both on growth performance, feed efficiency, digestive capacity, and distal intestine integrity in *Totoaba macdonaldi* juvenile fed with low fishmeal diets.

5.2 Materials and methods

5.2.1 Diet formulation

Four experimental diets were formulated to be isoproteic (501 g crude protein (CP) kg^{-1} diet) and isolipidic (116 g crude lipid (CL) kg^{-1} diet) based on adequate nutritional requirements of totoaba (Perez-Velazquez et al., 2016; Rueda-López et al., 2011), with a DHA/EPA ratio of 1.5 and DHA+EPA >1.2 % of diet (NRC, 2011). The four diet were formulated with poultry by-product meal (PBM, 68 % CP, 14 % CL, pet food grade, National Renderers Association, USA) and fishmeal (FM, 69 % CP, 6 % CL, Maz Industrial SA de CV, Mazatlán Sinaloa, México) in a 2:1 ratio according to Badillo et al. (2014). Soybean meal (SBM, 48 % CP, 6 % CL, Alimentos COLPAC, Sonora, México) was included at 240 g kg^{-1} diet in all diets. The basal diet, termed SBM, did not contain any functional nutrients and was used as a control diet. Three experimental diets were then formulated based on the basal diet but supplemented with 15 g kg^{-1} of glutamine (GLN, L-Glutamine, General Nutrition Corporation, Pittsburgh, USA), 10 g kg^{-1} of the prebiotic agavin (AGA,

donated by Instituto de Biotecnología, UNAM, Cuernavaca, México), or a combination of 15 g kg^{-1} of glutamine with 10 g kg^{-1} of agavin (GLN+AGA). All diets included 1 g kg^{-1} krill oil (Biogrow, ProAqua, México) as an attractant and 10 g kg^{-1} taurine (Insumos Nubiot, SA de CV, México) to compensate SBM low taurine levels (Table 21). Methionine (EVONIK, Degussa, México) was supplemented to meet totoaba requirements (Madrid et al., 2019). Lastly, glycine (Sigma Cat. 50046, Belgium) was added to adjust the nitrogen level in the diet so as to make all diets isonitrogenous (Cheng et al., 2012). Diets were manufactured in a mixer (Robot-Coupe, model R10, USA), pelleted at 7 mm in a meat grinder (Tor-Rey, Model M32-5, Mexico) and dried at 60 °C in a forced air oven for 24 h.

5.2.2 Experimental design, animals and facilities

Totoaba juveniles, obtained from the Centro de Reproducción de Especies Marinas del Estado de Sonora (CREMES) in Kino bay, Mexico, were transported to the facilities of the Marine Fish Culture Laboratory, at the Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE) in Ensenada, B.C., México. Five fish (167.7 ± 3.9 g) were randomly distributed into twelve 200-L cylindrical fiberglass tanks in a closed recirculating seawater system equipped with biofilter (Model BBF XF, Patent 5232586, AST, USA), heat pump (Delta Star DS-5 ½ hp, Aqualogic, USA) and UV sterilized (Model QL 25, Lifegard Aquatics, USA). All experimental treatments were evaluated in triplicate.

Water quality was monitored daily, with mean values for temperature equal to 23.8 ± 0.9 °C, dissolved oxygen equal to 6.1 ± 0.6 mg L^{-1} with an oxygen saturation up to 80 %, salinity equal to 35.3 ± 1.0 ‰, pH = 7.8 ± 0.2 and a water flow of 2.2 L min^{-1}. Every three days the total ammonia nitrogen, nitrite-nitrogen and nitrate-nitrogen levels were measured (Api Pharmaceutic Aquarium Kit) to keep values < 0.50 mg L^{-1}, < 0.75 mg L^{-1} and < 80 mg L^{-1}, respectively. Fish were kept under 12 h light: 12 h dark photoperiod schedule. Fish were hand-fed daily to apparent satiation at 08:00, 12:00 and 16:00 h for 56 days. Daily, all uneaten feed was removed within an hour of feeding and dry weighed and deducted from the quantity offered by hand.

5.2.3 Sampling

Growth response indexes and somatic indexes were calculated as previously described in section 2.2.3. Fish were harvest at the end of the experiment (i.e., day 56). For histological analysis, samples were processed using the protocol described in section 2.2.3.

Table 21. Formulation of the experimental diets. Dietary formulation is presented as g kg^{-1} on as fed basis and proximate composition in g kg^{-1} on a dry matter basis.

	Experimental diets			
Ingredients (g kg^{-1} DM)	**SBM**	**GLN**	**AGA**	**GLN+AGA**
Sardine meal (69 % CP)[a]	170.0	170.0	170.0	170.0
Poultry by-product meal (68 % CP)[b]	340.0	340.0	340.0	340.0
Soybean meal (48 % CP)[c]	240.0	240.0	240.0	240.0
Starch	45.0	45.0	35.0	35.0
Pregelatinized starch	100.0	100.0	100.0	100.0
Sardine oil[a]	20.0	20.0	20.0	20.0
Tuna head oil[a]	14.0	14.0	14.0	14.0
Rovimix for carnivorous fish[d]	30.0	30.0	30.0	30.0
Stay-C[d]	10.0	10.0	10.0	10.0
Taurine[e]	10.0	10.0	10.0	10.0
Methionine[f]	1.0	1.0	1.0	1.0
Attractant (krill oil)[g]	1.0	1.0	1.0	1.0
Sodium benzoate	2.5	2.5	2.5	2.5
Choline chloride	1.5	1.5	1.5	1.5
BHT	0.1	0.1	0.1	0.1
Glutamine[h]	0.0	15.0	0.0	15.0
Agavin[i]	0.0	0.0	10.0	10.0
Glycine[j]	15.0	0.0	15.0	0.0
Proximate composition (g kg^{-1} DM)				
Dry matter	981.3 ± 1.3	984.0 ± 1.4	986.2 ± 1.2	981.1 ± 0.9
Crude protein	499.7 ± 6.1	499.6 ± 9.4	504.5 ± 5.4	501.4 ± 6.1
Crude fat	120.8 ± 5.0	119.6 ± 0.2	112.6 ± 1.9	113.6 ± 3.0
Ash	116.5 ± 5.9	119.7 ± 0.8	117.5 ± 0.8	117.2 ± 2.5
NFE[k]	263.0 ± 4.7	261.1 ± 8.7	266.6 ± 5.1	267.8 ± 4.6

[a] Maz Industrial SA de CV, Mazatlán, Sinaloa, México.
[b] Pet food grade, National Renderers Association, USA.
[c] Alimentos COLPAC, Sonora, México.
[d] Rovimix; Stay-C, DSM, Guadalajara, México.
[e] Insumos NUBIOT SA de CV, México.
[f] Free aminoacids, EVONIK, Degussa, México.
[g] Biogrow, Proveedora de Insumos Acuícolas, SA de CV, Mazatlán, Sinaloa, México.
[h] L-Glutamine, General Nutrition Corporation, Pittsburgh, USA.
[I] Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, México.
[j] Sigma Cat. 50046, Belgium
[k] Nitrogen-free extract (NFE, %) = 100 - (% crude protein + % total lipid + % ash).

5.2.4 Analytical methods

Proximate analyses of the experimental diets and whole-body of fish were performed as previously described in section 2.2.4.

5.2.5 Distal intestine histology

The distal intestine samples for histology were process as previously described in the section 3.2.5.

5.2.6 Digestive enzyme activity

Digestive enzyme activity assays were performed following the protocols described in the section 2.2.6.

5.2.7 Statistical analyses

The assumptions of normality and homogeneity of variances were evaluated using the Shapiro-Wilks and Barlett test respectively (Zar, 2010). Prior to analysis, percentage data were arcsine transformed. Significant differences in performance indexes, somatic indexes, histological measures of the distal intestine, digestive enzyme activities, whole-body proximate composition, economic conversion ratio and fishmeal protein dependency ratio were analyzed by one-way ANOVA, followed by post-hoc Fisher's least significant difference rank test. The values of individual parameters and mean score of the semi-quantitative scoring system was analyzed by no parametric Kruskal-Wallis test, and the existing variation presented between all samples was determined with the pooled standard error (PSE). For all cases, statistical significance was set at $P<0.05$. Statistical analysis was performed using the software STATISTICA 8.0™ (StatSoft, Inc. USA).

5.3 Results

5.3.1 Growth performance, feed utilization, and somatic indexes

Significant improvements in all growth performance parameters and feed utilization values were found in totoaba fed diets with 1.5 % glutamine compared with fish fed with SBM or 1 % of agavin diets (Table 22). However, no significant synergetic effect was observed when fish were fed with diets containing both functional nutrients. PER and PPV were significantly higher in fish fed with the GLU diet compared to fish fed with other dietary treatments. Fish fed with GLN+AGA diet resulted in intermediate values among treatments and did not showed significant differences in growth performance and feed utilization with the other diets, except for the PER and PPV which resulted in significantly lower values compared to fish fed diet containing 1.5 % GLN. No mortality was observed during the experiment in any of the dietary treatments. No significant differences were observed in CF and FI among dietary treatments (Table 22).

Table 22. Growth performance and feed utilization of *Totoaba macdonaldi* fed diets supplemented with glutamine, agavin or a combination of both for 56 days. Different letters represent significantly differences values ($P<0.05$) within the same row.

	SBM	GLN	AGA	GLN+AGA	P value
Initial weight (g)	167.9 ± 5.5	167.5 ± 3.4	167.5 ± 3.6	167.8 ± 5.4	0.999
Final weight (g)	315.9 ± 10.0^{b}	345.4 ± 9.8^{a}	322.7 ± 13.5^{b}	329.4 ± 13.3^{ab}	0.015
TGC[1]	0.99 ± 0.01^{b}	1.15 ± 0.08^{a}	1.03 ± 0.06^{b}	1.07 ± 0.05^{ab}	0.008
WG[2] (g)	147.9 ± 4.7^{b}	177.9 ± 13.2^{a}	155.1 ± 12.1^{b}	161.6 ± 9.6^{ab}	0.007
RWG[3] (%)	88.1 ± 1.4^{b}	106.3 ± 10.0^{a}	92.6 ± 6.8^{b}	96.3 ± 4.9^{ab}	0.009
DWG[4] (g)	2.64 ± 0.08^{b}	3.18 ± 0.24^{a}	2.77 ± 0.22^{b}	2.89 ± 0.17^{ab}	0.008
FCR[5]	0.92 ± 0.02^{a}	0.84 ± 0.04^{b}	0.90 ± 0.04^{a}	0.90 ± 0.02^{ab}	0.014
PER[6]	2.17 ± 0.05^{b}	2.40 ± 0.12^{a}	2.21 ± 0.11^{b}	2.22 ± 0.05^{b}	0.014
PPV[7]	0.38 ± 0.01^{b}	0.43 ± 0.02^{a}	0.39 ± 0.02^{b}	0.39 ± 0.01^{b}	0.011
CF[7]	1.39 ± 0.03	1.43 ± 0.05	1.39 ± 0.04	1.37 ± 0.01	0.311
FI[8] (% day^{-1})	1.01 ± 0.04	1.03 ± 0.02	1.01 ± 0.06	1.04 ± 0.01	0.301

[1] TGC (Thermal Growth Coefficient) = [(final weight^{⅓} - initial weight^{⅓}) / (T°C x Days)] x 1000 (Jobling, 2003).
[2] Weight gain, g = final weight – initial weight
[3] Relative weight gain % = [(final weight – initial weight) / initial weight] x 100
[4] DWG (Daily weight gain) = final weight gain / experimental days
[5] FCR (Feed Conversion Ratio) = total feed consumed / wet weight gained.
[6] PER (Protein Efficiency Ratio) = weight gain / protein intake.
[7] PPV (Protein productive value) = protein retained / protein intake
[8] CF (Condition Factor) = final body weight x (body length)^{3} x 100 (Hardy and Barrows, 2002).
[9] FI, Feed Intake = FI (%/day) = 100 x (total amount of the feed consumed per fish / ((initial body weight + final body weight) / 2) / days).

Estimated somatic indexes HSI, VSI and ISI did not result in significant differences among dietary treatments at the end of the experiment (Table 23).

Table 23. Somatic indexes of *Totoaba macdonaldi* fed diets supplemented with glutamine, agavin or a combination of both for 56 days. Different letters represent significantly differences values (P<0.05) within the same row.

	SBM	GLN	AGA	GLN+AGA	P-value
HSI %[1]	1.23 ± 0.32	1.10 ± 0.21	0.96 ± 0.18	1.07 ± 0.35	0.229
VSI %[2]	3.40 ± 0.41	3.43 ± 0.33	3.20 ± 0.19	3.20 ± 0.42	0.351
ISI %[3]	0.47 ± 0.08	0.50 ± 0.05	0.47 ± 0.07	0.53 ± 0.06	0.264

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

5.3.2 Distal intestine morphology

The distal intestine of fish fed with 1.5 % glutamine and 1 % of agavin resulted in lower numbers of mucosal folds with wider sub-epithelial mucosa, except fish fed the GLN+AGA diet compared to fish fed the SBM diet (Table 24). Brush border height (BBH) was significantly higher in fish fed with diet supplemented with glutamine compared to fish fed the SBM diet, but did not present significant differences to fish fed the AGA and GLN+AGA diets. Although no significant differences were observed among dietary treatments, fish fed with glutamine present 11 % extra mucosal folds compared to fish fed the SBM diet (61.1 vs 54.5). Fish fed the SBM diet resulted in longer mucosal folds (15 % to 19 %) compared to fish fed the other dietary treatments.

Table 24. Morphometric measures of the distal intestine of *Totoaba macdonaldi* fed diets supplemented with glutamine, agavin or a combination of both for 56 days. Different letters represent significantly differences values (P<0.05) within the same row.

	SBM	GLN	AGA	GLN+AGA	P-value
MF number[1]	54.5 ± 6.3	61.1 ± 3.0	56.2 ± 1.2	56.5 ± 2.6	0.207
Length MF[2] (µm)	1153.0 ± 122.8	980.2 ± 136.1	951.7 ± 250.8	974.6 ± 105.7	0.383
SM[3] (µm)	25.0 ± 5.6	22.4 ± 3.7	21.7 ± 1.5	21.8 ± 3.0	0.628
EH[4] (µm)	16.8 ± 1.7	18.3 ± 0.4	19.2 ± 1.6	19.3 ± 2.0	0.254
% MF with wider SM[5]	29.5 ± 4.4^{a}	16.1 ± 7.8^{c}	20.9 ± 3.1^{bc}	24.4 ± 3.4^{ab}	0.005
BBH[6] (µm)	2.39 ± 0.19^{b}	2.93 ± 0.29^{a}	2.72 ± 0.21^{ab}	2.65 ± 0.16^{ab}	0.008

[1]MF number = mucosal fold number; [2]MF length = mucosal fold length; [3]EH = enterocyte height; [4]SM = sub-epithelial mucosa; [5]% MF with wider SM = percentage of mucosal folds with wider sub-epithelial mucosa; [6]BBH = brush border height.

At the end of the experiment the estimated parameters and mean score results of the semi-quantity scoring system did not result in any significant differences from the histological measurements of the distal intestine among dietary treatments (Table 25). Analysis of the histological images assessing goblet cells condition revealed that fish fed the different dietary treatments did not present signs of hyperplasia or

hypertrophy and the goblet cells were normally scattered within the mucosal folds and did show a reduction of supranuclear vacuoles in the basal area of the mucosal folds (Fig. 30).

Table 25. Individual and mean score the parameters evaluated to assess the degree of histological changes in the distal intestine of *Totoaba macdonaldi* fed diets supplemented with glutamine, agavin or a combination of both for 56 days. Different letters represent significantly differences values (P<0.05) within the same row.

	SBM	GLN	AGA	GLN+AGA	PSE	P-value
Mucosal folds	1.7	1.7	2.0	1.7	0.18	0.351
Supranuclear vacuoles	3.3	2.7	2.7	2.7	0.13	0.999
Goblet cells	2.7	2.7	2.3	2.3	0.19	0.999
Lamina propia	3.0	2.3	2.7	2.3	0.15	0.999
Sub-epithelial mucosa	2.7	2.3	2.3	2.0	0.14	0.391
Eosinophilic granulocytes	2.7	2.3	2.3	2.3	0.15	0.794
Mean score	2.7	2.3	2.4	2.2	0.11	0.794

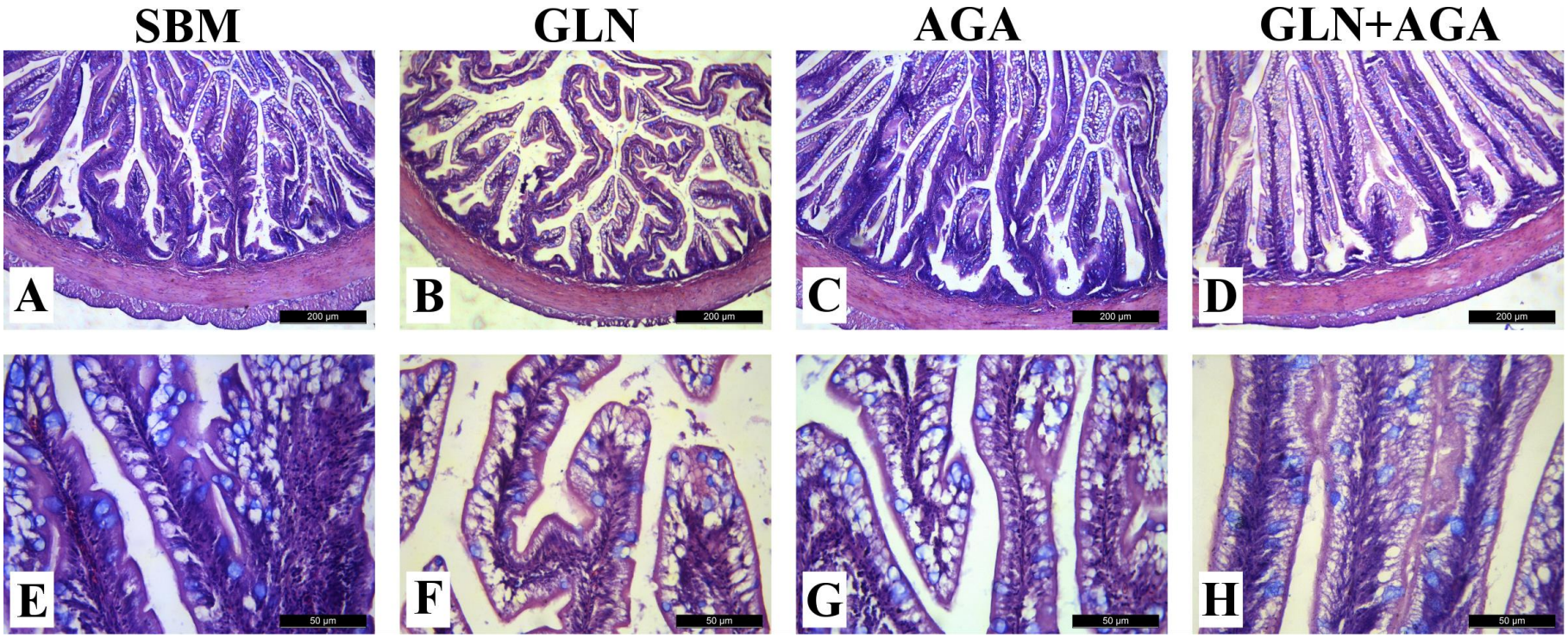

Figure 30. Light microscopy images of the morphological changes in distal intestine in *Totoaba macdonaldi* fed with basal diet (SBM, A and E), 1.5 % of glutamine (B and F), 1 % of agavin (C and G), and 1.5 % of glutamine in combination with 1 % of agavin (D and H) for 56 days. Figures from A to D bar = 200 µm, and from E to H bar = 50 µm.

5.3.3 Whole-body proximate composition

Fish fed with GLN and GLN+AGA diets resulted in significantly higher whole-body ash content compared to fish fed with SBM diets, while fish fed the AGA treatment resulted in intermediate ash content (Table 26). No significant differences were found among dietary treatments in moisture, crude protein, lipids or NFE of the fish whole-body composition.

Table 26. Whole-body proximate composition of *Totoaba macdonaldi* fed diets supplemented with glutamine, agavin or a combination of both for 56 days (mean ± SD, n=3).

	SBM	GLN	AGA	GLN+AGA	P value
Moisture	75.0 ± 0.3	75.2 ± 0.3	75.4 ± 0.3	75.0 ± 0.8	0.697
Crude Protein	17.7 ± 0.2	17.8 ± 0.2	17.4 ± 0.1	17.6 ± 0.3	0.280
Lipids	3.6 ± 0.2	3.1 ± 0.5	3.6 ± 0.2	3.6 ± 0.8	0.633
Ash	3.5 ± 0.1^{b}	3.7 ± 0.1^{a}	3.6 ± 0.0^{ab}	3.7 ± 0.1^{a}	0.047
NFE*	0.3	0.2	0.1	0.1	0.862

*Nitrogen-free extract (NFE, %) = 100 - (% crude protein + % total lipid + % ash).

5.3.4 Digestive enzyme activity

No significant difference in pepsin activity was found among dietary treatments (Fig. 31).

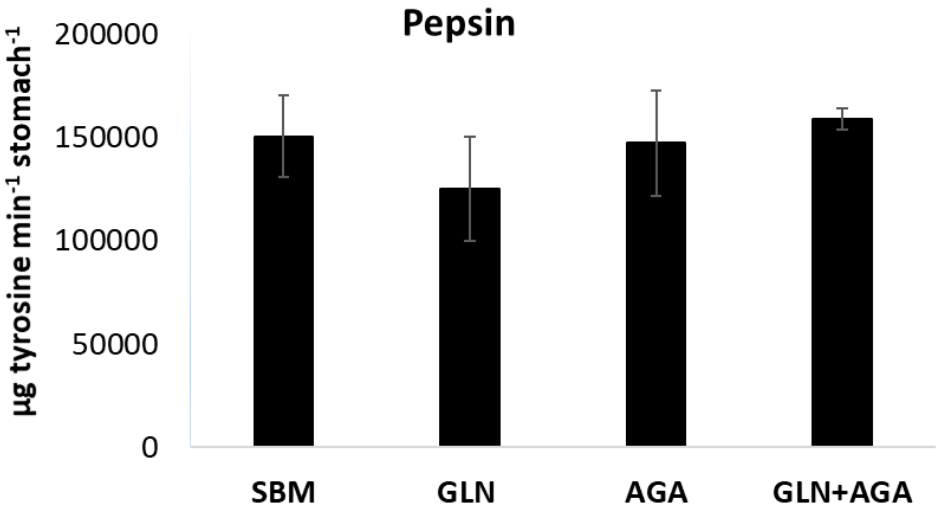

Figure 31. Acid protease (pepsin) activity in *Totoaba macdonaldi* fed diets supplemented with glutamine, agavin or a combination of both for 56 days.

Fish fed with the GLN diet resulted in significantly higher trypsin activity in the pyloric caeca and intestine compared to fish fed the SBM diet, but was not significantly higher compared to fish fed AGA and GLN+AGA diets (Fig 32). The same pattern was observed for the intestine total alkaline proteases activity. L-aminopeptidase activity in pyloric caeca and intestine was significantly lower in fish fed with the SBM diet compared to fish fed diets containing GLN or AGA, except LAP activity in the intestine of fish fed with GLN+AGA diet. No significant differences in activity for chymotrypsin, lipase and amylase activities were observed among dietary treatments.

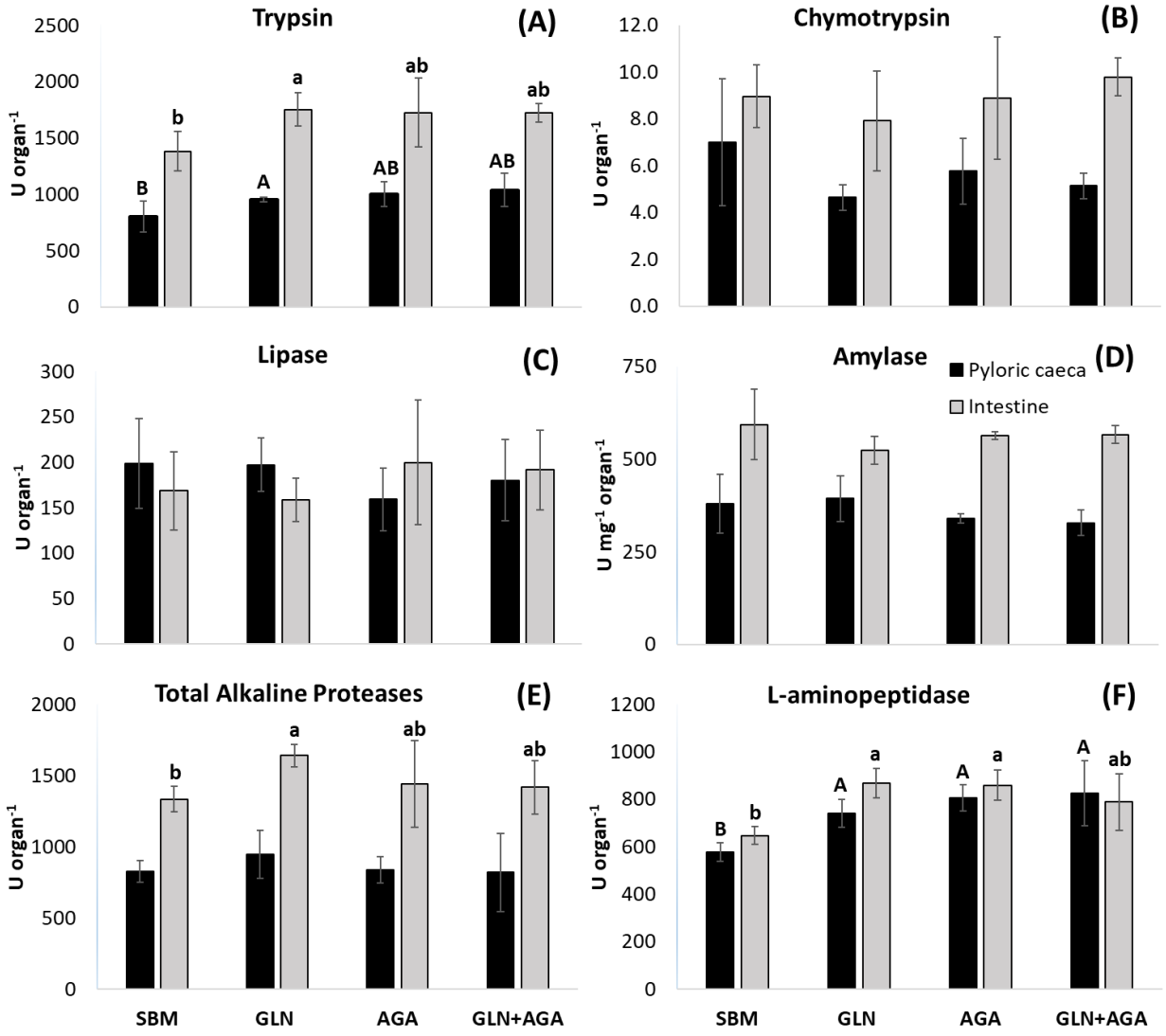

Figure 32. Total enzyme activity (U organ^{-1}) per pyloric caeca and intestine for trypsin (A), chymotrypsin (B), lipase (C), amylase (D), total alkaline proteases (E) and L-aminopeptidase (F) in *Totoaba macdonaldi* fed diets supplemented with glutamine, agavin or a combination of both for 56 days.

5.3.5 Fishmeal protein dependency (FPDR) ratio and economic conversion ratio (ECR)

Significantly lower FPDR in fish fed the 1.5 % of glutamine diet compared to fish fed the SBM, agavin and GLN+AGA diets (Fig. 33).

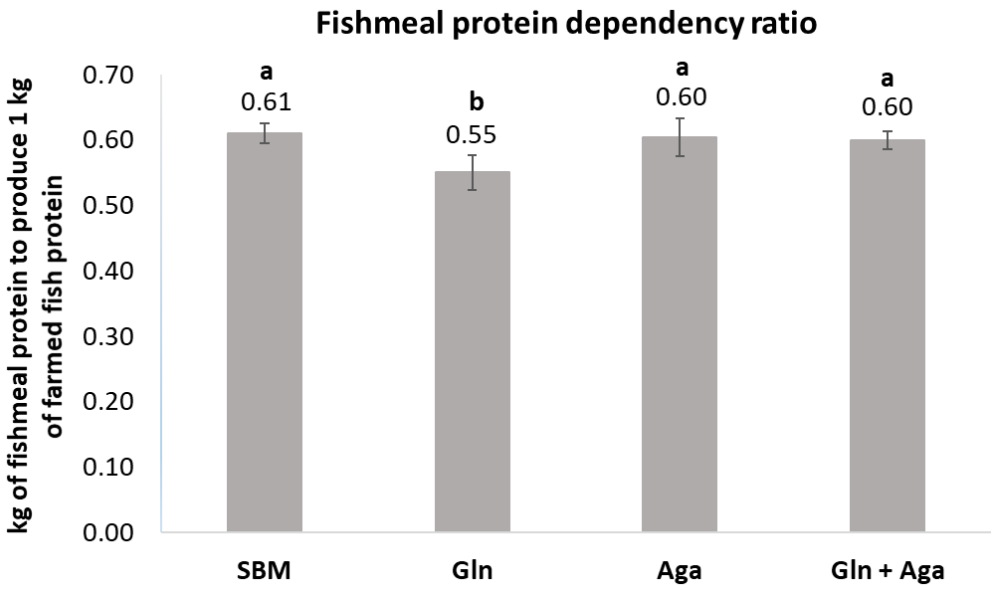

Figure 33. Fishmeal protein dependency ratio (FPDR) values for the experimental diets. Kilograms of fishmeal protein requires to produce 1 kg of farmed fish protein. Different lowercase letters represent significantly different values ($P<0.05$) among treatments.

The lowest ECR obtained was $0.70 USD needed to produce 1 kg of farmed totoaba in fish fed with the GLN diet. ECR from fish fed the SBM diet resulted in higher cost with an ECR equal to $0.78 USD, and a ECR of $0.76 USD for fish fed the AGA and GLN+AGA diets.

Table 27. Economic conversion ratio (ECR) of experimental diets.

	SBM	GLN	AGA	GLN+AGA
Feed conversion ratio (FCR)	0.92	0.84	0.90	0.90
Protein ingredients cost (USD$ kg^{-1})[z]	0.84	0.84	0.84	0.84
Economic conversion ratio (ECR)[y]	0.78	0.70	0.76	0.76

[z] Cost of fishmeal, poultry by-product meal and soybean meal

[y] ECR = FCR x Protein ingredients cost

5.4 Discussion

The use of functional nutrients as a nutritional strategy in fish fed with low fishmeal (FM) diets is a viable option to improve growth and feed utilization, by maintaining an adequate intestinal environment and structure, resulting in better digestion and absorption of nutrients when using alternative protein sources to fishmeal. To our knowledge, the present study is the first to evaluate the use of glutamine (Gln) and the combination of Gln plus agavin as a prebiotic, in totoaba juvenile fed with low FM diets.

Fish fed the diet supplemented with 1.5 % of Gln significantly improved growth performance and feed utilization compared with fish fed the SBM and AGA diets, but did not result in significant differences with fish fed the GLN+AGA diet, except for protein utilization (i.e., PER and PPV). These results are in line with several studies reporting positive effects on growth performance and feed utilization on cultured fish fed diets containing SBM, ranging from 20 % to 40 %, and supplemented with Gln. For example, in the hybrid sturgeon *Acipenser schrenckii* x *Huso dauricus* (initial weight (IW) 22.4 g) fed diets supplemented with 0.9 % to 1.5 % Gln for 56 days (Qiyou et al., 2011), the red drum *Sciaenops ocellatus* (IW 6.9 g) fed diets with 2 % Gln for 49 days (Cheng et al., 2011), hybrid strip bass *Morone chrysops* x *Morone saxatilis* (IW 4.1 g) fed diets with 1 % Gln for 56 days (Cheng et al., 2012) and the turbot *Scophthalmus maximus* (IW 7.6 g) fed diets with 1.5 % Gln for 56 days (Gu et al., 2017). Likewise, the supplementation of 1.2 % to 2 % Gln in FM-based diets enhanced the growth performance and feed utilization after 80 days in Jian carp (IW 7.8 g) (Yan and Qiu-Zhou, 2006). On the other hand, Zhang et al. (2017) fed turbot juveniles (IW 4.6 g) with 1 % and 2 % of Gln in diet containing 16 % of SBM and did not observed a positive effect on growth performance or feed utilization after 84 days. Similarly, Pereira et al. (2017) fed tilapia (IW 7.1 g) for 63 days with 2 % Gln in the diet containing 43 % of SBM and did not found significant differences in weight gain, feed intake, feed efficiency and protein efficiency, while supplementing with 1 % of Gln in the diet, resulted in a reduction in weight gain compared to the control diet. Similar results are reported by Pohlenz et al. (2012a) working with juvenile channel catfish (IW 6.1 g) fed diet containing from 0 % to 3 % GLN and did not find significant differences on growth performance after 70 days.

In vertebrates, Gln is considered a functional amino acid involved in multiple metabolic pathways and modulation of the expression of certain genes (i.e., antioxidative, antiinflamatory, intestinal barrier integrity, heat shock proteins,) in the intestine related to protein turnover and cell survival with beneficial effects on gut health in animals (Wu, 2009; Rhoads and Wu, 2009; Wu et al., 2015). Glutamine is highly catabolized (i.e., >60 %) by the intestinal mucosa, and is essential for the synthesis of purine and pyrimidine nucleotides in all cells, necessary for cell proliferation (i.e., skeletal muscle, enterocytes, immune cells)

(Wu et al., 1996; Burrin and Stoll, 2009; Li et al., 2009). Moreover, it has been documented that Gln regulates ion and nutrient transport, and triggers the cell signaling pathway of mTOR (mammalian target rapamycin) that plays a key role in regulation cell growth and metabolism in response to growth factors and nutritional status (Wullschleger et al., 2006; Bazer et al., 2012). Although little is known in teleost fish, in mammals, mTOR is a conserved serine/threonine kinase that regulates protein synthesis (i.e., initiate mRNA translation, ribosome synthesis, expression of metabolism-related genes, autophagy) in skeletal muscle and small intestine (Nakajo et al., 2005; Liao et al., 2008; Xi et al., 2012). The mTOR pathway is a "nutrient sensing system" stimulated by molecules that included specific amino acids (Bazer et al., 2012). Increasing concentrations of intracellular glutamine stimulates protein synthesis and inhibits proteolysis in muscle and enterocytes (Rhoads and Wu, 2009; Xi et al., 2011). For example, in piglets the supplementation of Gln is usually recommended since it helps the intestine meets its demand for Gln, and can spare the conversion of nutritionally essential amino acids for Gln synthesis by other tissues (i.e., skeletal muscle) (Wu et al., 2011). The latter authors reported that supplementation of Gln improved the efficiency of dietary amino acids utilization for protein synthesis. This may help explain the higher PER and PPV found in this study when Gln was supplanted in the diet resulting in better growth performance and feed utilization.

The benefits on intestinal integrity found in totoaba when supplementing Gln in the diet has been document in several studies evaluating its effect on cultured fish species. Increased mucosal fold length on the anterior, mid and distal sections of the intestine, higher enterocyte height and microvilli density have been reported in red drum (Cheng et al., 2011), hybrid striped bass (Cheng et al., 2012), channel catfish (Pohlenz et al., 2012a), hybrid sturgeon (Qiyou et al., 2011), Jian carp (Yan and Qiu-Zhou, 2006) and turbot (Gu et al., 2017). In accordance with these studies, morphometric measures made in the present study showed that totoaba supplemented with 1.5 % of Gln resulted in significantly higher brush border height (BBH) and lower percentage of mucosal folds with wider sub-epithelial mucosa (% MF with wider SM) compared to fish fed the SBM diet (Table 24). Although no significantly different among treatments, fish fed with Gln resulted in 11 % extra mucosal folds compared to fish fed the SBM diet (i.e., 61.1 vs 54.5). The latter morphological changes in the intestine can be associated with Gln supplementation in the diet since Gln exerts a powerful trophic effect on intestinal homeostasis (Dawood et al., 2017), and is the primary metabolic energy source, providing ATP through Krebs cycle for rapidly dividing cells during growth such as, enterocytes and immune cells (Burrin and Stoll, 2009; Kim and Kim, 2017). Rhoads and Wu, (2009) mentions Gln is a signal to intestinal cells to proliferate via mitogen-activated protein kinase (MAPK) pathway. Cell proliferation of the digestive tract occurs in the basal section of the mucosal folds and is followed by cellular migration to the top of the mucosal folds (Bakke-McKellep et al., 2007). Using

in vitro studies, Gln has been shown to significantly enhance cell growth proliferation and differentiation of enterocytes in the Jian Carp, increasing protein retention (Jiang et al., 2009). This may help explain the formation of mucosal folds observed in the present study. Furthermore, Gln has been shown to restore the function of Na^{+}-K^{+} ATPase, a trans-membrane protein essential for the adequate cell function after peroxide-induce challenge (Chen et al., 2009). In *in vivo* studies, the supplementation of 2 % Gln in the diet for channel catfish up-regulated enterocyte migration by 20 % (Pohlenz et al., 2012a). Likewise, the addition of 1.2 % Gln in the diets for Jian Carp, mitigate the negative effects of antinutritional factor, glycinin, found in SBM and helped partially restore the normal intestinal integrity, function and redox status, enhancing the growth performance and feed utilization (Jiang et al., 2015). Moreover, two independent experiments with turbot (IW ≈ 8 g) reported that fish fed with 1.5 % or 2 % Gln supplementation, alleviated soybean-induced enteropathy in diets containing 40 % SBM by enhancing intestinal integrity, increasing enterocyte tight junction proteins, and decreasing pro-inflammatory cytokine (TNF-α) gene expression, as well as altering intestinal microbiota (Gu et al., 2017; Liu et al., 2018). Therefore, the supplementation of Gln in totoaba diets can be used to protect the dynamic intestinal mucosa, as well as maintain or improve intestinal function to maximize nutrients digestion and absorption from alternative protein sources in low fishmeal diets.

The lack of differences on growth performance and feed utilization of fish fed with 1 % of agavin in their diet compared with fish fed the SBM diet, is in contrast to our previously reported results in Chapter 3, where the inclusion of 2 % agavin resulted in higher final weight and feed utilization compared to fish fed SBM diets. These differences can be partially explained by the larger initial size of the fish used in this last bioassay (IW 168 g), compared to the initial size of fish used in Chapter 3 (IW 61 g). Another possible explanation is the duration of the bioassay (i.e., 56 days), possibly not long enough to find significant differences in larger fish. Similar results were reported by González-Félix et al., (2018) in the same species when using fish with an initial weight of 215 g even when using longer feeding trials (i.e., 109 days). The authors did not find significant differences in growth performance and feed utilization when using 2 % of a commercial prebiotic based on yeast (GroBiotic®-A) in diet with 21 % SBM and fed for 109 days. These findings suggest that; 1) the smaller fish could be more susceptible to the level of SBM in the diet (i.e., 24 %) and, 2) that the larger fish might be capable of adapting to higher levels of SBM by modulating their intestinal morphology, microbiota or both to better counteract the negative effects of SBM in their diets. Interestingly, although not significantly different, fish fed with SBM diet resulted in a 15 % to 19 % longer mucosal folds compared to other treatments. This trend has been observed in the fish fed diets containing SBM as reported in Chapters 3 and 4.

The morphological changes on intestinal structure observed in the present study can be a fish "adaptation" to the diet by increasing the surface area of the intestine to enhance the digestive and absorption process. The scoring system to evaluated the degree of changes of intestinal mucosa related to enteritis did not reveal significant differences among the treatments. In vertebrates, the presence of certain secondary metabolites or some phytochemicals of plants have been shown to result in beneficial effects on the health of the host when they are supplied in low doses in the diet (Liu et al., 2003; Virgili and Marino, 2008; Chakraborty and Hancz, 2011; Chakraborty et al. al., 2014). In particular, although soy saponins from SBM have been suggested as responsible for inducing enteritis in Atlantic salmon (Krogdahl et al., 2015), there is evidence that the presence of saponins from other plants at low concentrations can enhance growth performance and feed utilization in fish. For example, Nile tilapia *Oreochromis niloticus* (IW 24 g) fed with six increasing levels of dietary ginseng herb from 0 to 250 mg kg^{-1} significantly improve growth performance, feed utilization and haematological indexes at week 17 (Goda, 2008). The authors suggest the better fish performance and feed utilization can be related to the saponins from ginseng herb (ginsenosides or panaxosides) that stimulate the immune system and inhibits the colonization of pathogenic bacteria (Goda, 2008). In a similar study, saponins derived from *Quillaja saponaria* increased growth in Nile tilapia (IW 1 g) when the diet was supplemented with 150 and 300 mg kg^{-1} and fed for 14 weeks. Fish fed 150 mg kg^{-1} grew more during the first 3 weeks, but at the end of the experiment, fish fed with 300 mg kg^{-1} resulted in higher weight gain and feed utilization. The authors suggested that the lower growth at the beginning of the experiment in the treatment with 300 mg kg^{-1} saponins could be explained by excessive mucosal damage, an assumption that was not analyzed by histology, and that the improvement at the end of the experiment is probably related to an adaptation of the fish to the saponin level through time (Francis et al., 2001b). Nonetheless, these hypotheses require further investigations evaluating several intervals of time and ANFs (i.e., saponins, phytosterols, oligosaccharides) levels assessing totoaba tolerance with respect to initial size and possibly elucidate the physiological mechanisms that are involved in the potential higher tolerance to SBM in the larger fish.

Similarly, in two separate bioassays using the same experimental conditions with different initial fish size (27 vs 283 g) with the European sea bass fed with FM-based diets containing low or high levels of soy saponins (0.1 % and 0.2 %; equivalent to the level found in 20 % and 40 % SBM inclusion), phytosterols (0.5 % and 1.0 %) and a combination of both soy saponins plus phytosterols (Couto et al., 2014; 2015). In fish with lower initial weight (27 g), histology analysis and the scoring system performed at 15 days found severe inflammatory changes in the intestine in higher levels of saponins, phytosterols or in the combination treatment, and tended to decrease in fish fed diets with lower inclusion levels. Interestingly at 59 days, no significant differences among treatments growth performance and feed utilization or the

scoring system used to evaluate intestinal enteritis. Although, the authors suggested that the observed results were due the high variability within groups, since the histological analysis shows that some fish were more susceptible to the ANFs and others were unaffected, and concluded that the ANFs seemed to cause gastrointestinal issues that may affect cultured fish under stressful conditions (Couto et al., 2014). Meanwhile, in European seabass fish with larger initial weight (283 g) showed high tolerance to dietary saponins and phytosterol, since histological analysis and scoring system were unaffected by the ANFs levels tested either after 15 or 59 days of the feeding trial and was reflected in similar growth performance among treatments at the end of the experiment (Couto et al., 2015). Nonetheless, the authors found a negative trend in feed efficiency, PER, and some histological alterations of the intestine and recommended the evaluation of the effects in longer bioassays that could potentially worsened the effects. Both studies demonstrated that fish with lower initial weight are more susceptible to the ANFs levels, similar to the results in growth and intestinal integrity found in Chapter 3 compared with the present feeding trial.

Taking all these into consideration, in feeding trials with larger fish and using growth as response variables, the use of immunological challenges, drastic environmental changes or a dietary stressor could be useful tools to evaluate potential benefits of supplementing diets with functional nutrients, in particular in diets with GLN+AGA, that in the present study resulted in intermediate benefits but could potentially be a functional diet when fish are exposed to stressful challenges. Furthermore, I hypothesized that in larger fish with higher initial size, higher levels of the prebiotic or functional nutrient should be more beneficial, but this assumption has to be evaluated in further investigations. On the other hand, it has been suggested that glycine added to the experimental diets to adjust the nitrogen content of the diets (i.e., to make them isonitrogenous) and evaluating the effect of Gln, may mask the effects on growth and physiological parameters, as reported for tilapia fed diets containing 43 % SBM and supplemented with Gln and arginine (Pereira et al., 2017). This could explain the similar results on growth and feed utilization observed between AGA and SBM diets in the present study. However, adding glycine to the AGA diet to adjust the nitrogen content of the diet did not result in any additional benefit.

Interestingly, fish fed with GLN and GLN+AGA in their diets resulted in significantly higher ash content in the whole-body proximate composition compared with those fish fed the SBM diet. The higher ash content in fish fed with the GLN diet can be associated to the trophic effect of glutamine on intestinal functionality by increasing the absorptive area of the intestine and thus improving mineral absorption. Moreover, it has been reported that supplementation of 1.5 % glutamine in diets containing 40 % of SBM modulated the microbiota and significantly increased the relative abundance of *Lactobacillus* and *Bacillus* in juvenile turbot (IW 8 g, Gu et al., 2017) adding to the beneficial effects already reported of feeding fish with diets

supplemented with prebiotics. Nonetheless, the underlying mechanisms by which glutamine can improve the absorption of minerals in fish is still unknown and should be further investigated. In a study with rainbow trout (IW 150 g) fed with increasing levels of dietary inulin and FOS (i.e., 0, 0.5 and 1 %) evaluating the effect on mineral content (i.e., Ca, P, Mg, Fe in the whole body) found a significant positive linear effect on the Ca content in fish fed with prebiotics. In humans and rats, it is well documented that the use of prebiotics stimulates mineral absorption resulting in increased bone mineral content (Scholz-Ahrens et al., 2007; Roberfroid et al., 2010). Several mechanisms have been proposed to explain the improved mineral absorption when prebiotics are used in the diet. For example, Ohta et al., (1995) reported that an increased production of SCFAs and organic acids resulted in reduced luminal pH, enhancing the solubility of Ca^{+2} and Mg^{+2}, as well as promoting cell growth mediated by the products of bacterial fermentation, predominantly lactate and butyrate, which benefits the proliferation of enterocyte cells and functional enhancement of the absorptive area (Scholz-Ahrens et al., 2007). In addition, increased degradation of the mineral complex "phytic acid" by the modulated microbiota making minerals (i.e., Ca, Mg, Fe, Cu) more available for absorption has been reported (Lopez et al., 2000). These arguments help explain the higher ash content in fish fed with the GLN+AGA diet containing the prebiotic agavin, while the lower ash content of fish fed with the AGA diet might be explained by the fact that glutamine exerts a greater benefit on the intestinal functionality, since it is a nutrient used directly by the enterocytes and can also modulate the microbiota adding extra benefits.

Digestive enzymes activities are typically used to assess digestion capacity of the fish. In the present study, both intestinal and pyloric caeca activity of trypsin, L-aminopeptidase and total alkaline proteases of the intestine were higher in fish fed diets supplemented with the functional nutrient compared to fish fed the SBM diet. These results are in agreement with those found in the previous studies (i.e., Chapter 3 and 4) with fish fed the AGA diet and the possible reasons were extensively discussed in each chapter. Nonetheless, in the present study the use of Gln in the diet and to a lesser degree in the GLN+AGA diet the enhanced digestion capacity resulted in better growth and feed utilization. Similarly, in the Jian Carp fed diets supplemented with Gln a positive correlation was found with the activity of intestinal proteases and lipases (Yan and Qiu-Zhou, 2006). Similar results were reported by Qiyou et al. (2011) who found higher activity of total proteases, lipases and amylases in hybrid sturgeon fed diets with functional nutrients. In both studies, Gln supplementation was correlated with higher growth performance and feed utilization. Although we did not observe significant difference in digestive enzyme activity among fish fed diet supplemented with a functional nutrient (i.e., GLN, AGA, and GLN+AGA), the protein spare effect of Gln in the diet might help explain the differences in growth observed in the present study.

The supplementation of Gln in the diet significantly reduced the estimated FPDR value compared to the other treatments and represents a 10 % decrease in costs with an increase of growth performance and feed utilization. These results suggest that Gln supplementation in the diet for totoaba requires 0.55 kg of protein from FM to produce 1 kg of farmed totoaba protein compared to fish fed the SBM diet that required 0.61 kg. Using AGA or GLN+AGA diets resulted in 0.60 kg of protein from FM and are similar to the values obtained for the SBM diet. Likewise, Gln supplementation in the diet resulted in reduced economic conversion ratio (ECR) from $0.78 USD to $0.70 USD. This represents a savings of 9 % in the cost of protein ingredients to produce 1 kg of totoaba compared to the SBM treatment, using AGA or GLN+AGA diets resulted in a reduction of only 2.5 % of the ECR.

In conclusion, supplementing Gln in the diets for totoaba improve fish growth performance, feed efficiency, digestive capacity and distal intestine integrity compared to fish fed with SBM or agavin in their diets. Fish supplemented with the prebiotic agavin and the potentially synergetic diet with Gln + agavin, should be evaluated in a longer bioassay or challenged using a stressor at the end of the feeding trial to better detect potential benefits of this functional nutrients. Glutamine supplementation significantly reduced the fishmeal protein dependency ratio and reduced the cost to produce 1 kg of totoaba protein using low fishmeal diets.

Chapter 6. Conclusions and recommendations

6.1 Conclusions

- SBM mixture (i.e., 16 % of SBM in combination with 6 % SPC) inclusion levels higher than 22 % causes a reduction in growth performance and feed utilization (i.e., feed efficiency and protein utilization) in totoaba juveniles after 56 days of feeding.

- After 28 days of feeding, totoaba fed with medium (44 %) to high (64 %) levels of SBM in their diet develop the typical characteristics associated with the inflammation of the intestinal mucosa (i.e, enteritis) and the degree of intestinal damage tended to increase as the level of SBM increased in the diet.

- After 56 days of feeding, inclusion levels of 44 % and 64 % SBM in totoaba diets was correlated with tissue disruption in the distal intestine characterized in the reduction of the number of mucosal folds and length and enterocyte height. In addition, digestive enzyme activity was reduced as well as an atrophy of the intestinal mucosa resulting in a reduction of the somatic indexes.

- The results of this study demonstrate a state of intestinal atrophy in totoaba caused by the exposure of medium to high dietary SBM (i.e., 44 % and 64 %) inclusion levels in the diet during the both experimental periods evaluated (i.e., 28 and 56 days).

- The inclusion of SBM mix in the diet increased the infiltration of lipid vacuoles to the pancreas and medium to high inclusion levels (i.e., 44 % and 64 %) resulted in large accumulation of vacuoles replacing part of the pancreatic tissue at 56 days reducing the production of pancreatic digestive enzymes.

- A direct relationship was found between dietary SBM content in the diet and pancreatic acini with eosinophilic coloration suggesting higher zymogen production with increasing SBM in the diet.

- The inclusion of the prebiotic agavin at 2 % in the diet with low fishmeal content formulated with 24 % SBM and 34 % PBM improved growth performance and feed utilization in totoaba juveniles after 44 days of feeding.

- The supplementation of 2 % agavin in the diet ameliorates the onset and sings of enteritis (associated to antinutritional factors from SBM) by increasing the brush border height, reducing

the number of mucosal folds with wider sub-epithelial mucosa, maintaining the presence of supranuclear vacuoles, improving intestinal somatic indexes, and avoiding goblet cells hypertrophy and hyperplasia.

- The mitigation of the adverse effects of SBM on the intestinal epithelium can be associated to the production of SCFAs from agavin by the modulated microbiota and should be further investigated.

- Supplementation of 2 % agavin to diets containing SBM and low fishmeal reduces by 75% the "fishmeal protein" necessary to produce 1 kg of farmed fish protein from 2.34 kg to 0.59 kg.

- The use of 2 % agavin in low fishmeal diets containing SBM improves the economic conversion ratio from $0.79 to $0.73 USD, thus reducing the cost of producing 1 kg of farmed totoaba by 7.5%.

- Independently of the agavin (i.e., 1 %, 2 % and 3 %) inclusion level in low fishmeal diets containing poultry by-product meal and soybean meal resulted in higher growth performance, protected the intestinal mucosa against antinutritional factors from SBM, and did not worsened feed utilization in terms of nutrient digestibility compared to the control diet without SBM (i.e., only PBM and FM).

- The use of 1 % agavin in practical diets for totoaba containing 24 % SBM reduced by 24% the "fishmeal protein dependency" from 0.72 to 0.55 kg and resulting in savings of $0.12 USD per kg of farmed totoaba.

- The supplementation of 1.5 % glutamine to totoaba diets improved fish performance and feed utilization, digestive enzyme activity, ash content in the whole-body proximate composition, and ameliorated changes in the distal intestine associated with SBM inclusion in the diet.

- Fish with initial weight of 168 g and fed with diets containing 24 % of SBM did not resulted in significant differences in growth performance and feed utilization compared with those fed with 1 % agavin. In contrast, fish with lower initial weight (61 g) and fed the SBM diets were more sensitive to SBM inclusion levels and resulted in lower performance.

- The addition of 1.5 % glutamine reduced "fishmeal protein dependency" ratio by 10 % from 0.61 to 0.55 kg and allowed the production of 1 kg farmed totoaba at a lower cost decreasing the economic conversion ratio from $0.77 to $0.70 USD of the protein ingredients.

6.2 Recommendations

- Evaluate enterokinase activity in the intestine trying to correlate the damage in the epithelium with the lower digestive enzymatic activity observed. The enterokinase secreted by enterocytes into the intestinal lumen is responsible for the trypsinogen activation initiating the activation cascade of pancreatic digestive enzymes.

- Evaluate the effect of agavin, glutamine or a combination of both in diets with lower crude protein content or digestible protein level than the recommended protein and amino acid profile. In addition, evaluate reducing the inclusion level of fishmeal in the diets to force the fish to optimize the utilization of alternative ingredients maintaining adequate growth performance and decreasing diet cost.

- Evaluate the activity of digestive enzymes in fasting and postpandrial (i.e., 4-8 h after feeding) to compare if there is a different response in enzyme activity when animals have been fed. The latter to determine an optimal sampling time and avoid a masking effect of sampling time.

- Perform morphometric analysis in the anterior, middle and distal intestine, as well as the pyloric caeca to obtain more information related to specific changes in each region. In the case of totoaba as a new emerging aquaculture species, this information is unknown and is necessary to documented possible alternations in gut integrity from using alternatives ingredients and to further evaluate the possible benefits of functional nutrients in other regions of the digestive tract.

- Use more appropriate tools such as image analysis programs to obtain more accurate measurements on the increase or reduction of the absorption surface (i.e., area) of the intestine.

- Perform an experiment with intermediate to high levels of soybean inclusion (i.e., > 24 % SBM) with different initial weights to determine if animals initial size is an important factor of the effects of SBM inclusion in the diets.

- Increase the duration of the experiments as much as possible to unequivocally detect a significant response in the variables being assessed in particular, growth performance, feed utilization, and intestinal integrity.

- When using totoaba larger than 150 g, one should use feeding trials that result a minimum 200 % weight increase to ascertain the effects being evaluated (i.e., 12 or more weeks).

- Characterize the intestinal microbiota in experiments with functional nutrients to identify the changes in populations of bacteria present in optimal conditions of growth, feed utilization and intestinal health, that allow elucidating how the presence of one or several bacteria and their metabolites might benefit the host.

- Based on the literature with terrestrial animals when evaluating the effects of prebiotics in fish, one should quantify the SCFAs produced by the modulated microbiota in the intestine and correlate this measures with growth, health and intestinal integrity measurements.

- In addition, when evaluating the supplementation of prebiotics to practical diets, one should measure as response variable the intermediary metabolism in the liver (i.e., enzymatic activity or specific genes) of lipid and carbohydrate metabolism to elucidate the underling mechanism (i.e., up or down-regulation of metabolic routes) and correlate with SCFAs production through microbiota modulation and the benefits in growth performance and feed utilization.

- Measurements of intestinal pH or the analysis of minerals content (i.e., Ca, P, Zn, Mg) of the whole body of the fish supplemented with prebiotics or glutamine can be used to elucidate if the changes in the microbiota and intestinal environment has a positive effect on mineral absorption.

- Sampling for histology of the distal intestine should be done with extreme care to avoid mechanical damage to the tissue that is not related to the experimental treatment and thus avoid masking the effect. In addition, in animals larger than 300 g, the histology of the intestine could be complicated due to the fact that in some experimental treatments, the intestine turns flaccid and the larger size of the intestine makes it difficult to place the sample in the cassette in the right position to perform the transversal sections. Additional better protocols for tissue fixation and preservation can help in this issue.

- Given the degree of assessment with new and current techniques on should evaluate the functionality of the intestine by making measurements of the enzyme activity of alkaline phosphatase (AKP), Na^+/K^+-ATPase, γ-glutamyl transpeptidase (γ-GT) and creatine kinase (CK); in liver alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) can be used to evaluated liver damage.

- It is important to measure the “redox status” by assessing the activity of glucose-6-phosphate dehydrogenase (G6PDH), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR),

glutathione peroxidase (GPX) which protect cells and other enzymes from oxidative damage (Jiang, Hu et al., 2015; Coutinho et al., 2016).

- With the current development of new techniques and the faster and cheaper molecular techniques one should quantify the genes related with the overall growth performance and health status of the fish. For example, in terms of intestinal inflammation: immunoglobulin M heavy chain (IgM), cluster of differentiation 8 (CD8), T-cell receptor beta (TCRβ), interleukin 1 beta (IL-1β), interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), interleukin 22 (IL-22), major histocompatibility complex I (MHC-I), major histocompatibility complex II (MHC-II), caspase 3 (Casp3).

- In terms of intestinal function and integrity: $Na^{+}K$-ATPase, proliferating cell nuclear antigen (PCNA), fatty acid binding protein (intestinal; FABP2), intestinal peptide transporter (PepT1); adenosine 5'-monophosphate-activated protein kinase (AMPK), mitogen-activated protein kinases (MAPKs), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF)-I, epidermal growth factor (EGF), mucin-2 (MUC-2), nuclear factor-kappa B (NF-κB), peroxisome proliferator-activated receptor-γ (PPAR-γ), heat shock proteins (HSP-70, HSP-25, HSP-72); tight junction: claudin-1, claudin-4, occludin, zonula occludens ((ZO)-1, ZO-2 and ZO-3) and permeability: aquaporins (Aqp-8ab, Aqp-10, Aqp-1a) (Couto et al., 2014; Hu et al., 2015; Gu etal., 2017; Kim and Kim, 2017; Liu et al., 2018).

- When evaluating the effects of glutamine supplementation in diets with alternative protein sources (i.e., soybean meal) one must assess the mammalian target gen know as rapamycin (mTor) which is known as important stimulator of the protein synthesis cascade and can be related with the better growth in fish.

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Annexes

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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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Histology

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.