Feeds, water quality, gut morphology and digestion in Nile tilapia (*Oreochromis niloticus*)

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Thesis

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I know that I know nothing

Socrates

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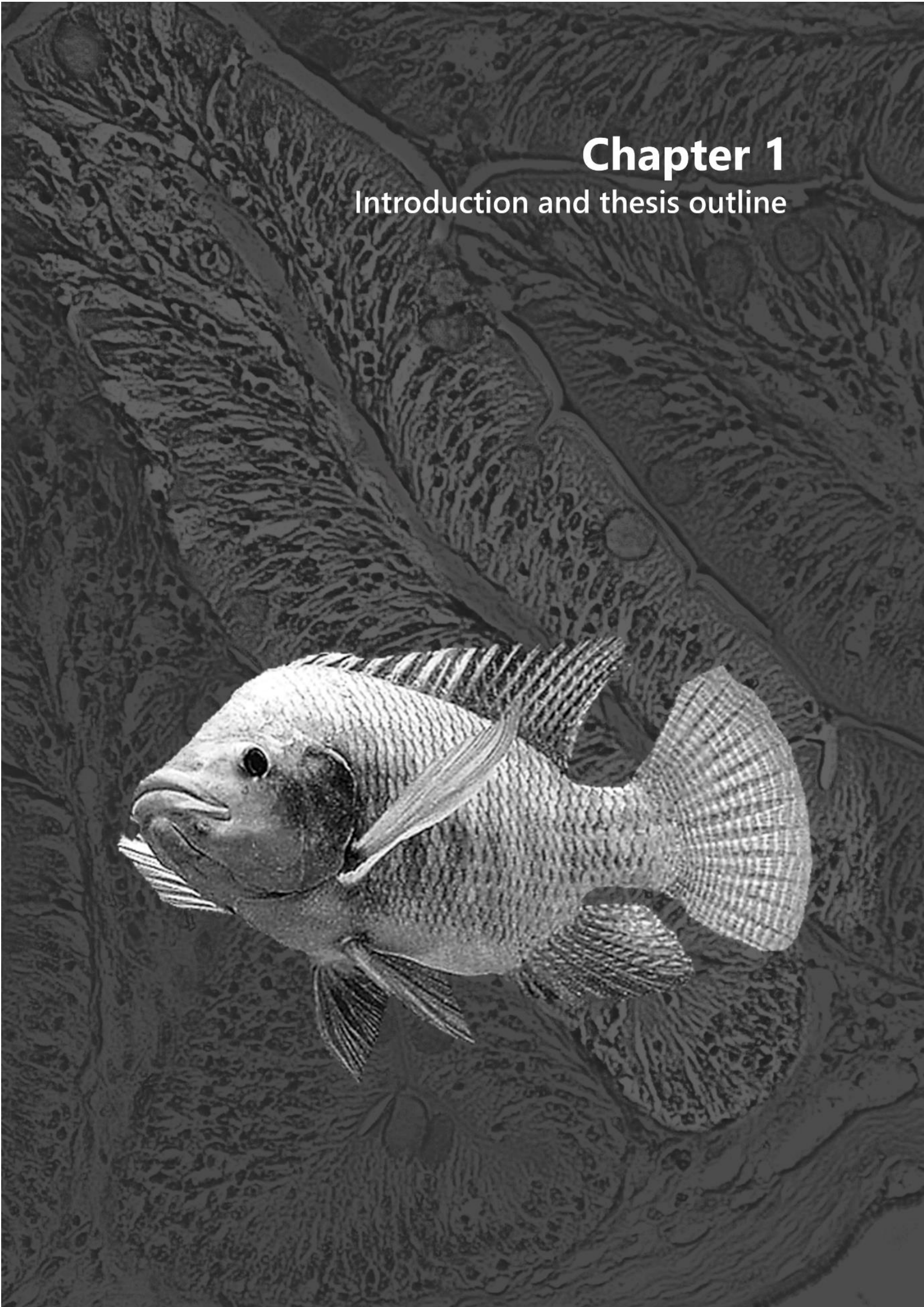
Chapter 1
Introduction and thesis outline



Aquaculture is currently the fastest-growing animal production sector in the world, expanding at an average annual rate of about 10.3% since 2010 (FAO, 2016). This extreme growth has raised concerns about environmental impacts such as water pollution, the spread of disease and the welfare and health of farmed fish. Although it can be minimized, fish are subjected to several handling processes and environmental changes during the production period that make stress unavoidable (Barton and Iwama, 1991). Stress can be caused by different factors, including malnutrition, adverse environmental conditions (low dissolved oxygen level, rapid change in salinity level, high NH_3 concentration), culture conditions and/or by physical, chemical and biological interference. This makes them sensitive for infections. As fish live in water, skin, gills and the intestine are the main entrance gates for pathogenic microorganisms (Ringø *et al.*, 2007). The intestine has long been recognized as one of the major routes of infection in fish (Sweetman *et al.*, 2010). However, before an infection can be established, pathogens must penetrate the primary barrier of the intestine (Ringø *et al.*, 2007). The primary defence mechanism of fish plays a vital role at the point of interaction of the environment and the physiology of the fish.

1.1 The intestinal paradox

The intestine is the site of food digestion and nutrient uptake; it regulates the ion and water balance and functions as a barrier against invading pathogens (Jutfelt *et al.*, 2007). The surface area of the intestine represents a selective permeable barrier that allows for absorption of nutrients, but excludes most toxic substances and pathogenic organism (Buddington *et al.*, 1997). The tight junction permeability is thereby regulated and modified according to the changing needs of the fish (Jutfelt, 2011). This is somewhat of a paradox: to protect the animal against invading pathogens (barrier function), the animal benefits from an intestine as impermeable as possible; however, to enable uptake of nutrients and exchange of ions and water, the intestine needs to be permeable to a certain extent.

Figure 1.1 shows a schematic overview of the interaction between the permeability/openness of the intestinal barrier and nutrient digestion. The nutrient digestion increases from normal to optimal depending on the permeability/openness of the intestinal barrier. However, if the tight junctions are too close or too open/permeable, nutrient digestion may also decrease. A too open barrier might increase the risk for pathogens, and antigens entering the circulation will hamper nutrient uptake. The ability of the protective barrier to alter the nutrient uptake would allow fish to adapt to changes in the diet or in the environmental conditions.

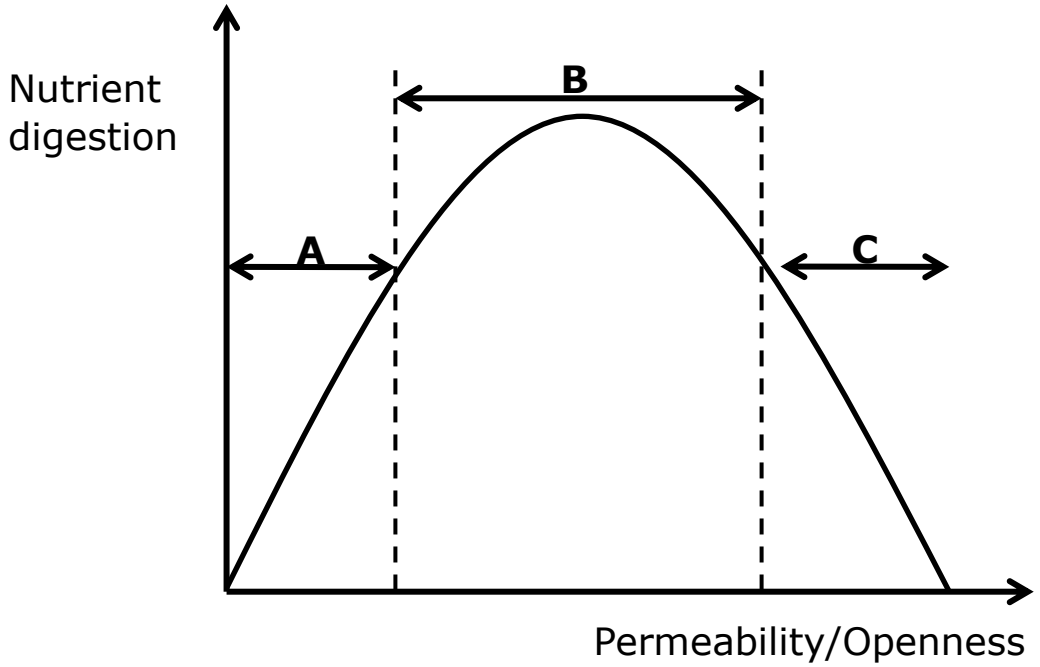


Figure 1.1 The schematic overview of the interaction between permeability/openness of the intestine and the nutrient digestion. Zone A shows that the permeability/openness is too close, which might hamper the nutrient uptake. Zone B shows that the variability of permeability/openness of the intestinal barrier with respect to nutrient digestion. Zone C shows that the increase in permeability/openness which leads to a decrease in nutrient digestion. This might be caused by the inflammation response of the intestine.

1.1.1 The barrier function of the intestine

The intestinal epithelial barrier can be subdivided into extrinsic, intrinsic and immunological parts (Jutfelt, 2011). Figure 1.2 shows a schematic overview of the three parts of the intestinal barrier. The first barrier that pathogens and toxins encounter is the extrinsic barrier. The extrinsic part consists of a mucus layer with antibacterial compounds such as lysozymes, complement factors and antibodies (van der Marel *et al.*, 2014). Mucus, a viscous fluid containing mucin glycoproteins, are secreted by goblet cells. The continuous flow of mucus from the epithelium into the lumen physically removes harmful agents from the epithelium as well as protects the epithelium from the mechanical wear (Fudge *et al.*, 2011). The intrinsic part is the major regulator of the intestinal permeability (Niklasson *et al.*, 2011). It starts from the luminal side, e.g., the epithelial monolayer with the tight junctional complex that creates the primary physical barrier between the lumen and lamina propria (Clayburgh *et al.*, 2004). Paracellular permeability of the epithelium is often quantified by passive diffusion of small hydrophilic marker molecules through tight junctions, while transcellular permeability is measured by lipophilic molecules diffusing over both the apical and basolateral membranes (Jutfelt, 2011). Changes in the tight junction can enhance intestinal permeability resulting in an expression of intestinal barrier damage (Gasbarrini and Montalto, 1999). Although the extrinsic and intrinsic barriers offer effective protection, they cannot always exclude both

the innate immune system (e.g. penetration of harmful agents. Therefore, an additional immunological barrier exists. Both the innate immune system (e.g. macrophages, neutrophils) and the adaptive immune system (the B- and T-cell like lymphocytes) are present in the intestinal wall. The epithelium and the lamina propria contain phagocytotic cells (e.g., macrophages and neutrophils), which engulf and digest harmful agents that have crossed the epithelium (Scott *et al.*, 2011).

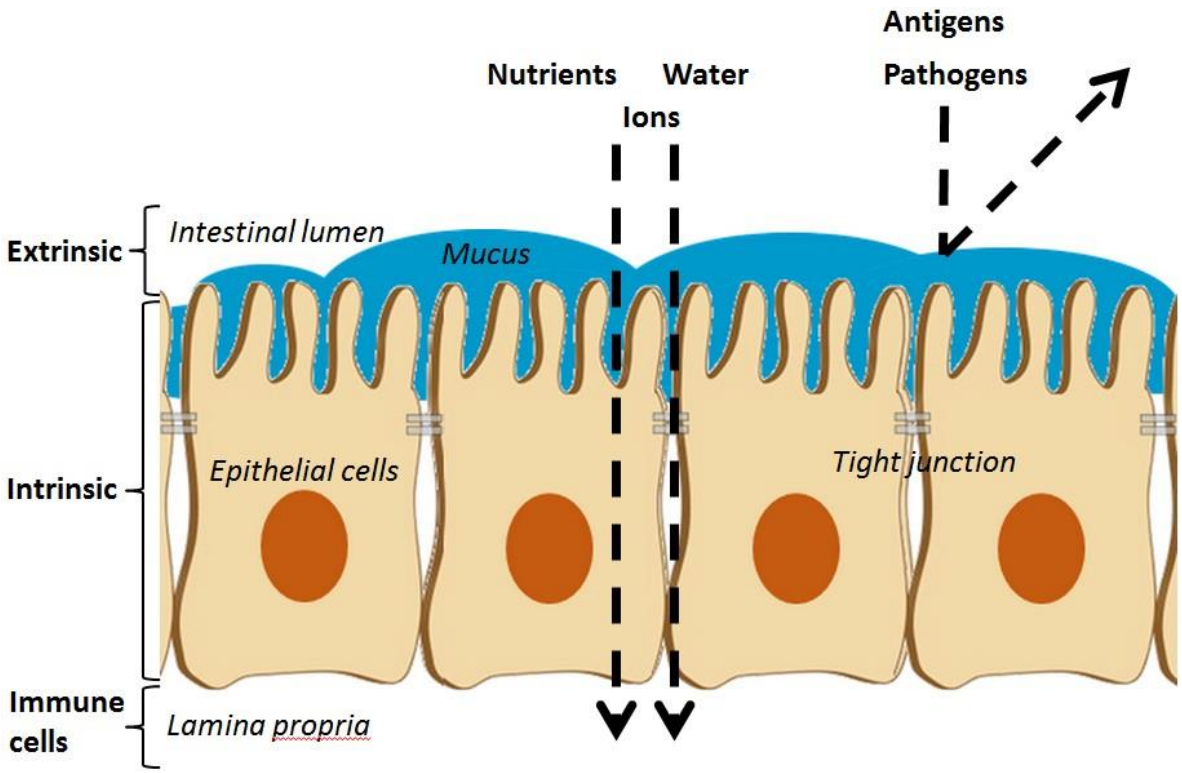


Figure 1.2 Schematic overview of the three parts of the intestinal barrier: 1) The extrinsic (outer) part consists of a mucus layer with antibacterial compounds. 2) The intrinsic part, made up by epithelial cells and the tight junctions, is the major regulator of the intestinal permeability. 3) The immune barrier (the internal defences line) includes the immune cells. Arrows indicate that the intestine "opens" for the nutrients, water, ions entering the circulation, but "closes" for the antigens and pathogens.

1.1.2 The function of the intestine for uptake/exchange of nutrients, water and ions

Another main function of the intestine is to take up food and to exchange water, to process the ingested food and water, to absorb water from the external medium, and to excrete water (Jutfelt, 2006). Large differences in the morphology and physiology of the intestine can be found between fish species with different feeding strategies (Chakrabarti *et al.*, 1995). Plant based ingredients in the diet are the major determining factor for intestinal length, and in general herbivorous fish tend to have longer intestines than carnivorous fish (Nagase, 1964). Functionally, the proximal part of the intestine is the

main nutrient-absorbing region (Nordrum *et al.*, 2000). The lumen in this region contains high nutrient concentrations, typically low numbers of bacteria, and is lined by an absorbing region (Nordrum *et al.*, 2000). The lumen in this region contains high nutrient concentrations, typically low numbers of bacteria, and is lined by an epithelium of high paracellular permeability (van der Marel *et al.*, 2014). In the distal part of the intestine, bacterial numbers are higher and the free nutrient concentration drops. As the nutrient content drops along the intestinal tract and bacterial toxins rise in concentration, the need for a tighter barrier increases (Jutfelt, 2011). Therefore, the barrier function changes between regions.

1.2 Effects of stress factors on the intestinal functions

In mammals, stress affects the intestinal functions and may also impair the barrier function. Consequently, antigens, such as bacteria, viruses and toxins may cross the epithelium (Groot *et al.*, 2000). Similarly, also in fish the intestinal barrier functioning can be affected by stress (Jutfelt, 2011). Actually, any disturbance to the general homeostasis of fish can affect the intestinal barrier. Many long-term stressors, including water hyperoxia and hypoxia, suboptimal water temperature, suboptimal diet composition and infection can impair the intestinal barrier function. (Jutfelt *et al.*, 2006, Ringø *et al.*, 2007, Jutfelt *et al.*, 2007, Sundh *et al.*, 2010) (Figure 1.3).

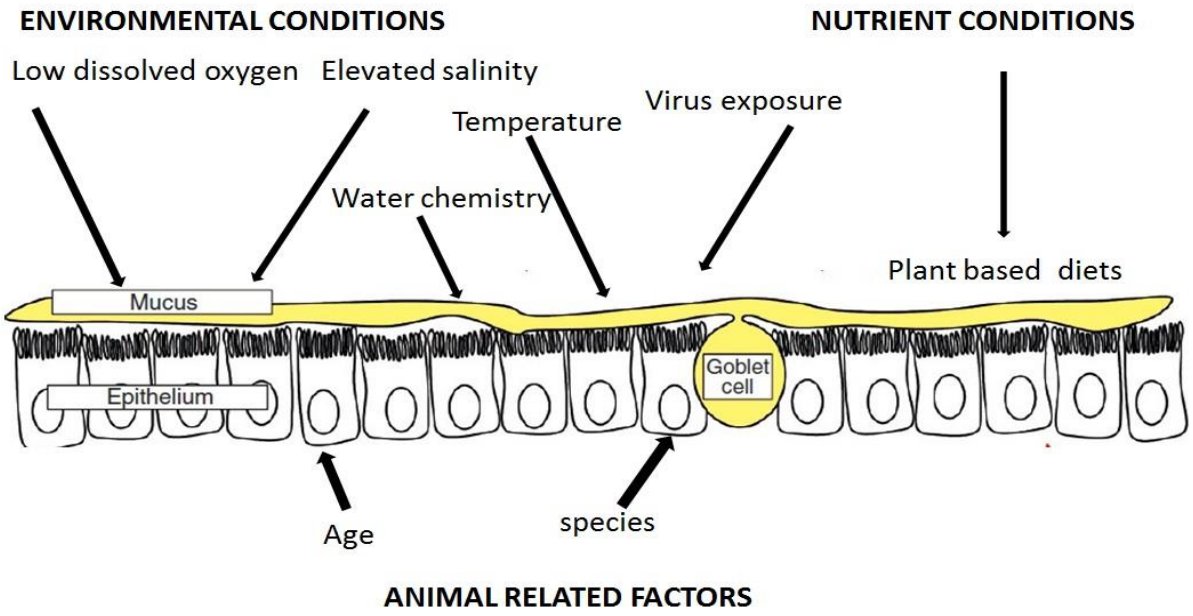


Figure 1.3 Summary of known and suspected factors that disrupt the integrity of the intestinal epithelium and that reduce the barrier function (Adapted from Jutfelt, 2011).

Impairment of the intestinal functions enables an increased exchange of materials between intestinal lumen and body in two ways: paracellularly through the tight junctions or transcellularly through transcytosis which enhances the susceptibility to infection. Larger hydrophilic molecules, and particles such as bacteria and virus are probably

transported through active transcytosis through epithelial cells, as they are too large for tight junction pores. Since, the intestine is in permanent contact with environmental water and may suffer morphological alterations under physical and chemical environmental challenge (Reis *et al.*, 2009, Yuan *et al.*, 2010) the intestinal barrier function can be a good experimental marker for evaluation of the impact of these environmental conditions on the health and welfare of the fish.

1.2.1 The effect of diets on the intestinal functions

Over the past decade, most aquafeed proteins were increasingly supplied by plant ingredients rather than fish meal or other animal protein. Most of the potential, alternative, plant-derived nutrient sources are known to contain a wide variety of anti-nutritional factors such as protease inhibitors in soybean meal, rapeseed meal, lupin seed meal, sunflower oil cake, sesame meal; phytic acid in soybean meal, rapeseed meal, cottonseed meal, sesame meal; saponins in soybean meal, peas, sunflower oil cake and alfalfa leaf meal; and tannins in pea seed meal and mustard oil cake (Francis *et al.*, 2001). However, the continuously increasing intensity of fish farming necessitates the supply of high quantities of sustainable protein ingredients for the required fish feed. Among the alternatives, plant ingredients are still the cheapest and most abundant. Moreover, they may have a suitable protein quality and will be available in the long term (Francis *et al.*, 2001, Gatlin *et al.*, 2007). In the case of tilapia, a wealth of alternative protein sources is available and already used in their feeds. A number of studies have estimated the apparent nutrient digestibility for some of these raw materials for example corn gluten meal, soybean meal (Köprücü and Özdemir, 2005), distiller dried grains with solubles (DDGS) (Schaeffer *et al.*, 2010), rice bran, sorghum and corn (Guimarães *et al.*, 2008b).

Next to their digestibility, the quality of these plant ingredients for their use in aqua-feeds is also assessed by a series of indicators, such as growth performance, biochemical composition, but also by assessing effects on disease and histopathology (Poleksic *et al.*, 2006, Poleksic *et al.*, 2010, Rašković *et al.*, 2011). Histological analysis of the digestive system is considered to be a good indicator of the nutritional status of fish (Caballero *et al.*, 2004). Histopathological changes in the intestine may vary depending on the species and feed used in the experiments. In salmonids, the replacement of fishmeal protein with soybean meal (SBM) causes histological, morphological and functional changes in the intestine. These changes in salmon may include: widening of the lamina propria and the increase of the presence of inflammatory cells such as lymphocytes, macrophages and leucocytes (Baeverfjord and Krogdahl, 1996); shortening of villi and microvilli (van den Ingh *et al.*, 1991). These SBM induced changes have been classified as an enteritis response of the distal intestine (Urán *et al.*, 2008b, Urán *et al.*, 2009). Some of these

changes have also been found in species other than salmonids, e.g., common carp (*Cyprinus carpio*) (Urán *et al.*, 2008a), gilthead sea bream (*Sparus aurata*) (Bonaldo *et al.*, 2008), summer flounder (*Paralichithys dentatus*) (Bone, 2013). In Nile tilapia, Mahmoud *et al.* (2014) reported that fish fed with a diet containing 43% SBM showed mild degeneration of the intestinal mucosa. In contrast, Egyptian sole (*Solea aegyptiaca*) can be fed with a diet containing up to 30% SBM without any induction of enteritis in the intestine (Bonaldo *et al.*, 2006). For Atlantic halibut (*Hippoglossus hippoglossus*) 36% full-fat soya may be added to the diets without negative effects on growth and intestinal morphology (Grisdale-Helland *et al.*, 2002), so these responses to SBM-based diets seem to be species specific but also dose dependent. Aside from SBM, there is growing evidence in the literature suggesting that other plant ingredients can also cause alterations in the morphology of the intestine in fish. In an experiment with lupin products, ulcer-like lesions were observed in salmon (Refstie *et al.*, 2006). Sitjà-Bobadilla *et al.* (2005) reported that increased supranuclear protein droplets, lipid vacuolisation of enterocytes and hypertrophied submucosa with eosinophilic infiltration in the distal intestine of juvenile gilthead sea bream fed diets in which 50, 75 or 100% of the fish meal was replaced by combination of plant proteins (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin). From the literature, the reports on whether, and to which extent plant ingredients affect the morphology of the intestine are still inconsistent for fish. The use of plant feedstuffs in diets may expose fish to cumulative effects of anti-nutritional factors and there may be late-onset or cumulative adverse effects resulting in a late manifestation of pathological conditions (Krogdahl *et al.*, 2010).

1.2.2 The interaction of environmental conditions and diet on the intestinal functions

The quality and quantity of food are important factors in the development of intestinal mass and the mucosal architecture (Buddington *et al.*, 1997), but environmental conditions can also play an important role (Lakani *et al.*, 2013). Environmental stress affects the intestinal barrier integrity in fish. For example, acute stress in Atlantic salmon caused immediate damage to the intercellular junctional complexes and occasionally total loss of intestinal cell content (Olsen *et al.*, 2002). Water oxygen levels are a key limiting factor in intensive fish farming and may affect health and welfare (Ellis *et al.*, 2002). Hypoxia is quite stressful for Atlantic salmon and creates long term disturbance of the physical intestinal barrier. Subjecting Atlantic salmon to hypoxia (50% DO saturation) tended to shorten villi height and to increase size of submucosa of the enterocyte layer in the distal intestine (Sundh *et al.*, 2010). In the tropics, intensive culture of tilapia in ponds often suffer from temporary fluctuations in the levels of environmental dissolved oxygen. Low oxygen levels had a negative impact on feeding behaviour, and growth rate

(Tsadik and Kutty, 1987) and a decreased respiratory frequency (Ishibashi *et al.*, 2002) in Nile tilapia. Therefore, it is hypothesised that low-oxygen levels may induce alterations in the intestinal barrier function of tilapia as well.

Next to dissolved oxygen, also salinity is an important abiotic environmental factor which affects fish growth (Mommsen, 1997). Changing water salinity also leads to a disturbance of the intestinal barrier function since the intestine plays a key role in osmoregulation (Baysoy *et al.*, 2013). Exposed to hyper-osmotic environments, fish lose water to, and gain salt from, the high ionic and hyper-osmotic environment. To get acclimated to the hypersaline water, fish need to replace water loss by drinking salt water and absorbing the salt and water via the intestine whereby water is retained in the body and excess salt is excreted via the gills (Li *et al.*, 2014). Nile tilapia exposed to elevated saline water showed changes in blood parameters (Verdegem *et al.*, 1997), immune function parameters (Choi, 2004), and histopathology (de Azevedo *et al.*, 2015, Hassaan *et al.*, 2014), which may make them more susceptible to infectious diseases. Therefore, it is hypothesised that fish may also suffer intestinal morphological alterations under a saline water stressor.

The long term effect of stress can result in an accumulation which may act as "wear and tear" on the intestinal barrier function, thereby reducing the capability of the fish to acclimate to sub-optimal environments (McEwen 1998). Therefore, it is expected that the physiological consequences of exposure to a combination of low water dissolved oxygen and elevated salinity will impact the intestinal barrier function.

The interaction between the diet and environmental conditions may lead to modifications of the integrity of different intestinal functions (Jutfelt, 2011). The use of antibiotics in animal production has been banned in the European Union and is increasingly under public scrutiny and criticism elsewhere. Consequently, a wide variety of products ranging from plant extracts, probiotics, prebiotics and organic acids have been evaluated as alternatives to antibiotics (Lim *et al.*, 2010). However, in literature the responses of the feed additives on fish performance are still inconsistent (reviewed by Ringø *et al.*, 2010, Welker and Lim, 2011, Ng and Koh, 2016). Testing feed additives is predominantly performed under optimal conditions (good water quality and balanced diets) which may explain why significant effects between treatments are often lacking. Therefore, it is hypothesised that the positive effect of diet may not be very noticeable when fish are cultured without being subject to stress. This concept, therefore, was applied to test the effect of feed additives on the intestinal barrier function. Organic acids were used as one of the possible additives to test the hypothesis whether the effects of feed additives on the intestinal barrier function should be investigated under the suboptimal conditions rather than under optimal conditions.

1.3 Aim of the thesis

Nutrients are important to consider for maintaining intestinal functions. Studies on both positive (using feed additives) and negative effects (using high inclusion of plant ingredients) of fish feeds are numerous, however, between studies results are often highly variable, both in type of response and in significance. In the present study, the central hypothesis is that adverse environmental conditions aggravate negative effects of plant ingredients on the intestinal functions to an extent that mild effects become severe and visible (Figure 1.4).

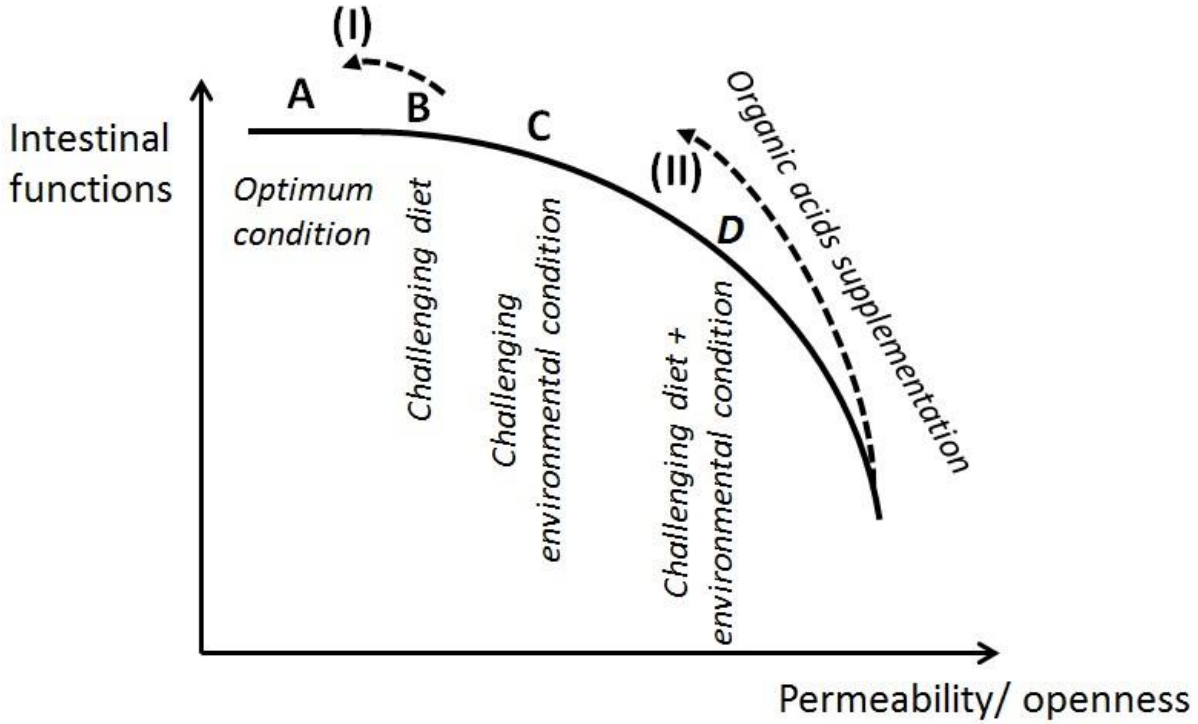


Figure 1.4 The central hypothesis. **Zone A** represents the optimum condition where environmental conditions and diet are optimal. **Zone B** indicates the area where fish are fed a hypothetically challenging diet under an optimal environmental condition. **Zone C** indicates the area where fish are fed an optimal diet but under a hypothetically challenging environmental condition. **Zone D** indicates the area where a challenging diet at zone B and a challenging environmental condition at zone C are combined. It is assumed that from zone A to zone B/C only a minor decrease in intestinal functions occur. However, if two factors of stress are imposed together, the decline of intestinal function can be more severe. If organic acids are supplemented at zone B (without a suboptimal environmental condition), a small impact is found, indicated by arrow I. If the organic acids are supplemented at zone D (a challenging diet and environmental condition combined), the impact of the organic acids is more pronounced, as indicated by arrow II.

In order to study the interaction between diet and environmental conditions, Nile tilapia, as a herbivorous species which is more tolerant to plant based diets, was chosen. It is expected that the response of the intestinal functions to dietary challenges would not immediately be visible in this species. However, in line with the central hypothesis, suboptimal environmental conditions could aggravate and make the effects of dietary

challenges visible. Therefore, Nile tilapia was considered as a suitable species for testing the interaction between challenging diets and suboptimal environmental conditions.

1.4 Outline of the thesis

In order to study the hypothesis, first, ingredients were tested to find which dietary ingredients are challenging for the intestinal functions. Therefore, in **Chapter 2**, six common raw materials including hydrolysed feather meal (HFM), soybean meal (SBM), rice bran (RB), rapeseed meal (RM), sunflower meal (SFM) and dried distillers grains with solubles (DDGS) were chosen to determine the effect of nutrient digestibility, nitrogen and energy balance, and changes in intestinal morphology. Furthermore, the correlation between nutrient digestibility/nutrient composition in diets and the changes in intestinal morphology were studied. The ingredient causing the most alterations in the intestinal morphology, was chosen for the following experiments. In **Chapter 3 and 4**, the intestinal responses on the contrast between high and low inclusion of SBM in the diet under different environmental conditions was measured. Soybean meal was selected because of the outcome of the Chapter 2 and the dietary contrast was expected to provide a difference in intestinal morphology. Oxygen (**Chapter 3**) and salinity (**Chapter 4**) were used to evaluate whether suboptimal environmental conditions (low dissolved oxygen in water or elevated salinity) may interact with a SBM based diet in nutrient digestion and intestinal morphology of tilapia. The results in Chapter 3 and 4 showed that an oxygen challenge had a larger impact on the intestinal functions than salinity. Fish cultured under an imbalanced diet and suboptimal environmental conditions were expected to change their intestinal functions; therefore, using some feed additives could possibly relieve these effect under those conditions. In **Chapter 5**, it was aimed to mitigate the impact of these challenging conditions using organic acids. The interaction between a challenging diet and suboptimal conditions was expected to amplify the change in the intestinal morphology to such an extent that the effect of organic acids may become measurable in reducing those changes. Attention was also paid to show the synergistic or additive effect in the combination of two organic acids. Finally in **Chapter 6,** the overall results obtained from the above mentioned are summarized and discussed, together with possible explanations for the effects of diet composition and environmental conditions on nutritional physiology and intestinal functioning of Nile tilapia.

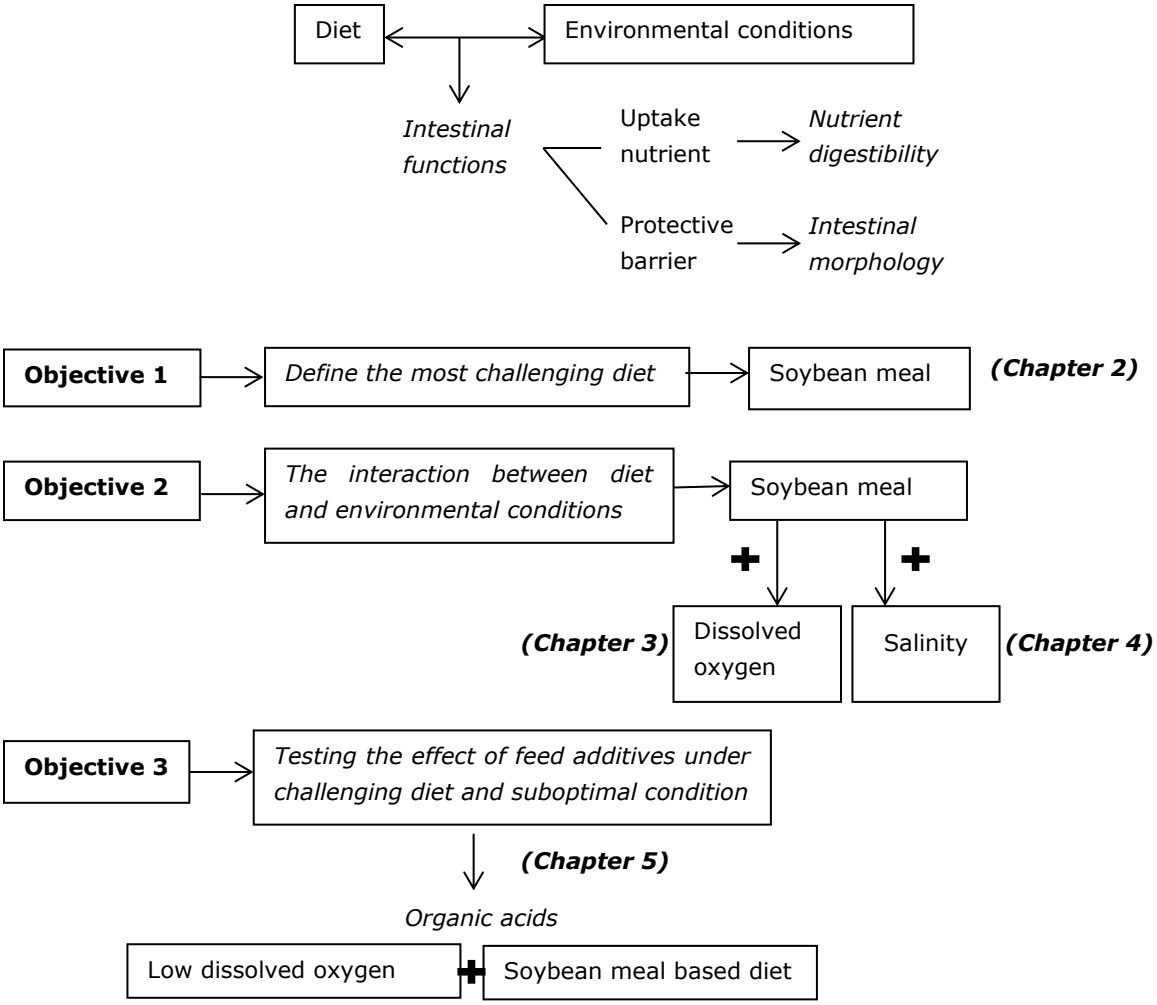
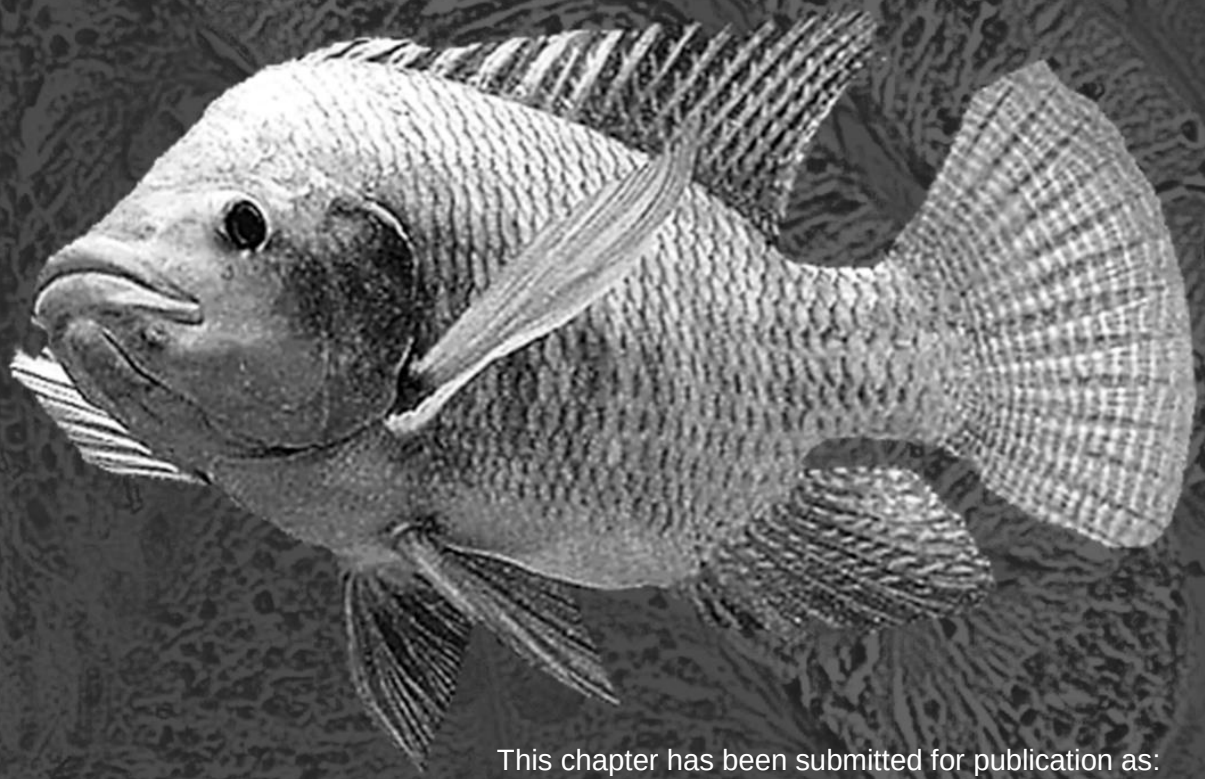


Figure 1.5 Overview of the design of the PhD thesis.

Chapter 2

Effects of feed ingredients on nutrient digestibility, nitrogen/energy balance and morphology changes in the intestine of Nile tilapia (Oreochromis niloticus)



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Abstract

Abstract

The present study assessed the effect of different feed ingredients on nutrient apparent digestibility coefficients (ADC), nitrogen/energy balance and morphology changes in the intestine of Nile tilapia. Changes in intestinal morphology were correlated with nutrient digestibility and also nitrogen/energy balance. Seven diets, varying by different protein sources, were tested by a 1x7 factorial design. Test ingredients were hydrolysed feather meal (HFM), soybean meal (SBM), rice bran (RB), rapeseed meal (RM), sunflower meal (SFM) and dried distillers grains with solubles (DDGS). Six fish per treatment were sampled for intestinal morphology analysis at the end of week 1, 3 and 6. The proximal, middle and distal intestine was processed for quantitative histology, counting the number of goblet cells (GC), and measuring the thickness of lamina propria (LP) and submucosa (SM). The study showed that the ADC of protein in raw materials were highest in SBM (92.2 %), followed by SFM (90.2 %), DDGS (89.2 %), RM (87.8 %), HFM (86.9 %) and RB (84.0 %). The nutrient ADCs had no correlation with intestinal morphology changes. Only the SBM diet caused noticeable changes in intestinal morphology such as an increase in the thickness of SM and LP and the number of GC. The diet composition however altered the protein efficiency and the maintenance energy requirement. Protein retention efficiency was the lowest in fish fed HFM and the highest in RB. The highest maintenance energy requirements were observed in HFM and SBM treatments. However, none of these changes in nitrogen/energy balance were correlated with the change in intestinal morphology.

2.1 Introduction

Over the last 20 years, aquaculture is growing more rapidly than all other animal food-production sectors (FAO, 2016). The expansion of aquaculture production has been accompanied by rapid growth of the aquafeed production. One of the challenges faced by the aquaculture industry is to identify alternatives to fish meal and fish oil on which many present aquafeeds are largely based. For many years, the aquafeed industry has recognised that a viable utilization of plant feedstuffs in formulated diets is an essential requirement for future development of aquaculture (reviewed by Gatlin et al., 2007). In the case of tilapia, a wealth of alternative protein sources are available and in use. For some of these ingredients, the apparent nutrient digestibility has been reported already, e.g. cottonseed meal and sunflower meal (El-Saidy and Gaber, 2003, Aanyu *et al.*, 2014), feather meal (Guimarães *et al.*, 2008a), dried distillers grains with solubles (DDGS) (Schaeffer *et al.*, 2010), rice bran (Guimarães *et al.*, 2008b), rapeseed (Borgeson *et al.*, 2006), and soybean meal (SBM) (Lin and Luo, 2011, Vidal *et al.*, 2015, Koch *et al.*, 2016). The substitution of fish meal coincides with an increased variability in dietary nutrient/ingredient composition. A larger variability in the nutrient digestibility is expected and hence the estimation of energy and nitrogen balance will also change. In a study by Schrama *et al.*, (2012) the authors stated that maintenance energy requirements of fish are dependent on the dietary macronutrient composition. For rainbow trout (*Oncorhynchus mykiss*), it was stated that changes in the dietary macronutrient composition and nutrient type (raw vs gelatinized starch) had an effect on energy and protein utilization (Kaushik and de Oliva Teles, 1985, Sanz *et al.*, 1994, Rodehutscord and Pfeffer, 1999). This indicates that any change in dietary ingredient/nutrient composition would have an effect on the energy balance. For Nile tilapia, information on the effect of different ingredients composition on energy and nitrogen balances is less investigated.

Plant ingredients contain often anti-nutritional factors such as protease inhibitors, phytates, glucosinates, saponins, tannins, non-starch polysaccharides, which can have negative impacts on the intestinal functions (Francis *et al.*, 2001). In the case of salmonids, SBM cause morphological and functional changes in the intestine such as widening of the lamina propria (Baeverfjord and Krogdahl, 1996), inducing enteritis in the distal intestine (Urán *et al.*, 2008b) and shortening villi and microvilli (van den Ingh *et al.*, 1991). These changes in intestinal morphology in fish fed SBM-based diets have been reported also for other species such as common carp (*Cyprinus carpio*) (Urán *et al.*, 2008a), rainbow trout (*Oncorhynchus mykiss*) (Nordrum *et al.*, 2000), gilthead sea bream (*Sparus aurata*) (Bonaldo *et al.*, 2008) and Nile tilapia (Mahmoud *et al.*, 2014, Ismaiel *et al.*, 2015). These changes in the intestinal morphology as a consequence of

feeding SBM-based diets seem to be species specific, just as its ability to recover from these changes when fed a non-SBM-based diet. In Atlantic salmon, no signs of intestinal recovery of this SBM-induced enteritis occur with time (Urán *et al.*, 2009). On the other hand, a recovery of the distal intestinal epithelium was observed in carp starting from 4 weeks onwards after continuously being fed a SBM-based diet (Urán *et al.*, 2008a). Although most of the intestinal morphology studies were conducted using SBM or soybean co-products only, there is growing evidences that other plant ingredients may also affect the intestinal morphology. In an experiment with lupin products, ulcer-like lesson were observed in salmon fed lupins (Refstie *et al.*, 2006). Sitjà-Bobadilla *et al.* (2005) reported increased supranuclear protein droplets, lipidic vacuolisation of enterocytes and hypertrophied submucosa with eosinophilic infiltration in the distal intestine of juvenile gilthead sea bream fed diets in which 50, 75 or 100 % of the fish meal was replaced by a combination of plant proteins (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin). Therefore, measurement of the alterations in the intestinal morphology induced by plant ingredients is also an important step in the evaluation process of the potential value of an ingredient in diets for fish (e.g., tilapia).

Intestinal morphology, digestibility and nitrogen/energy balanced are rarely quantified in one single study. These measurements were combined in the present study in order to assess if alterations in intestinal morphology, induced by ingredients, do relate to nutrient digestibility and/or nitrogen/energy balance.

2.2 Materials and methods

2.2.1 General desgins

The experiment was conducted at the experimental facility "Carus", Wageningen University, The Netherlands, testing 7 diets in a 1x7 factorial design. It was conducted in accordance with the Dutch law on experimental animals and approved by the Wageningen University Animal Experimental Committee (20013003.b).

2.2.2 Feed ingredients and diet reparation

An extruded reference diet (Table 2.1) was formulated to meet the nutrient requirements of Nile tilapia (NRC, 2011). Yttrium oxide (Y_2O_3) was added to the reference diet at a concentration of 0.02 %, and used as an inert marker. Test ingredients were obtained from Research Diet Services (Wijk bij Duurstede, The Netherlands), with the exception of hydrolysed feather meal which was supplied by Vionfood (Boxtel, The Netherlands).

The studied test ingredients, which are common in Nile tilapia feed, were hydrolysed feather meal (HFM), soybean meal (SBM; dehulled, solvent extracted), rice bran (RB)

rapeseed meal (RM; rapeseed solvent extracted), sunflower meal (SFM; decorticated, solvent extracted) and dried distillers grains with solubles, from wheat (DDGS). Six extruded test diets were formulated using 70 % reference diet and 30 % of each of the test ingredients as described by Foster (1999). Proximate analysed nutrient composition of the test ingredients and diets are shown in table 2.2 and 2.3, respectively.

2.2.3 Fish, housing condition and feeding

735 unfed (feed-deprived for about 24h) juvenile Nile tilapia were individually weighed (under sedation using 2-phenoxyethanol, 0.25 ml L^{-1} water) and randomly distributed among 21 aquaria (35 fish $aquarium^{-1}$). Each aquarium was assigned randomly to one of the seven diets forming triplicates per diet. Throughout the experiment, the culture conditions and the water quality parameters (mean ± SD) were maintained at the optimal conditions for Nile tilapia; tank volume (70 L $aquarium^{-1}$), water flow over each aquarium (7 L min^{-1}), photoperiod 12h light:12h dark, water temperature (28 ± 1 ^{0}C), pH (7.2 ± 0.2), dissolved oxygen (6.0 ± 0.3 mg L^{-1}) and total ammonia nitrogen (<0.5 mg L^{-1}). The fish were restrictively fed by hand at 3 % of the body weight. In case of persistent occurrences of feed refusals in one aquarium, the feed given to all the others was reduced to prevent differences in feed intake between aquaria.

To collect faeces for determination of nutrient digestibility, the outlet of each tank was connected to a swirl separator (44 cm height, 24.5 cm diameter; AquaOptima AS, Trondheim, Norway). The faeces were collected in a detachable 250 ml bottle placed at the bottom of each swirl separator. During faeces collection and in order to minimize the bacterial decomposition of faeces, the bottles were kept on ice. A different set of bottles was connected to the swirl separator during feeding to collect of uneaten feed pellets that were flushed out from the aquarium.

Table 2.1 Ingredient composition of the refence diets.

Ingredients (%)	Reference diet	Test diets
Fish meal	48.35	
Wheat	35.63	
Wheat bran	10	
Fish oil	2	
Soybean oil	2	
Vitamin and mineral premix*	2	
Yttrium oxide	0.02	
Reference diet		70
Test ingredient**		30
Total	100	100

*Vitamin and mineral premix (per kg of feed): vitamin A 6000 IU; D_3 2000 IU; E 100 mg; C 100 mg; K_3 10 mg; $B_1$15 mg; B_2 15 mg; B_6 15 mg; B_5 pantothenic acid 50 mg; B_3 niacin 60 mg; Biotine 0.2 mg; B_{12} 0.025 mg; Folic acid 3 mg; Fe 50 mg; Zn 100 mg; Co 0.1 mg; Cu 10 mg; Se 0.5 mg; Mn 20 mg; Mg 500 mg; Cr 1 mg; I 2 mg; Inositol 400 mg; Choline 2000 mg; Anti-oxidant 100 mg; Calcium proprionate 1000 mg.

**Test ingredients were hydrolysed feather meal, soybean meal (dehulled, solvent extracted), rice bran, rapeseed meal (rapeseed solvent extracted), sunflower meal (decorticated, solvent extracted) and dried distillers grains with solubles from wheat (DDGS).

Table 2.2 Nutrient composition of the ingredients used in the test diets.

Nutrient (% on dry matter)	Test ingredients					
	HFM	DDGS	SBM	RB	RM	SFM
Dry matter	96.80	90.50	89.02	89.22	89.94	91.29
Protein	87.21	33.38	55.34	15.22	39.52	37.68
Lipid	11.02	8.72	3.15	20.18	3.35	2.63
Ash	2.44	5.80	7.08	11.29	7.44	8.17
Phosphorous	0.37	0.80	0.76	2.65	1.31	1.37
Total carbohydrate	-	52.10	34.42	53.31	49.69	51.52
Gross energy (kJ g^{-1})	25.01	21.26	19.86	21.48	19.61	19.12

HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

Table 2.3 Nutrient composition of the reference and test diets.

Components (% in dry matter)	Reference diet	Test diets (70% reference + 30% test ingredient)					
		HFM	DDGS	SBM	RB	RM	SFM
Dry matter	93.96	94.59	94.35	93.17	94.25	92.61	94.13
Protein	43.75	56.53	39.53	45.74	34.72	42.02	41.57
Lipid	11.71	11.36	10.53	9.11	13.70	8.65	8.47
Ash	8.53	6.66	7.68	8.21	8.73	8.05	8.35
Phosphorous	1.28	1.00	1.12	1.12	1.66	1.27	1.29
Starch	25.3	17.30	18.20	18.70	23.4	18.70	18.70
Fibre	2.4	2.20	3.50	2.60	3.00	5.20	6.60
NSP	10.68	8.12	24.03	18.26	19.40	22.61	22.91
Total carbohydrate	36.01	25.46	42.26	36.94	42.85	41.29	41.61
Energy (kJ g^{-1})	21.27	22.25	21.11	20.63	21.25	20.57	20.44
Yttrium	0.016	0.016	0.017	0.017	0.017	0.017	0.017

NSP, non-starch polysaccharides; HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

2.2.4 Sampling and measurements

During the last week of trial (week 6), faeces were collected per aquarium twice a day, 1 hour prior to feeding for measuring nutrient digestibility, and stored individually at -20^{0}C until further analysis.

At the end of the experiment, and in order to determine the final biomass, fish from each aquarium were anaesthetized (2-phenoxyethanol, 0.25 ml L^{-1} water) and group weighed. To evaluate the intestinal morphology, at the end of week 1, 3 and 6, two fish from each aquarium (6 fish per treatment) were sampled. Fish were scooped gently out from each aquarium using hand dip net and euthanized by an over dose of 2-phenoxyethanol (1 ml L^{-1} water). Then, fish were weighed, dissected and the intestinal tract sampled for histological studies. The intestine was divided into three regions: proximal (from the pyloric part of the stomach to the spiral part of the intestine), mid (the spiral part of the intestine) and distal (from end spiral part of the intestine to 2 cm before anus) as described by Pirarat *et al.* (2011). One-cm portion of each of the three intestinal segments was fixed by immersion in Bouin's fixative solution.

After fixation, the intestinal sample slides were prepared and analysed under a light microscope for SBM-induced enteritis following the method described in Tran-Ngoc *et al*. (2016a). Briefly, the measurements were done on four random villi per slides and per intestinal segment for each fish. Three intestinal morphology parameters were assessed: a) the number of goblet cells (GC), b) the thickness of the lamina propria (LP) and c) the thickness of the sub-epithelia mucosa (SM) (Figure 2.1).

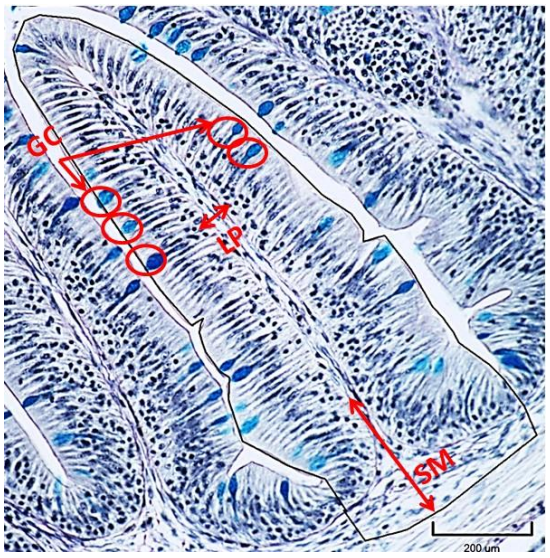

Figure 2.1 Intestinal proximal morphology of Nile tilapia after 6 weeks cultured at a normal reference diet. The submucosa (SM) is a thin layer of connective tissue between base of folds and stratum compactum. The lamina propria (LP) is thin and delicate core of connective tissue in simple folds. The goblet cell (GC) is type of mucus-secreting in the epithelium and scattered among the enterocytes. Staining: Haematoxylin/Eosin and Alcian blue, x40.

2.2.5 Chemical analyses

Chemical analysis of the feed, and faeces were done in triplicate. Dry matter (DM) was determined gravimetrically after drying at 103^0C for 4h, 4h and 24h until constant weight, respectively (ISO 6496, 1983). Ash was determined after incineration at 550^0C for 4h (ISO 5984, 1978). Crude protein (CP) was determined by the Kjeldahl method (ISO 5983, 1979). Crude fat was quantified after petroleum-diethyl ether extraction (ISO 6492, 1999). Energy content was measured by an adiabatic bomb calorimeter (IKA-C-700; IKA analysentechnik, Weitersheim, Germany). Starch content was enzymatically determined in feed and faecal samples by using amyloglucosidase after ethanol extraction and measuring glucose content as described by Goelema *et al.* (1998). Fibre was analysed according to the standard NEN 5417 and ISO-standard 549 methods. The yttrium and phosphorous (P) content of feed and faeces was analysed using inductively coupled plasma-mass spectrometry (ICP-OES) according to the standard NEN 15510 (2007) method. Total carbohydrate was calculated as dry matter – crude protein – crude fat – ash content. Non-starch polysaccharides (NSP) was calculated based on 100 - crude protein – crude fat – ash – starch.

2.2.6 Calculation

Specific growth rate was calculated as SGR (% bw d^{-1}) = $[(\ln W_f - \ln W_i)/t] \times 100$, where W_f and W_i are the final and initial weight, respectively; t is the experimental duration in days. Feed intake (FI_{bw}) of fish was expressed as a percentage of body weight (in % bw d^{-1}) = FI / BW_{mean} x 100, where FI (g d^{-1}) is the average feed intake per fish per day and BW_{mean} is the mean body weight, which was calculated as BW_{mean} (g) = (W_f + W_i) / 2. Feed conversion rate (FCR) was calculated as FCR (g g^{-1})= FI_{tot} / (W_f - W_i), where FI_{tot} (g) is the total feed intake per fish during the experimental period.

Apparent digestibility coefficients (ADC, in %) of dry matter, protein, fat, ash, phosphorus, total carbohydrate, and energy of the test and reference diets were determined as described by Cho *et al.* (1982)

$$ADC_{test\ diet}\ (\%) = 100 - 100 \times (\%\ Y_{feed} / \%\ Y_{faeces}) \times (\%\ Nutrient_{faeces} / \%\ Nutrient_{feed})$$

Where Y_{feed} and Y_{faeces} are the dietary and faecal yttrium oxide content and $Nutrient_{faeces}$ and $Nutrient_{feed}$ is the faecal and dietary nutrient content (all in % on dry matter basis).

Apparent digestibility coefficients of dry matter, protein, fat, ash, phosphorus, total carbohydrate, and energy of the test ingredients using the equation proposed by Forster (1999), mathematically simplified by Bureau and Hua (2006) and recently documented by the National Research Council (NRC, 2011).

$$ADC_{test\ ingredient}\ (\%) = ADC_{test\ diet} + [(ADC_{test\ diet} - ADC_{ref\ diet}) \times (0.7 \times D_{ref} / 0.3 \times D_{ingr})]$$

Where D_{ref} = % nutrient (or kJ g^{-1} gross energy) of reference diet mash (as is); D_{ingr} = % nutrient (or kJ g^{-1} gross energy) in test ingredient (as is).

Energy and nitrogen (N) balance parameters were calculated per tank and expressed respectively as, kJ $kg^{-0.8}$ BW d^{-1} and mg $kg^{0.8}$ BW d^{-1}. N balance calculations were as follows: gross nitrogen intake (GN) = FI x N_{feed}, where FI = feed intake of the fish (g feed $fish^{-1}$), N_{feed} = nitrogen content of the feed. Digestible nitrogen (DN) = (GN x ADC_{cp}) / 100, where GN = gross nitrogen intake, ADC_{cp} (%) = apparent digestibility coefficient of the crude protein in the feed. Faecal nitrogen losses = GN – DN. Branchial and urinary nitrogen loses (BUN) = DN – RN, where RN = retained nitrogen. RN = ((BW_t x CP) / 6.25) – ((BW_0 x CP) / 6.25), where BW_t = body weight of fish at the end of the experiment (kg), CP = crude protein content of the fish (g). Energy balance were calculated as follows: gross energy intake (GE) = FI x E_{feed}, where FI = feed intake of the fish (g feed $fish^{-1}$), E_{feed} = energy content of the feed. Digestible energy (DE) = (GE x ADC_E) / 100, where ADC_E (%) = apparent digestibility coefficient of the energy in the feed. Faecal energy losses (FE) = GE – DE.

Metabolizable energy (ME) = DE – BUE, where BUE = branchial and urinary energy losses. BUE = (BUN x 24.9) / 1000, where 24.9 kJ N g^{-1} = energy concentration of NH_3-N calculated by Bureau *et al.* (2003) and assuming that all N was excreted as (NH_3-N). Retained energy (RE) = (BW_t x E_t) – (BW_0 x E_0), where E_t = energy content of the fish at the end of the experiment, E_0 = energy content of the fish at the start of the experiment, BW_t = body weight of fish at the end of the experiment, BW_0 = body weight of fish at the start of the experiment. Heat production (HP) = RE –ME. Metabolizable energy for maintenance requirement (ME_{maint}) was estimated by ME_{maint} = ME – (RE_{pro} / 0.54) – (RE_{lipid} / 0.90); assuming k_P is 0.54 (cost of protein deposition) and k_L is 0.90 (cost of lipid deposition) (Lupatsch *et al.*, 2003). Retained energy as protein (kJ $fish^{-1}$) was calculated as retained protein (retained N x 6.25) multiplied by 23.7 (Brafield, 1985). Retained energy as lipid (kJ $fish^{-1}$) was calculated as the difference between total retained energy and the retained energy as protein, assuming total energy is equal to protein plus lipid.

2.2.7 Statistical analyses

Statistical analyses were performed using IBM SPSS 22 (SPSS Inc., Chicago, USA). Data were tested for normality and homogeneity (Shapiro Wilk and Levene test, respectively) and when necessary, transformed to achieve the required assumptions. Growth parameters and digestibility were subjected to one-way ANOVA. Histology data were analysed for effect of time and diet by two-way ANOVA. Pearson's correlation coefficients were calculated for the relationship between the enteritis symptoms and ADCs for nutrient and nutrient composition in diet. The results were considered statistically significant when *p-values* were below 0.05. When appropriate, the Tukey test was applied pair wise comparison of means.

2.3 Results

2.3.1 Fish performance

The effects on the different diets on growth are summarised in Table 2.4. Averaged over diets, the survival rate was 99% during the experimental period and was unaffected by dietary treatment.

Table 2.4 Growth performance of Nile tilapia during the experimental period.

	Ref. diet	Test diets (70% reference + 30% test ingredient)						
		HFM	DDGS	SBM	RB	RM	SFM	P-value
Experimental period (d)	42	42	42	42	42	42	42	
Tanks (n)	3	3	3	3	3	3	3	
Fish per tank (n)	35	35	35	35	35	35	35	
Survival (%)	100	98	100	98	99	100	100	
Initial BW (g)	10.83 ±0.2	10.93 ±0.4	10.73 ±0.3	11.13 ±0.5	11.17 ±0.4	10.9 ±0.3	11.0 ±0.2	ns
Final BW (g)	83.32 ±0.9^{b}	65.19 ±3.5^{a}	77.66 ±1.6^{b}	67.91 ±4.8^{a}	67.03 ±4.5^{a}	66.63 ±1.5^{a}	67.93 ±3.5^{a}	***
Feed intake (%bw d^{-1})	3.23 ±0.1	3.29 ±0.2	3.32 ±0.1	3.35 ±0.2	3.41 ±0.2	3.44 ± 0.1	3.42 ±0.2	ns
SGR (%bw d^{-1})	4.87 ± 0.1^{b}	4.27 ±0.1^{a}	4.70 ±0.1^{b}	4.30 ±0.1^{a}	4.27 ±0.3^{a}	4.30 ±0.0^{a}	4.33 ±0.1^{a}	***
FCR (g g^{-1})	0.88 ±0.0	0.97 ±0.1	0.92 ±0.0	0.98 ±0.1	1.00 ±0.1	1.00 ±0.0	0.99 ±0.1	ns

Results are presented as mean ± SD (n=3). Values on the same row with different superscripts (a,b) are significantly different (P<0.05); ns, no significant difference: *p<0.05; **p<0.01; ***p<0.001
HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; BW, body weight; SGR, specific growth rate; FCR, feed conversion rate.

Feed intake was similar for all treatments, but final body weight and SGR were affected by diet (P<0.001), being higher for reference and DDGS diet treatments. Mean FCR ranged from 0.88 to 1.00; however no significant differences occurred among treatments.

2.3.2 Digestibility

Apparent digestibility coefficients (ADCs) of nutrient and energy in the experimental diets and test ingredients for Nile tilapia are shown in Table 2.5 and 2.6, respectively.

Table 2.5 Apparent digestibility of nutrients in the test diets.

% on dry matter	Reference diet	Test diets (70 % reference + 30 % test ingredient)						
		HFM	DDGS	SBM	RB	RM	SFM	P-value
Dry matter	80.1 ±0.1^{c}	78.1 ±0.2^{c}	73.7 ±0.9^{b}	78.4 ±1.7^{c}	71.5 ±0.9^{ab}	71.5 ±0.5^{ab}	69.2 ±2.1^{a}	***
Protein	92.3 ±0.5^{c}	87.4 ±0.5^{a}	89.9 ±0.8^{b}	92.2 ±1.0^{c}	87.4 ±1.0^{a}	88.8 ±0.4^{ab}	90.7 ±0.4^{bc}	***
Fat	94.1 ±1.0^{c}	84.8 ±2.4^{a}	92.0 ±0.9^{bc}	93.5 ±0.6^{bc}	86.9 ±1.3^{a}	90.5 ±0.4^{b}	91.3 ±0.5^{bc}	***
Ash	46.8 ±2.0^{bc}	32.3 ±8.3^{ab}	50.7 ±2.3^{c}	43.8 ±12.4^{abc}	29.2 ±0.9^{a}	37.1± 1.8^{abc}	38.9 ±4.5^{abc}	**
Starch	99.6±0.1^{a}	99.9 ±0.1^{a}	99.7 ±0.2^{a}	99.9 ±0.8^{a}	99.6 ±0.3^{a}	99.9 ±0.1^{a}	99.9 ±0.2^{a}	ns
NSP	-5.5±1.7^{a}	-4.6 ±1.7^{a}	26.8 ±2.5^{cd}	30.0 ±3.2^{d}	17.2 ±3.4^{bc}	20.9 ±2.1^{cd}	8.1 ±7.2^{b}	***
Total carbohydrate	68.6 ±0.6^{e}	66.5 ±0.6^{de}	58.2 ±1.5^{bc}	65.4 ±1.6^{de}	62.3 ±1.5^{cd}	56.7 ±1.2^{b}	49.4 ±3.9^{a}	***
Phosphorous	56.1 ±2.8^{d}	55.7 ±0.6^{d}	61.7 ±3.1^{d}	55.1 ±5.0^{cd}	37.0 ±1.3^{a}	46.5 ±0.6^{b}	47.7 ±3.1^{bc}	***
Energy	85.1 ±0.3^{d}	81.6 ±0.9^{c}	78.1 ±1.0^{b}	83.5 ±0.6^{cd}	77.1 ±1.2^{b}	77.0 ±0.7^{ab}	74.3 ±1.6^{a}	***

Results are presented as mean ± SD (n=3). Values on the same row with different superscripts (a,b,c) are significantly different (P<0.05); ns, no significant difference; *p<0.05; **p<0.01; ***p<0.001; HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; NSP, non-starch polysaccharide.

Table 2.6 Apparent digestibility of nutrients in the test inregdients.

% on dry matter	Test ingredients						
	HFM	DDGS	SBM	RB	RM	SFM	P-value
Dry matter	77.7 ±0.3^{b}	72.3 ±1.1^{b}	78.0 ±2.1^{b}	69.4 ±1.1^{ab}	69.5 ±0.6^{ab}	66.7 ±2.6^{a}	***
Protein	86.9 ±0.6^{b}	89.2 ±1.0^{bc}	92.2 ±1.1^{d}	84.0 ±1.7^{a}	87.8 ±0.4^{bc}	90.2 ±0.5^{cd}	***
Fat	82.6 ±2.9^{a}	91.3 ±1.2^{cd}	93.0 ±1.2^{d}	85.9 ±1.5^{ab}	87.4 ±0.7^{bc}	88.3 ±1.0^{bc}	***
Ash	21.1 ±14.6^{a}	52.1 ±3.1^{b}	42.9 ±15.9^{ab}	26.0 ±1.1^{a}	34.5 ±2.3^{ab}	37.0 ±6.1^{ab}	*
Total Carbohydrate	91.1 ±6.2^{d}	56.5 ±1.7^{bc}	64.6 ±2.0^{c}	61.2 ±1.7^{bc}	54.6 ±1.4^{ab}	46.3 ±4.5^{a}	***
Phosphorous	55.4 ±1.1^{cd}	63.9 ±4.2^{d}	54.6 ±7.1^{cd}	34.8 ±1.5^{a}	44.3 ±0.7^{ab}	45.8 ±3.7^{bc}	***
Energy	81.0 ±1.1^{c}	76.4 ±1.2^{b}	83.1 ±0.8^{c}	75.3 ±1.5^{b}	74.9 ±0.8^{ab}	71.5 ±2.0^{a}	***

Results are presented as mean ± SD (n=3). Values on the same row with different superscripts are significantly different (P<0.05). *p<0.05; **p<0.01; ***p<0.001; HFM, hydrolysed feather meal; DDGS, drain distiller grain with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

In general, most of the ADCs values of nutrients and energy were affected by the composition of the test diets (P<0.001, Table 2.5 and 2.6). Table 2.5 shows that protein and energy digestibility were similar for the reference and soybean diets and significantly higher than for the other diets. There was less variability in NSP digestibility for diets with high inclusion of animal products (reference and hydrolysed feather diets; range -5.5 to -4.6 %) than in NSP digestibility for diets in which plant products were included (range from 8 – 30 %).

The ADCs of nutrients and energy differed between test ingredients: protein and fat digestibility were highest for soybean meal and lowest for rice bran and hydrolysed feather meal, respectively (P<0.001, Table 2.6). Energy digestibility was significantly higher for hydrolysed feather and soybean meal and lowest for sunflower meal. The ADC of protein was general high for all ingredients (84 – 91 %), while that for ash was low (21 – 52 %).

2.3.3 Nitrogen and energy balance

Nitrogen and energy balances are presented in table 2.7. All the nitrogen balance parameters were affected by diet (P<0.001). Gross nitrogen intake and digestible nitrogen intake reached the highest value in fish fed on hydrolysed feather meal diet while the highest value of retained nitrogen was reached in fish fed the reference and DDGS diet. All the other diets showed no significant difference in nitrogen retention. Protein retention efficiency ranged between 34 and 53 % and was the lowest in fish fed the hydrolysed feather meal diet even though this diet showed the highest digestible nitrogen intake.

Regarding the energy balance data, all parameters were also significantly affected by test diets. Retained energy was different between diets caused by the differences in metabolized energy intake (P<0.001). Retained energy as protein followed the same trend as retained nitrogen and was the highest in the reference and DDGS diets. Maintenance requirements for fish fed hydrolysed feather diet was about 90 kJ $kg^{-0.8}$ BW d^{-1} and was significantly higher than maintenance requirements predicted for fish fed the rice bran and sunflower diets.

The correlation between nitrogen/energy balance and the changes in intestinal morphology were also addressed. However, none of intestinal morphology parameters were correlated with any of the nitrogen/energy balance parameters (data not shown).

Table 2.7 Nitrogen and energy balance in the reference and test diets for Nile tilapia.

	Ref. diet	Test diets (70% reference + 30% test ingredient)							
		HFM	DDGS	SBM	RB	RM	SFM	SEM	P-value
N balance (mg kg$^{-0.8}$BW d^{-1})									
Gross nitrogen intake	1652^{c}	1943^{d}	1488^{b}	1599^{bc}	1242^{a}	1496^{b}	1501^{b}	43.1	***
Digestible nitrogen intake	1524^{d}	1699^{e}	1338^{b}	1474^{cd}	1085^{a}	1328^{b}	1361^{bc}	38.9	***
Branchial urinary nitrogen losses	816^{bc}	1116^{d}	659^{ab}	853^{c}	514^{a}	711^{bc}	742^{bc}	15.9	***
Retained nitrogen	708^{b}	583^{a}	679^{b}	621^{a}	571^{a}	617^{a}	619^{a}	45.9	***
Protein efficiency	47^{bc}	34^{a}	51^{cd}	42^{b}	53^{d}	47^{abc}	46^{bc}	1.8	***
Energy balance (kJ kg$^{-0.8}$ BWd^{-1})									
Energy intake	502^{c}	478^{abc}	497^{bc}	451^{a}	475^{abc}	458^{ab}	461^{abc}	12.3	**
Digestible energy intake	427^{d}	390^{c}	388^{c}	376^{bc}	366^{abc}	352^{ab}	343^{a}	9.5	***
Branchial urinary energy losses	20^{bc}	28^{d}	16^{ab}	21^{c}	13^{a}	18^{bc}	18^{bc}	1.1	***
Metabolisable energy intake	407^{d}	362^{bc}	371^{c}	355^{bc}	354^{bc}	335^{ab}	324^{a}	8.5	***
Heat production	163^{ab}	175^{b}	144^{ab}	165^{ab}	132^{a}	146^{ab}	133^{a}	10.8	***
Retained energy	244^{b}	187^{a}	228^{b}	190^{a}	222^{b}	189^{a}	192^{a}	6.7	***
Retained energy as protein	105^{b}	86^{a}	101^{b}	92^{a}	85^{a}	91^{a}	92^{a}	2.4	***
Retained energy as fat	139^{b}	101^{a}	127^{b}	98^{a}	137^{b}	98^{a}	100^{a}	4.8	**
Maintenance energy requirement	57.7^{ab}	90.5^{b}	43.7^{a}	76.3^{ab}	44.3^{a}	57.0^{ab}	43.3^{a}	12.5	*

Values on the same row with different superscripts are significantly different (n = 3; P<0.05). *p<0.05; **p<0.01; ***p<0.001; HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; N, nitrogen; SEM, standard error; BW, body weight; d, day.

2.3.4 Intestinal morphology

Average values of the intestinal morphological parameters per diet and over time are described in table 8. The results show that the intestinal morphology of Nile tilapia was altered by the diets being fed. Soybean meal had a negative effect on the thickness of the SM showing this parameter reached the highest value in the proximal and middle part of the intestine (P<0.01; Table 2.8). The effect of soybean meal on the thickness of the LP (P<0.01) and on the number of GC (P<0.05) followed a similar trend as that of the thickness of SM, with the highest values seen in the proximal and distal parts of the intestine. In contrast, the impacts on the intestinal morphology of the reference diet, hydrolysed feather meal and DDGS diets were neutral and showed minor differences

between these diets. The other plant ingredient based diets (rice bran, rapeseed and sunflower) had an intermediate effect on the intestinal morphology (Table 2.8).

In addition to the diet effect, many of the intestinal parameters were affected by time. The thickness of SM showed a significant time effect, whereas the time effect on the thickness of the LP and the number of GC was only observed in the mid and proximal intestine, respectively. The thickness of the SM increased with time in the proximal, middle and distal intestine (Figure 2.2) during the course of the experiment.

A significant interaction effect between diet and time was present for the parameters LP and GC. The effect of soybean meal on LP in the proximal intestine aggravated over time, while for the other diets that impact time was not present (Figure 2.3). The same trend was found for the thickness of LP in the distal intestine. On the other hand, the number of GC in the proximate intestine significantly increased over time for fish fed plant protein diets with the exception for DGGS and rice bran (Figure 2.4). Fish fed the hydrolysed feather meal did not show the difference in GC over time and fish fed the reference diet showed an reduction in number of GC over time (Figure 2.4).

Table 2.8 Effect of tested ingredients on morphological parameters at different sections of the intestine in Nile tilapia.

	Test diets (70 % reference + 30 % test ingredient)								P-value		
	Ref. diet	HFM	DDGS	SBM	RB	RM	SFM	SEM	D	T	D x T
Submucosa (µm)											
Proximal	376	353	434	458	365	363	421	23	**	***	ns
Mid	367	336	344	422	355	382	278	25	**	*	ns
Distal	432	468	414	428	418	400	422	41	ns	***	ns
Lamina propria (µm)											
Proximal	64^{ab}	61^{a}	61^{a}	79^{b}	74^{ab}	75^{ab}	67^{ab}	4	**	ns	**
Mid	97	91	94	108	99	93	86	9	ns	**	ns
Distal	115^{a}	116^{a}	144^{ab}	154^{b}	117^{a}	147^{ab}	127^{ab}	8	**	ns	***
Goblet cell (10^{-3} cells μm^{-1})											
Proximal	17.9^{a}	18.3^{a}	19.8^{ab}	23.2^{b}	21.5^{ab}	19.7^{ab}	20.1^{ab}	1	*	**	***
Mid	26.8	22.8	26.6	27.2	25.2	22.6	22.4	2	ns	ns	ns
Distal	20.0	18.0	24.8	28.2	26.1	27.0	27.0	2	*	ns	ns

Values on the same row with different superscripts are significantly different (n = 6; P<0.05); ns, no significant difference, $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$; HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal; SEM, standard error; D, diet; T, time; D x T; interaction diet and time.

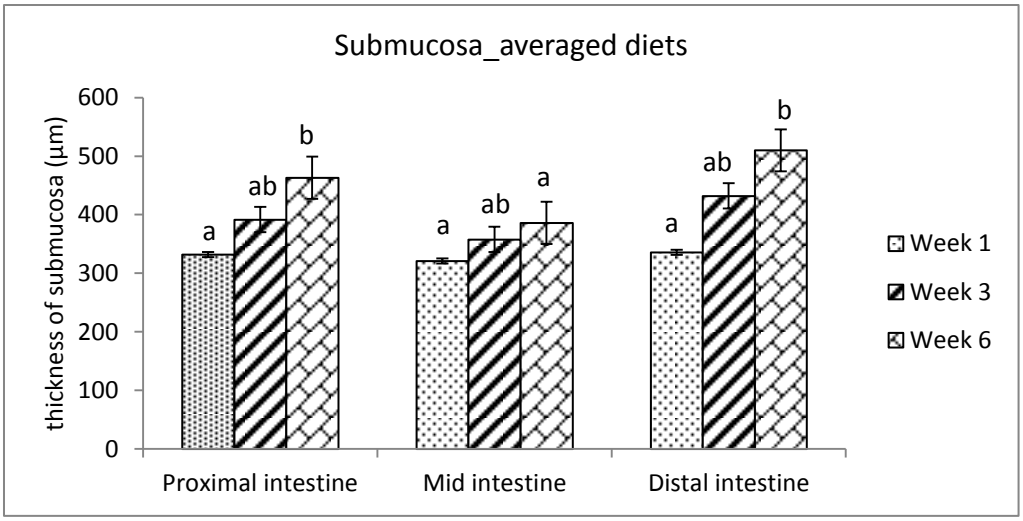

Figure 2.2 The effect of time on the submucosa thickness average over all seven experimental diets in different parts of the intestine of Nile tilapia. Each bar shows overall mean of the thickness of submucosa for each week with standard deviation represented by error bar. Bars within each region of intestine having no common letter are significantly different (n=42; $p<0.05$)

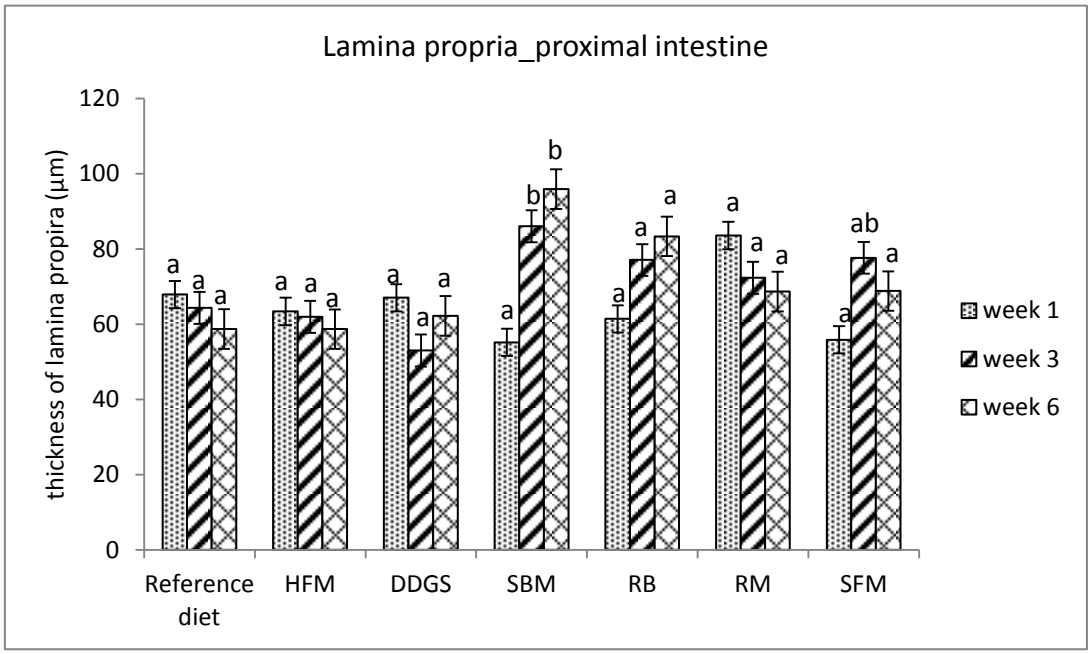

Figure 2.3 Effect of diet composition on the thickness of lamina propria in proximal intestine of Nile tilapia over time. Each bar shows overall mean the time effect for each diet composition with standard deviation represented by error bar. Bars within diets lacking a common letter are significantly different (n=6; $p<0.05$). HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

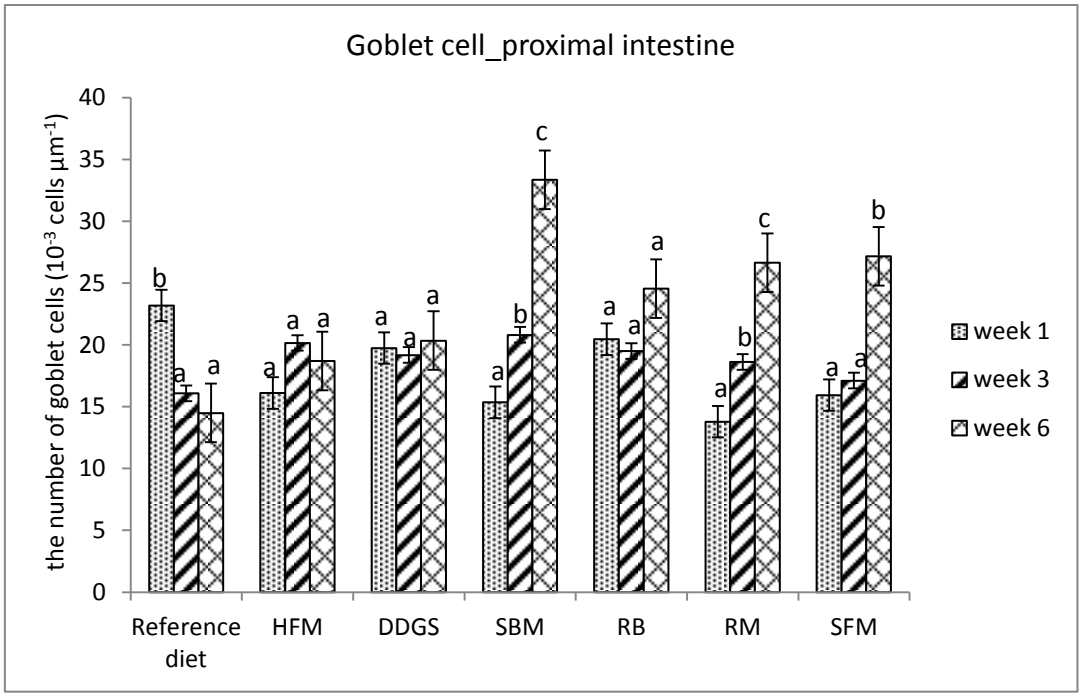

Figure 2.4 Effect of diet composition on the number of goblet cells in the proximal intestine of Nile tilapia over time. Each bar shows overall mean the time effect for each diet composition with standard deviation represented by error bar. Bars within diets lacking a common letter are significantly (n=6; $p<0.05$). HFM, hydrolysed feather meal; DDGS, dried distillers grains with solubles; SBM, soybean meal; RB, rice bran; RM, rapeseed meal; SFM, sunflower meal.

2.3.5 The correlation between intestinal morphology and digestibility of nutrient

Table 2.9 shows the correlation between the changes in intestinal morphology and the nutrient concentrations in the diet. With the exception of energy, there were no correlations between nutrient concentrations of the diet and intestinal morphology in the proximal and mid intestine. However, in the distal intestine, dietary NSP showed a positive relationship with LP and GC, whereas energy, protein and lipid content had a negative relationship.

Table 2.9 Correlations between the intestinal morphology and the nutrient correlations

	Proximal intestine			Mid intestine			Distal intestine		
Nutrition composition in diet	SM	LP	GC	SM	LP	GC	SM	LP	GC
Protein	-0.14	0.26	-0.28	-0.03	-0.03	-0.16	0.21	-0.10	-0.47*
Fat	-0.38	-0.19	-0.11	0.02	0.07	0.19	0.07	-0.53*	-0.34
Ash	0.14	0.42	0.30	0.10	0.10	0.21	-0.17	0.01	0.42
Phosphorous	-0.23	0.32	0.19	-0.07	0.02	0.01	-0.13	-0.27	0.27
Energy	-0.43	-0.48*	-0.38	-0.06	-0.03	-0.02	0.20	-0.48*	-0.65**
NSP	0.37	0.28	0.36	-0.10	-0.43	-0.34	-0.22	0.47*	0.64**

SM, submucosa; LP, lamina propria; GC, goblet cell; NSP, non-starch polysaccharides; $^{*}p<0.05$; $^{**}p<0.01$.

Furthermore we tested if intestinal morphological parameters correlated with the digestibility of the nutrients (Table 2.10). Most nutrients digestibility did not correlate with morphological parameters. However, there was one exception, NSP digestibility, correlated strongly with morphological parameter changes in the proximal and distal intestine. In addition, the thickness of SM in the proximal intestine was positively related to the digestibility of protein and lipid.

Table 2.10 Correlations between intestinal morphology and the digestibilty of nutrients

	Proximal intestine			Mid intestine			Distal intestine		
ADC	SM	LP	GC	SM	LP	GC	SM	LP	GC
Protein	0.52*	0.05	0.12	0.23	0.14	0.40	-0.15	0.30	0.17
Fat	0.52*	0.12	0.13	0.25	0.14	0.33	-0.21	0.35	0.33
Ash	0.36	-0.19	-0.12	0.23	-0.21	0.25	-0.001	0.35	0.05
Phosphorus	0.30	-0.42	-0.23	0.13	-0.06	0.27	0.04	0.26	-0.22
Energy	0.07	-0.09	-0.08	0.50*	0.22	0.37	0.04	-0.07	-0.31
NSP	0.50*	0.44*	0.58**	0.37	0.15	0.19	-0.20	0.62**	0.63**

ADC, Apparent digestibility coefficients ; SM, submucosa; LP, lamina propria; GC, goblet cell; NSP, non-starch polysaccharides; $^{*}p<0.05$; $^{**}p<0.01$.

2.4 Discussion

The quality of the diet/ingredient can be assessed/evaluated by various criteria, like: the nutrient digestibility values, the nitrogen/energy balance parameters; but also alteration in intestinal morphology.

2.4.1 Digestibility and introgen/energy balance

The ADC values found in this study are in line with literature. It is clear that ADC of the respective nutrients depends on the source of ingredients used ($P<0.001$). The ADC of dry matter provides a measure of the total quantity of a feed ingredients that is digested and absorbed (Fagbenro, 1999). The highest ADCs of dry matter (ranging from 77-78 %) were observed in hydrolysed feather meal and soybean meal based diets, which might be related to the low carbohydrates content in these ingredient. The protein ADC values of the tested ingredients are in agreement with other studies (Sklan *et al.*, 2004a, Guimarães *et al.*, 2008a, Tram *et al.*, 2011). The ADC of protein was highest in soybean meal (92.2 %) followed by sunflower meal, DDGS, rapeseed meal, hydrolysed feather meal, and rice bran in decreasing order. This indicates that these ingredients, especially soybean meal, have the potential for being used as a protein source in Nile tilapia feeds. Phosphorous present in form of phytate is known to be unavailable to fish due to the lack of endogenous or microbial phytase in their intestinal tract, which is required to digest phytate (Lall, 1991). With the exception for DDGS, ADC of phosphorous in plant protein ingredients was lower than in animal protein ingredients, which is in agreement with the results found in hybrid tilapia (Zhou and Yue, 2012) and cobia (Zhou *et al.*, 2004). Although the phosphorous concentrations were higher in rice bran, rapeseed and

sunflower meal, the ADCs of phosphorous of those ingredients were lower than those in hydrolysed feather meal, DDGS and soybean meal. These results indicate that the lower dietary concentration of phosphorous is associated with a better digestibility. Similar findings were also reported for hybrid tilapia (Zhou and Yue, 2012), rainbow trout (Burel *et al.*, 2000) and cobia (Zhou *et al.*, 2004). The ADCs of energy were lower in plant ingredients than in animal ingredients (feather meal), with the exception for soybean meal. Virtually all of the total gross energy in hydrolysed feather meal originates from protein and lipid, so an efficient utilization of the gross energy in hydrolysed feather meal was expected. Similar results were reported by Zhou and Yue (2012) and Sklan et al. (2004a) for hybrid tilapia and other species such as hybrid striped bass (Sullivan and Reigh, 1995) or cobia (Zhou *et al.*, 2004). In general, the current results demonstrated that the digestibility of plant protein can even be higher than of animal protein depending on the source of raw materials.

The nitrogen balance revealed that hydrolysed feather meal had the lowest nitrogen retention in spite of having a high protein digestibility (87 %) and the highest digestible nitrogen intake. This was also reflected in a very low protein efficiency (34 %). Most probably, the protein from the hydrolysed feather meal diet was used as an energy source. This was substantiated by the very high amount of branchial and urinary nitrogen losses in this diet diets. However, also the imbalanced amino acids of feather meal (Lee, 2002, Guimarães *et al.*, 2008a) could partially explain the low nitrogen retention. Fish fed the rice bran diet had a similar nitrogen retention as the other diets, but had the highest protein efficiency. Keeping in mind that the rice bran diet had about 35 % protein content compared to 44 % protein content in the reference diet, it means that Nile tilapia utilized the rice bran diet more efficiently than the reference diet. The same trend was also observed with fish fed the DDGS diet. Except for hydrolysed feather meal, the protein efficiency values reported in the current study are comparable to other studies for Nile tilapia (Kaushik *et al.*, 1995, Tran-Duy *et al.*, 2008b, Figueiredo-Silva *et al.*, 2013).

Energy balance data showed that differences between diets in terms of energy retained can be attributed to the differences in maintenance energy requirements. For different fish species, energy maintenance requirements seem to be dependent on culture conditions such as water temperature (Lupatsch and Kissil, 2005, Pirozzi *et al.*, 2010) dissolved oxygen level in water (Glencross, 2009) and stocking density (Lupatsch *et al.*, 2010). However, it is not clear to which extent dietary macronutrients composition has an effect on maintenance requirements. Glencross *et al.* (2008) showed that energy maintenance requirements for rainbow trout did not differ when fish were fed on a 15 % or 30 % lupin kernel meal diet compared to a fish meal based diet. On the other hand, changing dietary mineral levels affected the maintenance requirements in Nile tilapia

(Saravanan *et al.*, 2013) and in African catfish (Dersjant-Li *et al.*, 2001). Moreover, in pigs changing the fibre fraction also affected the maintenance requirements through changes in the physical activity (Schrama *et al.*, 1998). Our study shows that the fish fed the hydrolysed feather meal and soybean meal had higher energy maintenance requirements than fish fed other diets. Schrama *et al.* (2012) showed that dietary ingredients composition have altered maintenance requirements. It becomes clear from our results that it is indeed the changes in dietary ingredient composition which caused the differences in maintenance requirements.

The changes in energy maintenance requirements lead to trade-offs with other energy-demanding processes such as the functioning of the primary epithelia barrier in the gastrointestinal (GI) tract (Segner *et al.*, 2012). In other words, the intestinal morphology alterations induced by an inflammation response is often considered an energy cost. However, in this study, we could not detect a correlation between protein efficiency and energy balance with the degree of intestinal morphology parameters alterations (data not shown).

2.4.2 Intestinal morphology

In fish, the GI tract is the place for food digestion and nutrient uptake as well as a primary barrier that prevents entrance of harmful agents (Niklasson *et al.*, 2011). Several studies have shown that different feed ingredients can affect intestinal morphology (Baeverfjord and Krogdahl, 1996, Urán *et al.*, 2009). When animal protein ingredients are replaced by plant-based ingredients, fish get exposed to a series of "foreign" components such as starch and anti-nutritional factors that can interfere with the natural processes occurring in the intestine (Steiner and Encarnacão, 2010). In Nile tilapia, several reviews have dealt with the subject of replacing fish meal by plant protein based diets (Tram *et al.*, 2011, Zhou and Yue, 2012, Vidal *et al.*, 2015, Figueiredo-Silva *et al.*, 2015), and more recently, Tran-Ngoc *et al.* (2016a) showed that soybean meal combined with an environmental factor had negative effects on the intestinal morphology of Nile tilapia, but the information regarding how the intestinal morphology is affected by other plant-based ingredients is still scare.

The use of plant ingredients in diets can expose fish to cumulative effects of anti-nutritional factors resulting in a late manifestation of pathological conditions (Krogdahl *et al.*, 2010). In comparison with the other tested ingredients, soybean meal caused significant negative changes in the intestinal morphology of Nile tilapia such as widening of the SM and LP, and an increase of the number of GC. Soybean is widely known to contain adverse anti-nutritional compounds which induce intestinal disorders in Atlantic salmon (van den Ingh *et al.*, 1991, Baeverfjord and Krogdahl, 1996, Urán *et al.*, 2009), rainbow trout (Heikkinen *et al.*, 2006, Venold *et al.*, 2012), or summer flounder (*P.*

dentatus) (Bone, 2013). In Nile tilapia, the observed alterations in intestinal morphology were less severe than in salmonids. In addition, the alterations in the intestinal morphology were mainly found in the proximal region. In Nile tilapia, the mucosa of the intestinal proximal section is longer and has more branched villi than the middle and distal region (Gargiulo *et al.*, 1998) and therefore might be more vulnerable to intestinal disorder.

It is particular interesting to see that the other plant ingredients such as rice bran, rapeseed, sunflower and DDGS meal did not induce severe changes in the morphology of the intestine in spite of the fact that they also contain anti-nutritional factors (ANFs). Several investigations indicate that alcohol-soluble substances (van den Ingh *et al.*, 1996, Francis *et al.*, 2001), especially soya saponins (Knudsen *et al.*, 2008, Knudsen *et al.*, 2007, Krogdahl *et al.*, 2010) are factors which may potentially induce the morphological changes. Bone (2013) suggested that pathological changes observed in fish fed soybean meal may be due to additive or synergistic impacts of several anti-nutritional factors. Saponin levels in SBM range between 5–7 g kg^{-1} (Knudsen *et al.*, 2006), but are absent or at much lower levels in other plant ingredients. They are usually insufficient to induce pathological changes in the gastrointestinal tract (Gatlin *et al.*, 2007). This could be confirmed by the work of Madalla (2008) who showed that histopathological changes in the intestine could not be detected after feeding Nile tilapia with morning leaf meal, cassava leaf meal and cassava root meal. More recently, Aanyu *et al.* (2014) showed also that a diet with sunflower and cotton seed cake caused no significant negative changes in the number and length of intestinal folds. Couto *et al.* (2016) found the inclusion of 30 % whole cereal meal in diets for gilthead seabream (*Sparus aurata*) did not affect the intestinal morphology. Also in Atlantic salmon, the intestinal morphology were not affected in fish fed diets containing either cellulose, native or extruded NSPs (Kraugerud *et al.*, 2007). In contrast, Sitjà-Bobadilla *et al.* (2005) reported a hypertrophied submucosa of intestine when gilthead sea bream were fed with the combination of plant proteins (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin). The increase of the thickness of the SM found in that study is similar to the alteration in intestinal morphology induced by soybean in Nile tilapia, but other features of the alteration in intestinal morphology were not observed. Therefore, aside from soybean meal, there is still scant evidence suggesting that the other plant ingredients used in the present study cause alteration in the morphology of the GI tract.

2.4.3 The correlation between nutrient content, nutrient digestibility and intestinal morphology

The morphology of the GI tract is influenced by the type of feed eaten by the fish (El-Bakary and El-Gammal, 2010, Delashoud *et al.*, 2010). The efficiency of which hydrolysed nutrients are absorbed can be assessed using the morphology of the intestine since it is the main site for nutrient absorption (Rodrigues *et al.*, 2009). This study attempted to investigate the effect of nutrient composition on the alteration in the intestinal morphology and indirectly on the nutrient digestibility.

In general, nutrient composition did not have implications for the intestinal morphology in the proximal and mid intestine, with the exception of energy. However, in the distal part, we found a negative correlation between nutrient concentration (protein, lipid and energy) and the number of GC and the thickness of LP. This might be related to the fact that high dietary concentration of protein and lipid go together with low dietary levels of carbohydrates (especially NSP). This would result in a higher amount of NSP in the distal intestine, which might have affected the integrity of the distal intestinal epithelium. When the intestinal epithelium is disturbed, an increased mucus flow stimulates the removal of pathogens from the entire subsequent intestine, which might prevent pathogens from entering the damaged intestinal epithelium (van der Marel *et al.*, 2014). In Nile tilapia, the GC increased in density in the distal intestine of the intestine where mucus secretion may facilitate excretion increased amounts of undigested materials (Sklan *et al.*, 2004b). Consequently, in this study, we found a positive correlation between the number of goblet cells in the distal intestine and the NSP concentration in the diet.

We found that the nutrient digestibility is positively related with the thickness of SM in the proximal and mid intestine. The thicker the submucosa in the proximal and mid intestine, the better the digestibility of protein, lipid and energy. Most probably, a thicker submucosa increased the surface area for absorption leading to the increased digestibility of protein and lipid that we observed. Sitjà-Bobadilla *et al.* (2005) also reported an increased thickness of SM in the intestine of gilthead sea bream fed a mixture of plant protein sources and it did not impair feed conversion. Furthermore, the proximal intestine is the major site where proteins enter the absorptive epithelium by pinocytosis and is enclosed within the cytoplasmatic vacuoles where they are digested by hydrolytic enzymes (Gargiulo *et al.*, 1998). This might explain why we found a correlation between nutrient digestibility and the thickness of SM in the proximal intestine.

If this assumption is true, care should be taken in interpreting changes in the intestinal morphology. Baeverfjord and Krogdahl (1996) described "non-infectious subacute enteritis" in fish by changes in the intestinal morphology: shortening of intestinal villi, loss of supranuclear vacuolization of the enterocytes, widening of lamina propria of villi,

and infiltration of inflammatory cells in the LP. We use three of these intestinal parameters to describe SBM-induced enteritis in Nile tilapia (Tran-Ngoc *et al.*, 2016a). However, the thickness of SM may also be larger because of the nutrient absorption in the proximal part of the intestine, resulting in the proliferation of the intestinal cells (Gargiulo *et al.*, 1998). The thickness of LP and the number of GC are strong indicators for the alteration in intestinal morphology since the correlation between nutrient composition and nutrient digestibility, especially for NSP digestion, was predominantly found in LP and GC.

NSP hamper digestion in fish (Sinha *et al.*, 2011). Enzymes such as ß-glucanase or ß-xylanases that digest NSPs are scarce or even non-existent in fish (Kuz'mina, 1996). Therefore, the dietary NSPs remain indigestible and cannot be used as an energy source. In Nile tilapia, a reduction nutrient digestibility was associated with an increased digestion viscosity (Leenhouwers *et al.*, 2007a). The delay of digestion passage in the intestinal tract, as a result of increase in viscosity, may stimulate microbial fermentation of NSPs in the intestine with the production of volatile fatty acids (VFA) as an end product (Sinha *et al.*, 2011). Administration of NSPs in the diet of tilapia and African catfish (*Clarias gariepinus*) increases VFA levels in the intestinal tract mainly acetic acid, propionic and butyric acid (Amirkolaie *et al.*, 2006, Leenhouwers *et al.*, 2007a, Leenhouwers *et al.*, 2007b). Scheppach (1994) showed that in domestic animal, VFAs stimulated the colonic sodium and fluid absorption and exerted a proliferative effect on the colonocytes. In fish, the beneficial effects of VFA resulted in strong antimicrobial activity, growth promotion (Elala and Ragaa, 2015, Koh *et al.*, 2016, Ng *et al.*, 2009, Zhou *et al.*, 2009) and improvement in villi development in the intestine, leading to a better nutrient utilization (Robles *et al.*, 2013). Our study showed a positive correlation between NSP content and the widening of the LP and the increase of the number of GC. In addition, the better NSP were digested, the stronger were the alterations in intestinal morphology. These outcomes are opposite to the ones we would expect. At this moment, a plausible explanation is lacking.

2.5 Conclusions

This study demonstrates that feed ingredients have impact on the alteration in intestinal morphology parameters but also on the nutrient digestibility and the nitrogen/energy balance. Soybean meal caused the most obvious alteration in intestinal morphology although it was well digested. The digestibility of protein was highest in soybean meal followed by sunflower meal, DDGS, rapeseed meal, hydrolysed feather meal, and rice bran in decreasing order. The lowest protein retention efficiency was found in fish fed the hydrolysed feather meal and the highest in rice bran. Hydrolysed feather meal and soybean meal resulted in the highest predicted maintenance energy requirement. The

alterations in intestinal morphology was not related to the nutrient digestibility nor to nitrogen/energy balance parameters. However, NSP digestibility was an exception, which was positively correlated with the intestinal morphology. The higher dietary NSP concentration was also related with the intestinal morphology.

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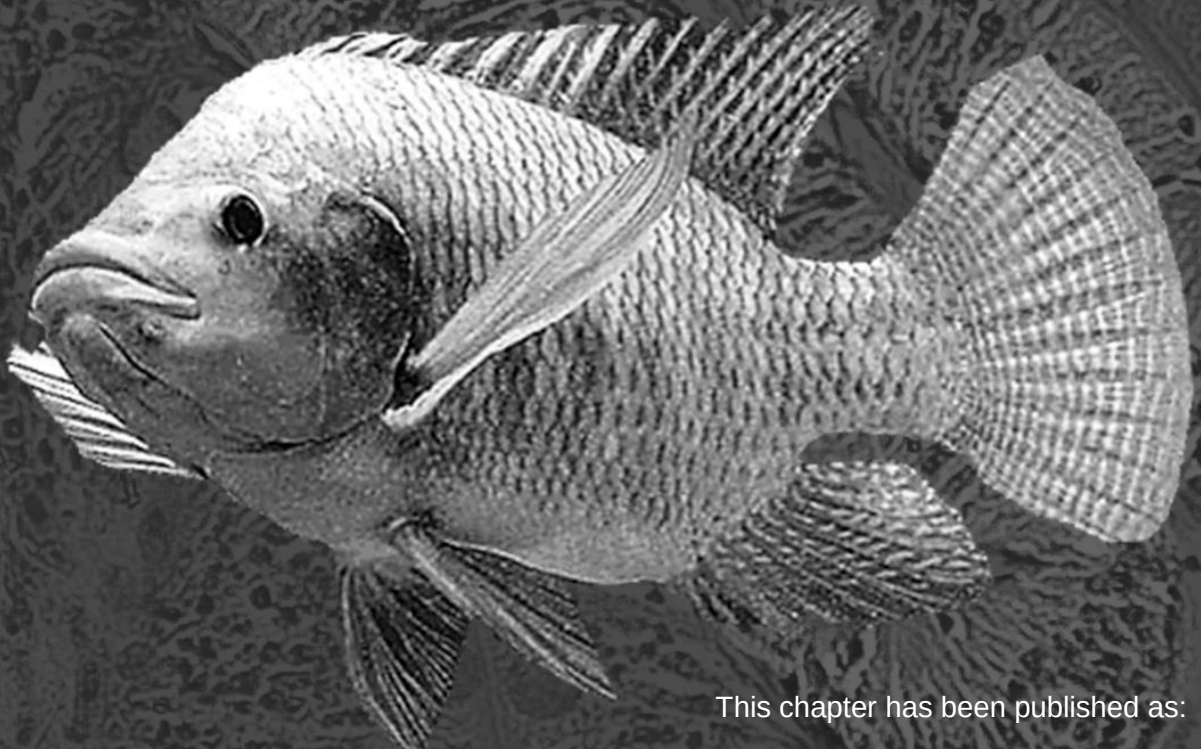
Chapter 3

Interaction between dissolved oxygen concentration and diet composition on growth, digestibility and intestinal health of Nile tilapia (Oreochromis niloticus)



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Abstract

Abstract

The present study was undertaken to evaluate the individual and combined effects of oxygen concentration and diet composition on the growth, nutrient utilization and intestinal morphology of Nile tilapia (*Oreochromis niloticus*). Two recirculating aquaculture systems were used to create differences in oxygen concentration: normoxia (6.9 mg L^{-1}) and hypoxia (3.5 mg L^{-1}). Two diets were formulated using a different soybean meal (SBM) content to create a contrast in the potential to affect the gut barrier function. Triplicate groups of 35 fish with initial mean body weight of 23 gram were fed "Control" diet containing 20 % fish meal and "Test" diet containing only plant protein at normoxia and hypoxia for 8-weeks. Six fish per treatment were sampled for intestinal morphological analysis at the end of week 1, 4 and 8. The proximal, middle and distal intestine were processed for quantitative histology, in order to count goblet cells (GC) and eosinophilic granulocytes (EG); and to measure the thickness of lamina propria (LP) and sub-epithelial mucosa (SM). The study showed that growth was best in the "Control" diet under normoxia, while no interaction between oxygen and diet composition was found. Hypoxia reduced nutrient digestibility significantly ($P<0.05$). For the "Test" diet, the decline in digestibility was larger than for "Control" diet over time. Both diet composition and oxygen level induced changes in intestinal morphology of Nile tilapia. We observed a thickening of the LP and SM caused by an increased infiltration of inflammatory cells, and an increased number of GC and EG among the enterocytes. The negative effect of increased soybean meal on intestinal morphology was enhanced at low oxygen level, and aggravated in time. The SBM enteritis-like symptoms were more pronounced in the proximal than in the distal intestine of Nile tilapia.

3.1 Introduction

Gut barrier function is vital for gut health and maintaining the general health of the fish (Jutfelt, 2011, Sundh, 2009). The gut acts as a physical and chemical barrier providing the first line of defence against invading organisms entering the body via consumed feed or through ingestion of water (Cain and Swan, 2010). Impairment of the gut barrier function enables an increased exchange of materials between gut lumen and body in two ways: paracellularly through the tight junctions or transcellularly through transcytosis which enhances the susceptibility to bacterial infection. The gut barrier function is influenced by several factors like dietary composition, environmental challenges, gut microbial population and immune functioning of the fish (Jutfelt, 2006).

In intensive rearing of salmonids, fluctuating concentrations of dissolved oxygen (DO) is known to create prolonged stress disrupting the tight junctions in the intestinal wall, therefore increasing the permeability (Olsen *et al.*, 2002, Sundh *et al.*, 2010). Paracellular permeability increased in both the proximal and distal intestine of Atlantic salmon (*Salmo salar*) subjected to 50 % DO saturation (Sundh *et al.*, 2010, Niklasson *et al.*, 2011). Several reports were published on the reactions of fish to hypoxia in terms of growth, digestibility and oxygen consumption such as blue tilapia (*Oreochromis aureus*) (Papoutsoglou and Tziha, 1996), Nile tilapia (*Oreochromis niloticus*) (Ishibashi *et al.*, 2002), European sea bass (*Dicentrarchus labrax*) (Thetmeyer *et al.*, 1999) and juvenile turbot (*Scophthalmus maximus*) (Pichavant *et al.*, 2000). The effects on the gut barrier function were not analysed. In general, data on impacts of oxygen concentration on gut barrier function in other fish species than salmonids are lacking.

Also dietary composition is associated with an impaired intestinal barrier (Jutfelt, 2011). In salmonids, for example, plant proteins and especially soybean meal cause histological, morphological and functional changes in the gastrointestinal tract (GI). These changes may widen the lamina propria and increase the presence of inflammatory cells (a mixed cell population, including lymphocytes, macrophages and polymorphonuclear leucocytes) (Baeverfjord and Krogdahl, 1996); induce enteritis in the distal intestine (Urán *et al.*, 2008b, Urán *et al.*, 2009); and shorten villi and microvilli (van den Ingh *et al.*, 1991). Soy saponins in combination with other plant protein ingredients are suspected to be the possible inducers of this intestinal inflammation (Knudsen *et al.*, 2008). Some of these changes have also been found in species other than salmon, e.g., common carp (*Cyprinus carpio*) (Urán *et al.*, 2008a), rainbow trout (*Oncorhynchus mykiss*) (Nordrum *et al.*, 2000) and Gilthead sea bream (*Sparus aurata*) (Bonaldo *et al.*, 2008), summer flounder (*Paralichithys dentatus*) (Bone, 2013). In Nile tilapia, a recent study suggested a mild enteritis after feeding a diet with 43 % soybean meal (Mahmoud *et al.*, 2014).

In the present work, it is hypothesized that one single stressor might have a minor impact on the intestinal morphology and digestion whereas the impact might increase when two stressor are combined. In the current study, the combined impact of oxygen stress and a dietary challenge on digestion and intestinal morphology was examined. Moreover, these impacts was assessed over time.

3.2 Materials and methods

3.2.1 General design

The experiment was conducted at the Fisheries Faculty of Nong Lam University, Ho Chi Minh city, Vietnam. Juvenile all sex reversed male Nile tilapia were obtained from a local hatchery. A 2 x 2 factorial design was used to evaluate the effects of diet and DO concentration in water on growth, digestibility and intestinal morphology of Nile tilapia. Two diets were formulated and tested in triplicate tanks under normoxia and hypoxia conditions. To study for temporal effects on digestibility and intestinal morphology, faeces and intestinal tissues were collected and analysed at different time points (after 1, 4 and 8 weeks). The experiment lasted for 56 days.

3.2.2 Housing

Two identical recirculating aquaculture systems were used to create differences in DO concentration. Each system, was composed by a large water tank (1 m^3) connected to six cylindrical tanks (150 L) holding the fish. Each cylindrical tank was equipped with a small bio-filter. Water was pumped from both the water storage tanks via the small bio-filters to the fish tanks (total N=12). Each tank was stocked with 35 Nile tilapia with an initial mean body weight of 23 ± 0.3 g.

In the normoxia treatment, the DO concentration was aimed to be closed to 100 % saturation. Saturation was achieved by 1) aerating water in the water storage tank with air stones before supplying the fish-tanks; and 2) adding air stones inside each fish-tank. In the hypoxia treatment, the aeration was reduced to reach approximately 50 % saturation in the storage tank by adjusting the output of air stones, and the fish-tanks were not aerated. DO concentration was measured daily using an oxygen meter (MW600 model, Milwaukee Instruments Inc., Rocky Mountain, NC, USA). The measured DO concentration inside the fish-tanks at the normoxia and hypoxia treatments were 80 % (6.9 ± 0.2 mg L^{-1}) and 40 % (3.5 ± 0.4 mg L^{-1}), respectively. During the trial, the water temperature was at 27 ^{0}C and pH at 6.7. Total ammonia nitrogen (TAN) was remained below 2 mg L^{-1} in both systems. TAN was measured with a Sera kit (SERA GmbH, Heinsberg, Germany) and pH with a pH meter (digital mini-pH meter, BASF, Kuantan, Malaysia). The rate of system water renewal was calculated based on TAN and DO concentration. One third of volume of the storage tank was renewed if TAN was above 2

mg L^{-1} and/or DO concentration below 6 and 3.5 mg L^{-1} at the normoxia and hypoxia, respectively. Minimally, every three days, water was added to compensate water losses due to faces collection and evaporation.

3.2.3 Diets

Two diets were formulated using a different soybean meal content to create a contrast in the potential effect on the gut barrier function. The "Control" diet contained 20 % fish meal and the "Test" diet contained only plant proteins. Both diets were formulated to be isoproteic and isolipidic. In both diets, the amino acids and other nutrients were exceeding the minimum requirements of Nile tilapia (NRC, 2011). Extruded pellets (2 mm diameter) were produced and the marker chromium oxide (Cr_2O_3) was added to the diets at 1.0 g kg^{-1} to measure digestibility coefficients. Formulation and diet analyses are shown in Table 3.1.

Table 3.1 Formulation and nutrient content of experimental diets.

	Diets	
	Control	Test
***Ingredient* (%)**		
Fish meal	20	-
Soybean meal	21.3	54.5
Rice bran	18	10
Distillers dried grains with soluble	20	10.7
Cassava	13	13
Fish oil	0.5	0.5
Soybean oil	2.2	4.3
DL-Methionine	1.0	1.0
Dicalcium phosphate	1.0	3.0
Vitamin and mineral premix*	2.0	2.0
Chromic oxide	1.0	1.0
Analysed nutrient content on DM basis (g kg^{-1})		
Dry matter (DM; g kg^{-1} diet)	924	930
Crude protein	302	314
Crude fat	81	86
Crude fiber	44	45
Total carbohydrates[1]	517	518
Ash	100	82
Phosphorus	11	10
Chromic oxide	16	15

[1]Calculated as, total carbohydrates = 1000 – (crude protein + crude fat + ash)

* Vitamin and minerals premix (per kg of feed): contain vitamin A 40,000 IU; D_3 9600 IU; E 300mg; C 700mg; K_3 60mg; B_1 54mg; B_2 64mg; B_6 64mg; Niacine 96mg; Pantothenic acid 132mg; Choline 60% 800mg; Fe 259.2 – 336.8mg; Cu 43.2 – 52.8mg; Zn 1060 – 1540mg; Mn 216 – 264mg; Co 0.44 – 0.52 mg; I 17.28 – 21.12 mg; Se 2.16 – 2.64 mg; Folic acid 20mg; Biotin 1mg; Inositol 192mg; Carrier.

3.2.4 Experimental procedures

Fish were fed initially with the same amount of feed corresponding to 3 % of body weight per day; twice daily at 9.00 hrs and 16.00 hrs. In case of fish refusing feed in any tank, the feeding of all tanks was reduced to prevent uneaten feed. Feed intake, growth, feed

conversion ratio and survival were assessed at the end. Feed intake was adjusted every 2 weeks.

Faeces were collected twice a day, prior to feeding, using sedimentation columns as described by Cho *et al*., (1982). Faecal collection bottles were placed in a thermostatic box with ice to avoid the bacterial degradation of nutrients in faeces. To follow the changes in digestibility over time, the faeces collected during week 1, 4 and 8 were kept separately and stored at -20 ^{0}C until analysis.

Samples for gut histology were taken three times during the experiment, at the end of week 1, 4 and 8. At each moment, two fish from each tank (6 fish per treatment) were sacrificed with an overdose of 2-phenoxy-ethanol (1.0 ml L^{-1}) and the entire intestinal tract was subsequently dissected. The intestinal tract was divided in three parts: proximal (from the pyloric part of the stomach to the spiral part of the intestine), middle (the spiral part of the intestines) and distal (from end spiral part of the intestines to 2 cm before anus) (Pirarat *et al.*, 2011). One-cm portions of each these segments were fixed by immersion in Bouin's fixative.

3.2.5 Light microscopy

After fixation, gut samples were dehydrated in graded ethanol solutions (70 %, 80 %, 90 % and 96 %) before equilibration in xylene and embedding in paraffin (Dimitroglou *et al.*, 2010). Next, 5 µm transverse sections were cut and stained using Alcian blue periodic acid-Schiff staining technique (AB-PAS) (Grethen, 1962). Alcian blue staining enhances the contrast between goblet cells (GC) and the supranuclear vacuoles. Each slide contained 2 sections of a complete cut of an annular ring of proximal, middle, and distal intestine. Sections were photographed with an INFINITY 2 CCD digital camera (Lumenera®, Sony, Japan) connected to a Meiji microscope (Meiji Techno, Japan). The pictures were processed and analysed using the Infinitive Analyse software (Lumenera®, Sony, Japan). Based on literature on salmonids and carp (Urán *et al.*, 2008a, Urán *et al.*, 2008b, Urán *et al.*, 2009) that measured enteritis response by an increase of inflammatory cells like eosinophilic granulocytes (EG) and GC, and an increase in submucosa (SM) and lamina propria (LP) thickness, we selected the same enteritis indicators in this study. First, qualitative histological analyses were done to observe main treatment and temporal effects. Based on those qualitative analyses, quantitative measurements were defined. For quantitative measurements, four villi were randomly selected per slide and per gut segment for each fish. For each selected villus, a surface area was defined by drawing the outer boundary of the villus (the apical site of enterocytes) as depicted in Figure 3.1. At the basal part, the lowest point of the enterocytes lining on each side of the villus was interconnected. The surface of the marked area was recorded. Within each marked area the following measurements were

made: a) counting the GC (expressed in number per μm^2 of villi); b) counting EG as inflammatory cells (expressed in number per μm^2 of villi); c) measuring LP thickness (µm) as half way the length of the villi in the marked area; d) measuring SM (µm) as the distance between inside of the loose connective tissue and the point where both enterocytes lining contacted each other. Values of these four parameters were averaged per fish and per gut segment.

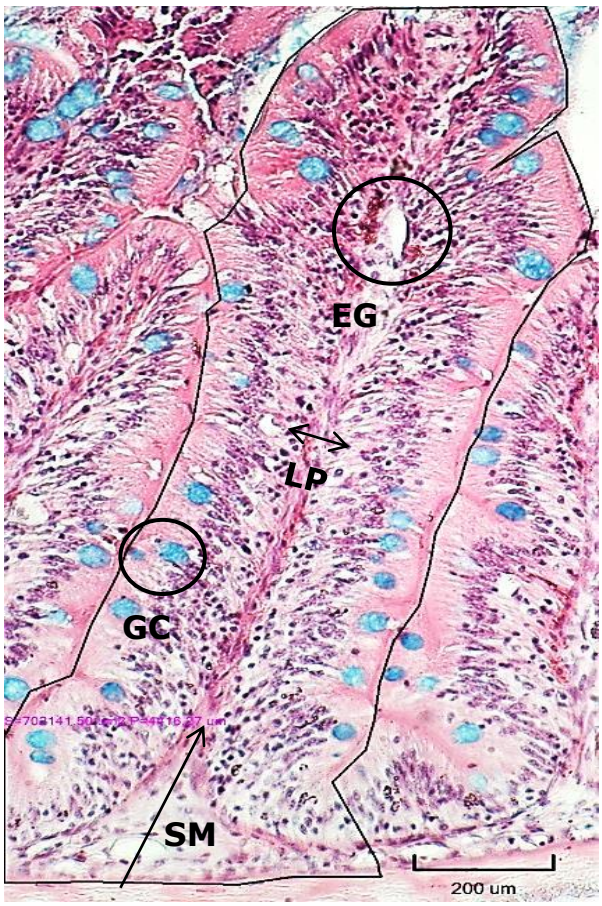


Figure 3.1 Morphological appearance of the proximal intestine of Nile tilapia fed control diet under normoxia condition. The submucosa (**SM)** is found between the basal part of the folds. The lamina propria (**LP)** is a delicate and single thin layer of cells underlying the epithelium. The goblet cells (**GC)** are present in a basal amount and scattered among the enterocytes. The eosinophilic granulocytes (**EG)** are present few in SM basal small quantity (not shown) or occasionally migrate into LP. The density of GC, EG (cells μm^{-2}), the thickness of LP and SM (µm) on marked area of a selected villi were measured. (Haematoxyline/Eosin and Alcian blue staining, x40).

3.2.6 Chemical analyses

Representative samples of diets and faeces were analysed following the standard laboratory procedures (AOAC, 2005): dry matter (DM) was measured after drying samples in an oven at 105 ^{0}C for 24 h; ash was calculated from weight loss by incineration of the samples for 24 h at 550 ^{0}C in a muffle furnace; crude protein (N x 6.25) and crude fat was measured using the Kjeldahl and acid hydrolysis method, respectively. Chromic oxide was determined according to Furukawa (1966). Total

carbohydrate was calculated as 1000 – (crude protein + crude fat + ash). Organic matter was calculated as 100 - % Ash (Thompson *et al.*, 2008).

3.2.7 Calculation

Specific growth rate was assessed as SGR (% bw d^{-1}) = [($\ln W_f$ - $\ln W_i$)/t] x 100, where W_f and W_i are the final and initial weight, respectively; t is the experimental duration in days. Feed intake (FI_{bw}) of fish was expressed as a percentage of body weight (in % bw d^{-1}) = FI / BW_{mean} x 100, where FI (g d^{-1}) is the average feed intake per fish per day and BW_{mean} is the mean body weight, which was calculated as BW_{mean} (g) = (W_f + W_i) / 2. Feed conversion ratio (FCR) was calculated as FCR (g g^{-1})= FI_{tot} / (W_f - W_i), where FI_{tot} (g) is the total feed intake per fish during the experimental period.

Apparent digestibility coefficients (ADC, in %) of dry matter, crude protein, crude fat, total carbohydrate, ash and phosphorus were calculated as described by Cho *et al*. (1982):

ADC of nutrients (%) = 100 – 100 x (%Cr_{feed}/%Cr_{faeces}) x (%$Nutrient_{faeces}$/%$Nutrient_{feed}$)

Where Cr_{feed} and Cr_{faeces} are the dietary and faecal chromic oxide content and $Nutrient_{faeces}$ and $Nutrient_{feed}$ are the faecal and dietary nutrient content (all in % on dry matter basis)

3.2.8 Statistical analyses

Statistical analyses were performed using IBM SPSS 22 (SPSS Inc., Chicago, USA). Growth parameters were analysed by 2-way ANOVA. ADC was repeatedly measured over time for each tank. Therefore, the effect of diet, oxygen and time were analysed by repeated measurements analysis. Histology were analysed for effect of time, diet and oxygen by 3-way ANOVA. The results were considered statistically significant when *P*-values were below 0.05.

significant when *P*-values were below 0.05.

3.3 Results

3.3.1 Fish performance

The survival rate was not significantly (P<0.05) affected by any of the treatments (Table 3.2). Feed intake was similar for all treatments, but final body weight and SGR were affected by diet (P<0.05) being lower on the "Test" diet containing only plant proteins. DO concentration affected the final body weight, SGR, and FCR (P<0.01). At hypoxic conditions, final body weight and SGR were lower and FCR higher when compared to fish kept at normoxia conditions. No interaction effect between oxygen and diet were observed for any of the performance parameters (P>0.05; Table 3.2).

Table 3.2 Growth performance of Nile tilapia during 56 days experimental period.

	Diet				P-value			
	Control		Test					
	Normoxia	Hypoxia	Normoxia	Hypoxia	SEM	O	D	OxD
Experimental time (d)	56	56	56	56	-	-	-	-
Tanks (n)	3	3	3	3	-	-	-	-
Fish per tank(n)	35	35	35	35	-	-	-	-
Survival (%)	98	100	97	96	1.1	ns	ns	ns
Initial BW (g)	22.7	22.9	22.8	22.8	0.3	ns	ns	ns
Final BW (g)	82.0	73.6	78.7	69.8	1.2	***	*	ns
Feed intake (%bw d^{-1})	2.37	2.45	2.35	2.37	0.03	ns	ns	ns
SGR (%bw d^{-1})	2.3	2.1	2.2	2.0	0.04	**	*	ns
FCR (g g^{-1})	1.17	1.30	1.19	1.31	0.03	**	ns	ns

SEM, Standard error mean; O, oxygen; D, diet; OxD, interaction effect between oxygen and diet; SGR, specific growth rate; FCR, feed conversion rate; ^{ns}P>0.05; *P<0.05; **P<0.01; ***P<0.001, 0.05<$^{\#}$P<0.1.

3.3.2 Digestibility

Table 3.3 showed the average values of apparent nutrient digestibility for the whole period, statistics on the main effects of oxygen, diet and their interaction, and finally statistics on time effects. The results showed that ADC of dry matter, crude protein, ash and carbohydrate were higher in normoxia than in hypoxia (P<0.05). Diet also influenced the ADC of dry matter, organic matter, crude protein and crude fat with better results being achieved with the "Test" diet. However, no significant interaction between oxygen and diet composition on digestibility could be found during the experimental period.

Oxygen concentration had an effect on digestibility, but that effect was changed over time (P_{Week} < 0.05, P_{WxO} < 0.05; Table 3.3). This time effect was most significant for ADC of protein. Figure 3.2 showed that ADC of protein was not different between normoxia and hypoxia at week 1 and week 4. However, at week 8, ADC of protein was clearly reduced at hypoxia condition.

Table 3.3 Effect of the experimental diets and oxygen level on apparent digestibility of nutrients

ADC (%)	Diet				SEM	Main effects			[1]Time effects			
	Control		Test									
	N	H	N	H		O	D	OxD	W	Wx O	Wx D	WxOxD
DM	85.5	85.0	87.2	85.9	0.5	**	**	ns	**	***	ns	ns
OM	86.9	86.7	88.5	88.8	1.2	ns	*	ns	*	*	ns	ns
CP	94.7	94.4	96.4	95.8	0.2	*	***	*	***	***	***	*
CF	96.7	96.8	97.7	97.8	0.2	ns	***	ns	**	ns	ns	ns
Ash	71.9	70.5	73.7	70.8	1.2	*	ns	ns	ns	**	ns	ns
Carbo.	81.0	80.4	82.1	80.3	0.7	*	ns	ns	**	***	ns	ns$^{\#}$

ADC, apparent digestibility coefficient; DM, dry matter; OM, organic matter; CP, crude protein; CF, crude fat; Carbo., carbohydrates; SEM, standard error; N, normoxia; H, hypoxia; O, oxygen; D, diet; W, week; WxO, interaction effect between week and oxygen; WxD, interaction effect between week and diet; WxOxD; interaction effect between week, oxygen and diet; ^{NS}P>0.05; *P<0.05; **P<0.01; ***P<0.001; 0.05<$^{\#}$P<0.1.
[1]Time effects are related to Figure 3.2.

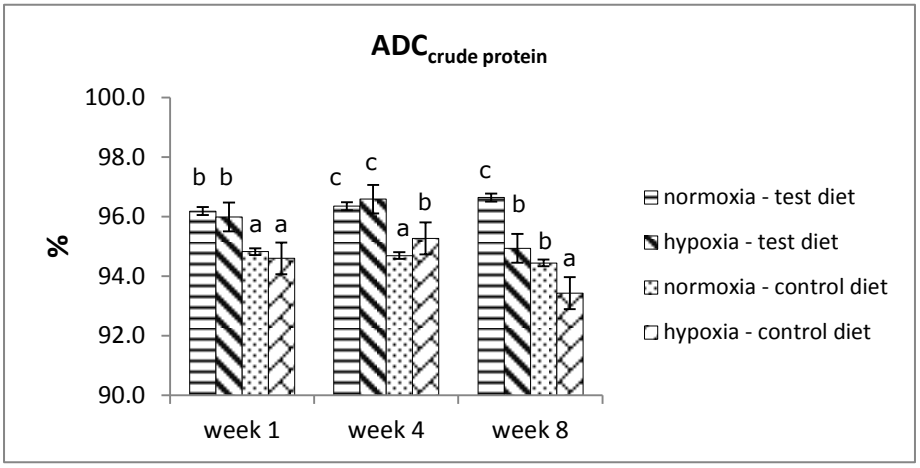


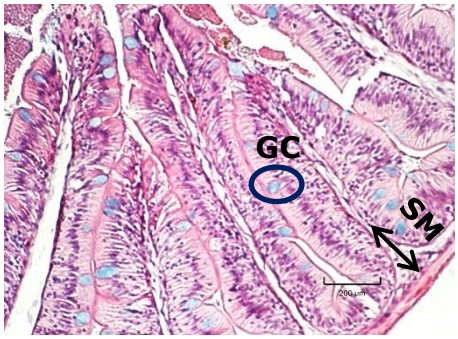
Figure 3.2 Effect of diet composition and oxygen levels on ADC of crude protein over time in Nile tilapia. Each bar shows overall mean main effects between oxygen and diet for each week with standard deviation represented by error bar. Bars within weeks having different lower case letters are significantly different (*n* = 3; P<0.05).

3.3.3 Intestinal morphology

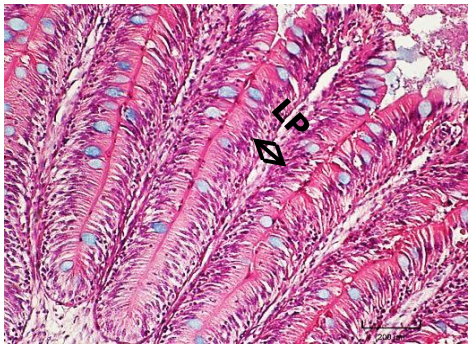
Morphological changes were primarily found in the proximal intestine of Nile tilapia (Figure 3.3).

3.3.3.1 Main effects of oxygen, diet and their interaction

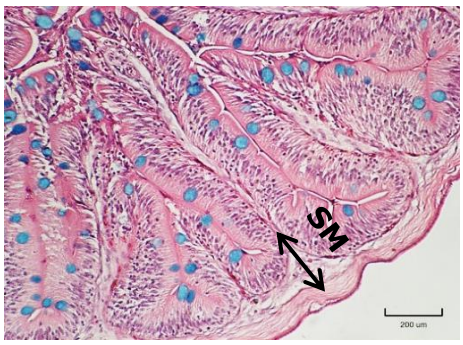
Table 3.4 showed the average values per diet and oxygen concentration (2x2) of intestinal parameters over the whole period. The results showed that hypoxia had a negative effect on SM and LP by increasing of the thickness of SM and LP. The effect of diet was found to be significant on all four intestinal parameters. Fish fed the "Test" diet exhibited both an increase of the thickness of SM and LP, and also the number of GC and EG. The interaction between oxygen and diet was only found at SM and EG in proximal and distal section, respectively.



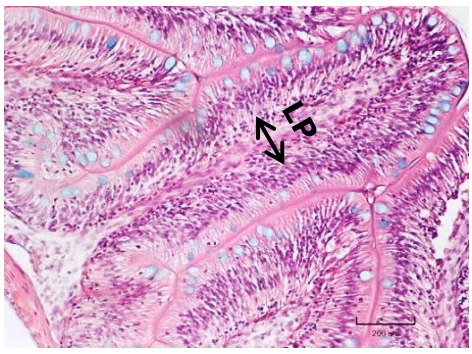
A) Normoxia – Control diet – week 1



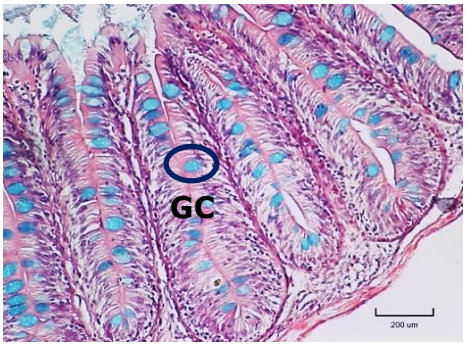
B) Normoxia – Control diet – week 8



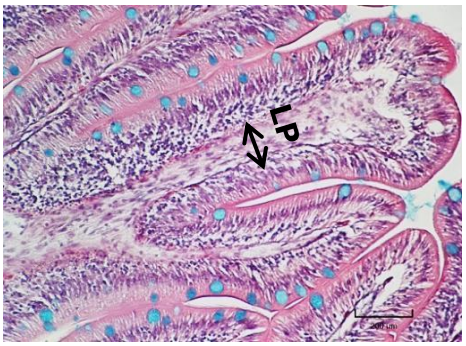
C) Hypoxia – Control diet – week 1



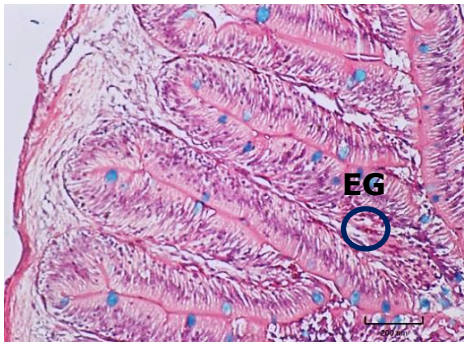
D) Hypoxia – Control diet – week 8



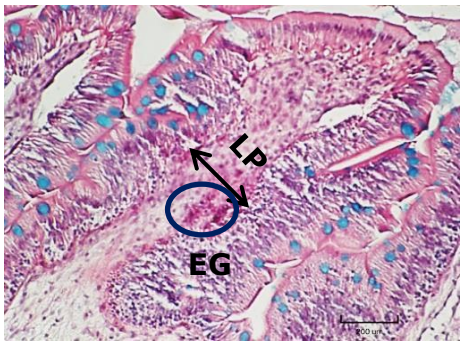
E) Normoxia – Test diet – week 1



F) Normoxia – Test diet – week 8



G) Hypoxia– Test diet – week 1



H) Hypoxia– Test diet – week 8

Figure 3.3 The morphology of the proximal intestine of Nile tilapia fed the "Control" and "Test" diet under normoxia and hypoxia at week 1 and 8. Haematoxylin/Eosin and Alcian blue staining, x40. Fish fed the "Control" diet under normoxia did not significantly show morphological intestinal changes during 8 weeks: the goblet cells (**GC**) were observed to be scattered among the enterocytes, the eosinophilic granulocytes (**EG**) were found in submucosa and the lamina propria. Lamina propria (**LP**), a thin layer of cells, was not widening and the submucosa (**SM**), between the basal part of the folds and muscularis mucosa, was not enlarged. The morphological changes were more obvious at week 8, especially in fish fed the "Test" diet under hypoxia.

3.3.3.2 Time effects related to intestinal morphological changes

We found that DO concentration and diet composition had main effects on intestinal morphology, but those effects were not constant over time. Those time effects (P_{WxDxO}<0.05; Table 3.4) can be seen in LP (Figure 3.4) and GC (Figure 3.5) in the proximal intestine and EG (Figure 3.6) in the distal intestine. The thickness of LP in the proximal section was similar among treatments at week 1. However, the thickness of LP increased over time in fish fed the "Test" diet, and this tendency was greater under hypoxia at week 4 and week 8 (Figure 3.4). The number of GC in the proximal intestine of fish fed the "Test" diet at hypoxia was significantly higher compared to other treatments at week 1. At week 4 and 8, there was no significant differences in the number of GC in the proximal intestine among the treatments (Figure 3.5). The number of EG in the proximal intestine increased at week 1 of fish fed the "Test" diet at hypoxia. This EG number decreased at week 4 and levelled off until the end of week 8 (Figure 3.6).

Table 3.4 The effect of diet (Control vs. Test) and oxygen levels (normoxia vs. hypoxia) on morphological parameters in Nile tilapia at different sections of the intestine.

	Diet				SEM	*Main effects*	*[1]Time effect*
	Control		Test				
	N	H	N	H		O, D, OxD	W, WxO, WxD, WxOxD
Submucosa (µm)							
Pro.	208.9	235.4	250.6	353.9	32.8	O**, D***, OxD*	W***
Mid.	211.4	234.2	270.0	366.4	40.9	O*, D***	-
Dist.	262.2	302.9	332.3	399.0	33.2	O**, D***	W***, WxD*
Lamina propria (µm)							
Pro.	38.7	50.4	44.6	72	7.7	O***, D**	W***, WxD, WxDxO*
Mid.	37.8	52.9	65.9	89.2	11.2	O**, D***	W*
Dist.	61.7	76.9	122.1	146.6	30.5	D***	-
Goblet cells (10^{-5} cells µm^{-2})							
Pro.	3.7	3.3	3.3	4.1	0.6	OxD$^{\#}$	WxDxO*
Mid.	4.1	3.6	2.9	3.0	0.6	D*	W**
Dist.	3.3	3.4	3.8	3.6	0.7	-	W*
Eosinophilic granulocytes (10^{-5} cells µm^{-2})							
Pro.	0.3	0.3	0.3	0.3	0.1	-	W*
Mid.	0.3	0.2	0.4	0.6	0.2	D*	
Dist.	0.5	0.3	0.2	0.8	0.2	OxD**	W**, WxDxO**

Pro., proximal intestine; Mid., mid intestine; Dist., distal intestine; N, normoxia; H, hypoxia; SEM, standard error; O, oxygen; D, diet; OxD, interaction effect between oxy and diet; W, week; WxO, interaction effect between week and oxygen; WxD, interaction effect between week and diet; WxOxD; interaction effect between week, oxygen and diet; ^{NS}P>0.05; *P<0.05; **P<0.01; ***P<0.001; 0.05<$^{\#}$P<0.1

[1]Time effects are related to Figure 3.4, 3.5 and 3.6.

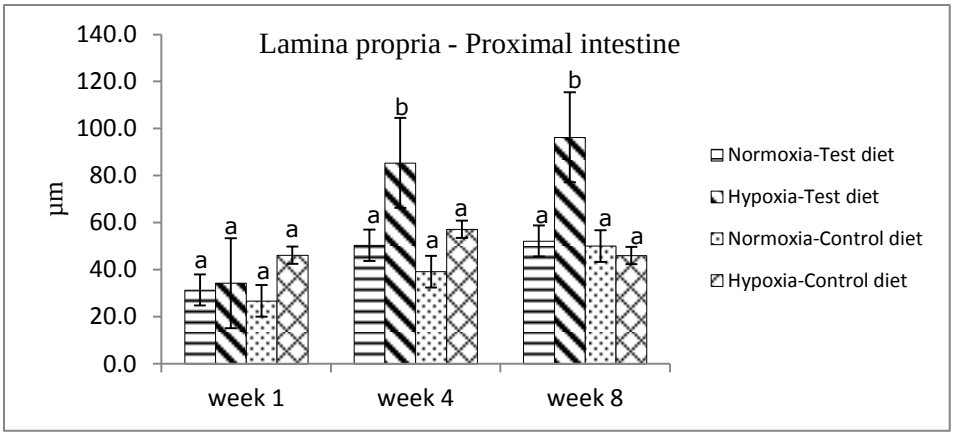


Figure 3.4 Effect of diet composition and oxygen levels on the thickness of lamina propira in proximal intestine over time in Nile tilapia. Each bar shows overall mean the main effects between oxygen and diet for each week with standard deviation represented by error bars. Bars labelled with different lower case letters are significantly different for each week (*n*= 6; P<0.05).

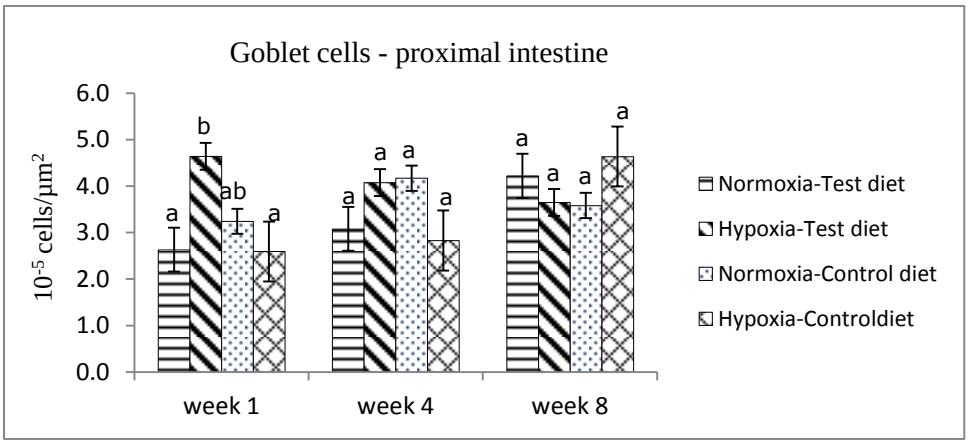


Figure 3.5 Effect of diet composition and oxygen levels on the number of goblet cells in proximal intestine over time in Nile tilapia. Each bar shows overall mean the main effects between oxygen and diet for each week with standard deviation represented by error bars. Bars within weeks lacking a common letter are significantly different for each week (*n*= 6; P<0.05).

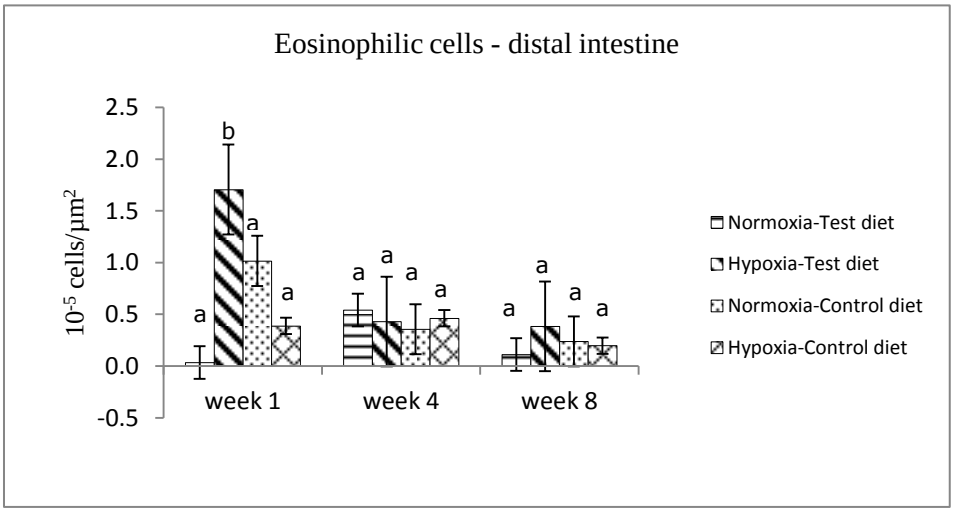


Figure 3.6 Effect of diet composition and oxygen levels on the number of eosinophilic cells in distal intestine over time in Nile tilapia. Each bar shows overall mean the main effects between oxygen and diet for each week with standard deviation represented by error bars. Bars labelled with different lower case letters are significantly different for each week (*n* 6; P<0.05).

3.4 Discussion

3.4.1 Diet effect on intestinal morphology

Diets containing a high level of soybean meal (SBM) may induce an inflammatory response in the distal intestinal epithelium of carnivorous fish species (Krogdahl *et al.*, 2003, Nordrum *et al.*, 2000). Baeverfjord and Krogdahl (1996) described this condition as "a non-infectious sub-acute inflammation of the distal intestine" that causes shortening of intestinal villi, loss of supranuclear vacuolization of the enterocytes and widening of the LP. In Nile tilapia, many studies have shown considerable success in partially or totally replacing fish meal with SBM in diets although the literature on the occurrence of SBM and other plant protein-induced enteritis is scare (El-Sayed, 1999, El-Saidy and Gaber, 2002). In the present study, the "Test" diet rich in SBM (55 %) caused significant negative changes in the intestinal morphology of Nile tilapia especially in the proximal intestine. The increase in thickness of SM and LP in the proximal intestine and the proliferation in number of GC and EG in the distal intestine may suggest that these parameters are good indicators to detect for SBM-induced enteritis like symptoms in Nile tilapia. This is supported by Urán *et al.* (2009) who used those parameters to assess the degree of SBM-induced enteritis on Atlantic salmon. Carnivorous fish such as Atlantic salmon are believed to be less adapted to diets containing ingredients of plant origin (Urán, 2008). Therefore, the severity of SBM-induced enteritis in Atlantic salmon is clearly understood. Changes in intestinal morphology in Nile tilapia as observed in this study, were mild to moderate. Many plant-derived nutrient sources contain anti-nutritional factors (Francis *et al.*, 2001) that may lead in some species to an intestinal inflammation. Soya-saponins, oligosaccharides and alcohol-soluble anti-nutrients components in the soybean are all candidates to cause changes in the histological appearance which may be involved in triggering the enteritis response in Atlantic salmon (van den Ingh *et al.*, 1996, Knudsen *et al.*, 2007), rainbow trout (Penn, 2005) and summer flounder (Bone, 2013). In Nile tilapia, previous studies could not detect histopathological changes in the intestine after feeding the fish with plant ingredients such as moringa leaf meal, cassava leaf meal and cassava root meal (Madalla, 2008). Recent work also indicated that a diet with sunflower and cotton seed cake did not negatively affect the number and length of intestinal folds in Nile tilapia when compared to the control diet containing fish meal (Aanyu *et al.*, 2014). In contrast, Mahmoud et al. (2014) reported that the intestine of Nile tilapia fed a diet with 43 % SBM showed mild degeneration of the intestinal mucosa. The result is in line with our current study that feeding Nile tilapia with diets containing high levels of soybean meal to caused SBM-enteritis like symptoms.

3.4.2 Dissolved oxygen concentration effect on intestinal morphology

The quality and quantity of food are important factors in the development of the intestinal mass and the mucosal architecture (Buddington *et al.*, 1997, Jutfelt, 2006), but environmental conditions can also play an important role (Lakani *et al.*, 2013). DO is considered one of the most influential environmental factors on the development and growth of fish (Diaz and Breitburg, 2009). In Atlantic salmon, hypoxia is known to be stressful and to create long-term disturbances of the physical intestinal barrier. Subjecting Atlantic salmon to hypoxia (50 % DO saturation) tended to shorten villi height, to display altered appearance of the intestinal segments, and to increase size of SM of the enterocyte layer in the distal intestine (Sundh *et al.*, 2010). In Nile tilapia, low oxygen level may affect feeding behaviour, growth rate (Tsadik and Kutty, 1987), stress response and energy metabolism (Ishibashi *et al.*, 2002). To the best of our knowledge, the occurrence of intestinal disorders in Nile tilapia under hypoxia has never been reported earlier. The present study demonstrated that intestinal morphology of Nile tilapia was negatively affected when fish were exposed to a low (40 % saturation) DO concentration, with an increasing cell infiltration into the SM and LP in the proximal intestine.

In most fish groups the GI tract is functionally and morphologically divided into distinct regions, and the barrier function varies between the regions (Jutfelt, 2011). The typical signs of SBM-induced enteritis was described in the distal intestine of Atlantic salmon due to the complex structure in the distal intestine compared to the proximal intestine (Sundh *et al.*, 2010). In Nile tilapia, however, the mucosa of the proximal intestine is longer and has more branched villi than the distal intestine (Gargiulo *et al.*, 1998) and is therefore more vulnerable for intestinal disorders. As a result, in Nile tilapia the enteritis-like changes seemed to be particularly present in the proximal intestine, whereas in Atlantic salmon these changes occurred in the distal intestinal segment (van den Ingh *et al.*, 1991).

3.4.3 The effect of diet and DO concentration on performance and digestibility

Hypoxia or an imbalanced diet can induce stress response in Nile tilapia. A combination of these factors can have a synergetic effect. Although growth was not affected by the interaction of both factors, the ADC of crude protein and crude fat was affected.

The current findings showed that the final body weight and SGR were significantly lower and FCR was higher to fish exposed to hypoxia condition. This response is in agreement with previous data obtained by Tran-Duy (2008a) that observed lower growth of Nile tilapia at hypoxia (3.0 mg L^{-1}) than at normoxia (5.0 mg L^{-1}). Similar reduced growth

responses were also seen for juvenile turbot and sea bass when exposed to hypoxic conditions of 3.2 and 4.5 mg O_2L^{-1}, respectively (Pichavant *et al.*, 2001).

Next to oxygen, growth was also affected by diet composition. In this study, the final mean body weight and SGR were significantly lower in "Test" than in the "Control" diet under normoxia conditions. This agrees with results of Lin an Luo (2011) who found that a 100 % SBM protein substitution level from fish meal could significantly decrease weight and SGR in hybrid tilapia (*O. niloticus x O. aureus)*. Other studies showed that higher inclusion levels of solvent-extracted soybean and full-fat soybean meal also reduced growth performance and feed utilization in Nile tilapia (Riche *et al.*, 2001, Riche and Garling, 2004).

Besides assessing the interaction between oxygen and diet composition on intestinal morphology and growth in Nile tilapia, it is also important to know the mechanisms behind the observed performance such as nutrient digestibility. In this study, at both diets the ADC averaged over time (week 1, 4 and 8) of dry matter, crude protein, ash and carbohydrates were considered decreased in Nile tilapia subjected to hypoxia. Tran-Duy (2008) suggested that the reduced nutrient digestibility at hypoxia is caused by hampering of the energy expenditure. Limited oxygen availability restricts energy expenditure and consequently reducing the processing of absorbed nutrients. In order to prevent accumulation on absorbed nutrient in the blood and to reduce the energy expenditure on digestion, the ADC might be reduced. Various studies (Mahmoud, 2009, Rašković *et al.*, 2011) suggested intestinal morphological changes as an explanation for differences in ADC between diets. An alternative explanation for the reduced nutrient digestibility at hypoxia might be due to changes in intestinal morphology. This is supported by the observation that the effect of hypoxia on ADC of crude protein was only present in week 8 (Figure 3.2), which parallels with the time related alteration in intestinal morphology especially in the proximal part of intestine (Figure 3.4). The proximal intestine is the major site where proteins enter the absorptive epithelium by pinocytosis and become enclosed within the cytoplasmatic vacuoles where they are digested by hydrolytic enzymes (Gargiulo *et al.*, 1998). This might explain why the ADC of crude protein at hypoxia was significantly lower than those at normoxia when a widening of the LP was observed at the proximal intestine.

The protein quality of dietary ingredients is the leading factor affecting fish performance and digestibility (Harlıoğlu, 2012). In this study, the ADCs of dry matter, crude protein, organic matter and crude fat were affected by diet ($P<0.05$). At the same environmental conditions, the ADC of the "Test" diet was higher than the "Control" diet. If Nile tilapia are reared under normoxic conditions, as much as 100 % of the fish meal protein can be replaced by plant protein source without reducing the digestibility. The ADC of crude

protein of SBM of Nile tilapia was 93 % (Sintayehu *et al.*, 1996), 87.4 % (Köprücü and Özdemir, 2005) and 92.1 % (Tran-Ngoc *et al.*, 2016b).

3.4.4 The interaction between diet and DO concentration in time on intestinal morphology

It was hypothesized that the interaction between diet and environmental condition on intestinal morphology of Nile tilapia would be stronger over time. We observed that the interaction was not constant over time. At week 4, minor intestinal morphology changes were found in fish fed "Test" diet at hypoxic condition. A slight increase in cellularity of the connective tissue in the LP and SM was seen in some individuals. At week 8, these changes were fully developed. The SBM enteritis-like symptoms were not only depending on the diet and DO concentration, but in time those symptoms became worse. Contrary to previous observations made with respect to common carp only exposed to a diet with high SBM (Urán *et al.*, 2008a), Nile tilapia did not show any recovery signs after feeding SBM under hypoxic condition. The mechanism why intestinal barrier function of Nile tilapia was more severely affected by the combination of diet and DO concentration is yet unclear to us and requires further study.

3.5 Conclusions

The current study showed that changes in intestinal morphology of Nile tilapia were affected by both diet composition and DO concentration. The negative effect on intestinal morphology of increased plant protein inclusion was enhanced at low oxygen level. The interaction between diet and DO concentration on intestinal morphology was aggravated with time. SBM enteritis-like symptoms were more pronounced in the proximal intestine than in the distal intestinal of Nile tilapia, as opposed to the situation in Atlantic salmon. Finally, the SBM enteritis-like symptoms coincided with a reduction on growth performance and protein digestibility.

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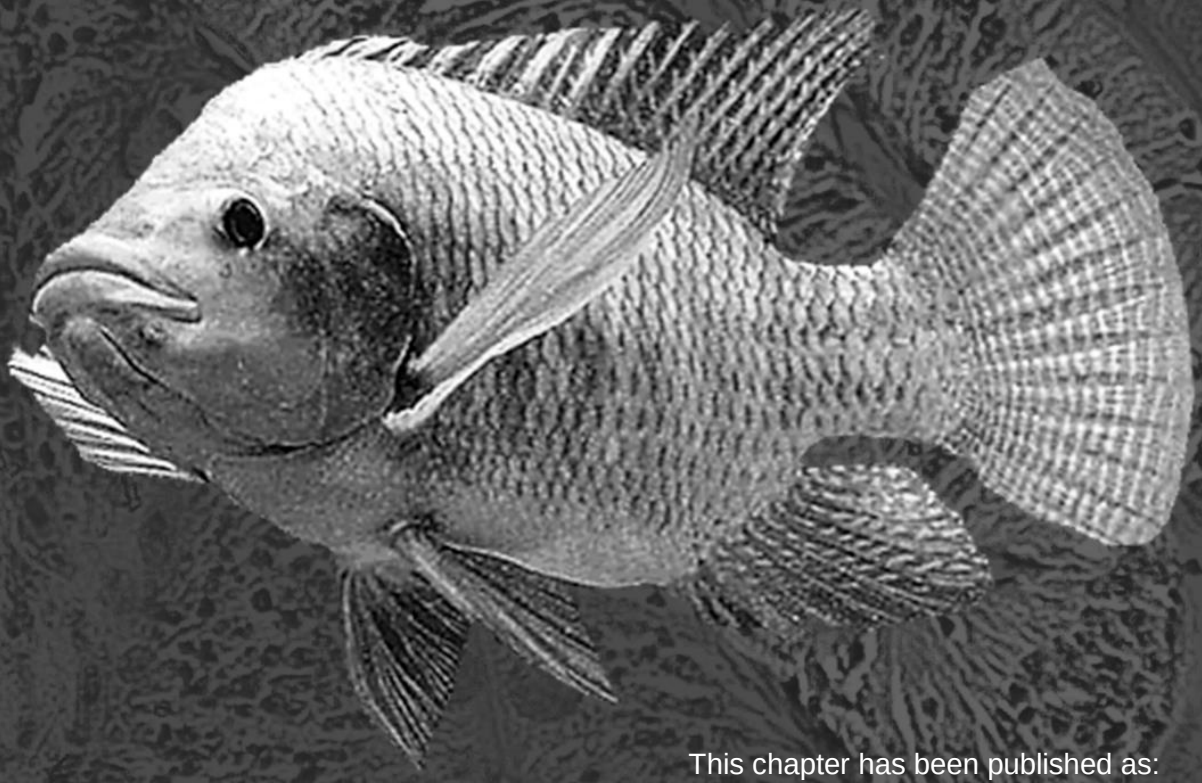
Chapter 4

Salinity and diet composition affect digestibility and intestinal morphology in Nile tilapia (Oreochromis niloticus)



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Abstract

Abstract

An increase in salinity of the aquatic habitat can be an environmental stress factor for fresh aquatic organism, including fish. The present study investigated the impact of salinity and diet composition on digestibility and intestinal morphology of Nile tilapia. Triplicate groups of 35 fish weighing 35 gram were fed a "Control" and "Test" diet and kept at salinity of 0 ‰ and 15 ‰ for 8 weeks. The diets were formulated using a different soybean meal content to create a contrast in alterations in intestinal morphology. Six fish per treatment were sampled for intestinal morphology analysis at the end of week 1, 4 and 8. The proximal, middle and distal intestine were processed for quantitative histology, in order to count the number of goblet cells and eosinophilic granulocytes; and to measure the thickness of lamina propria and sub-epithelia mucosa. The study showed that a salinity of 15 ‰ increased the nutrient digestibility; however this enhanced digestibility diminished over time. The intestinal morphology was influenced by both the salinity as well as the dietary soybean meal content. For soybean meal, the impact on morphology was largest in the proximal region of the intestine, whereas for salinity the largest impact was in the distal region. The negative effect of increased soybean meal on the lamina propria thickness was enhanced at a salinity of 15 ‰ and aggravated in time. Difference in nutrient digestibility induced by the salinity seemed not to be related to alterations in the intestinal morphology.

4.1 Introduction

The salinity of coastal aquatic and terrestrial ecosystems may increase due to rising sea levels and more frequent typhoons (Baroiller, 2015). Salinity changes can cause stress to the fish due to interference with physiological homeostasis and routine biological processes (Kültz, 2015). Freshwater fish such as red tilapia (*Oreochromis mossambicus* x *O.niloticus)* (Hepher *et al.*, 1983), rainbow trout (*Oncorhynchus mykiss)* (Oorschot and Boon, 1993) need more energy for osmoregulation maintenance when cultured in a saline environment. Nile tilapia, exposed to saline water showed changes in blood parameters (Verdegem *et al.*, 1997), immune function parameters (Choi, 2004), and histopathology (Hassan *et al.*, 2013, de Azevedo *et al.*, 2015), which is suggested to make them more susceptible for infectious disease. The gills and gastrointestinal tract are important organs that interact with the environment and play a key role in osmoregulation (Baysoy *et al.*, 2013). In hyper-osmotic environments, fish loose water to, and gain salt from, the high ionic and hyper-osmotic environment. The hypersaline water-acclimated fish need to replace water loss by imbibing salt water and absorbing the salt and water via the gastrointestinal tract whereby water is retained in the body and excess salt is extruded via the gill (Li *et al.*, 2014). Water absorption is driven by active uptake of ions by the gastrointestinal tract, particularly Na^+ and Cl^- absorption across the intestinal epithelium (Yan *et al.*, 2013). Furthermore, identification of tight junction proteins with intestinal barrier functions combined also play an important role in osmoregulation (Marshall and Grosell, 2006). Therefore, the intestine is in contact with environmental water and may suffer morphologic alterations under physical and chemical environmental challenge (Reis *et al.*, 2009, Yuan *et al.*, 2010). It can be hypothesised that environmental challenges, like elevated salinity, may also alter nutrient digestion through the induced morphological alterations in the intestine. Nutrient digestibility in most fish species have been studied in relation to nutritional/dietary factors (Köprücü and Özdemir, 2005, Tram *et al.*, 2011, Zhou and Yue, 2012). Only a few studies addressed the impact of environmental factors such as temperature in Atlantic salmon (*Salmo salar*) (Ng *et al.*, 2004) or oxygen concentrations in Nile tilapia (Tran-Ngoc *et al.*, 2016a) on nutrient digestibility. In general, several studies on tilapia spp. have evaluated the influence of salinity on the performance and gill histopathology (Avella *et al.*, 1993, Kang'ombe and Brown, 2008, de Azevedo *et al.*, 2015), but information on the impact of salinity on digestibility and intestinal morphology is still scarce.

Exposure to stressors such as diet composition can also weaken the intestinal barrier function (Jutfelt, 2011). The inclusion of soybean meal (SBM) in the diet of the carnivorous Atlantic salmon was associated to the reduction of the integrity of the intestinal barrier function (Baeverfjord and Krogdahl, 1996, Krogdahl *et al.*, 2003,

Knudsen *et al.*, 2008). Also in Nile tilapia, an omnivorous species, recent studies have shown that the intestinal morphology can be affected by the presence of SBM in the diet causing mild to moderate enteritis (Mahmoud *et al.*, 2014, Tran-Ngoc *et al.*, 2016a).

Most studies focus on just one single stress factor and how it affects intestinal morphology. Recently, it was demonstrated in Nile tilapia that the impact of dietary SBM inclusion on digestion as well as intestinal morphology was enhanced when fish were exposed to hypoxia compared to normoxia (Tran-Ngoc *et al.*, 2016a). The potential impact of the interaction of different stress factors received less attention thus far. In the present study, we assessed the impact of salinity on the intestinal morphology and nutrient digestibility. Furthermore it was studied if dietary-induced stress (created by different inclusion levels of SBM) interacts with the impact of salinity on intestinal morphology and nutrient digestion.

4.2 Materials and methods

4.2.1 General design

A 2 x 2 factorial design was used to evaluate the effects of salinity and diet composition on growth, digestibility and intestinal morphology of juveniles of Nile tilapia. The experiment was conducted at the Fisheries Faculty of Nong Lam University, Ho Chi Minh City, Vietnam. The all-male juveniles Nile tilapia were obtained from a local hatchery.

4.2.2 Diets

Two different experimental diets were produced using a different SBM content to create a contrast to affect the intestinal morphology. A "Control" diet contained fishmeal, soybean meal and dried distillers grains with solubles as main protein sources (in equal amount). A "Test" diet contained only plant protein originating from soybean meal (~55 %) and dried distillers grains with solubles (11 %). Extruded pellets (2 mm diameter) were produced. The inert marker Cr_2O_3 was added to the diet at 1.0 % to measure digestibility coefficients. Formulation and diet analyses are shown in Table 4.1.

4.2.3 Housing condition and feeding

Two identical recirculating aquaculture systems were used. The design and water management of the systems were identical, but the systems differed in the water salinity concentration, 0 versus 15 ‰. We chose 0 ‰ as a normal condition for fish growing and 15 ‰ to be slightly above the isosmotic point in order to induce a moderate amount of stress. Six cylindroconical fish tanks with 150 L capacity per system (i.e., 12 in total) were used during the experimental period. The tanks were connected with a water filtration unit containing sponges, bio balls, and ceramic rings. Thirty five Nile tilapia with

initial mean body weight of 34.5 ± 0.3 gram were stocked in each tank. The experimental salinities were prepared by using either freshwater or sea water (36 ‰) diluted with freshwater to achieve the final concentration of 15 ‰. Throughout the experiment, the environmental/water quality parameters (mean ± SD) were maintained under optimal conditions for Nile tilapia: photoperiod 12 light: 12 dark h; water temperature (27.0 ± 0.2 ^{0}C); pH (7.1 ± 0.1); dissolved oxygen of water inside the fish-tanks (5.5±0.5 mg L^{-1}); and total ammonia nitrogen (<0.5 mg L^{-1}).

Table 4.1 Ingredient composition and analysed nutrient content of the experimental diets.

	Diets	
	Control	Test
***Ingredient* (%)**		
Fish meal	20	-
Soybean meal	21.3	54.5
Rice bran	18	10
Dried distillers grains with solubles	20	10.7
Cassava	13	13
Fish oil	0.5	0.5
Soybean oil	2.2	4.3
DL-Methionine	1.0	1.0
Di-calcium phosphate	1.0	3.0
Vitamin and Mineral Premix [a]	2.0	2.0
Chromic oxide	1.0	1.0
Analysed nutrient content on DM basis (g kg^{-1})		
Dry matter (DM; g kg^{-1} diet)	924	930
Crude protein	302	314
Crude fat	81	86
Crude fibre	44	45
Total carbohydrates [b]	517	518
Ash	100	82
Phosphorus	11	10
Chromic oxide	16	15

[a]Vitamin and minerals premix (per kg of feed): contain vitamin A 40,000 IU; D_3 9600 IU; E 300 mg; C 700 mg; K_3 60 mg; B_1 54 mg; B_2 64 mg; B_6 64 mg; Niacin 96 mg; Pantothenic acid 132 mg; Choline 60 % 800 mg; Fe 259.2 – 336.8 mg; Cu 43.2 – 52.8 mg; Zn 1060 – 1540 mg; Mn 216 – 264 mg; Co 0.44 – 0.52 mg; I 17.28 – 21.12 mg; Se 2.16 – 2.64 mg; Folic acid 20 mg; Biotin 1 mg; Inositol 192 mg; Carrier.
[b] Calculated as, total carbohydrates = 1000 – (crude protein + crude fat + ash).

Fish were fed initially with the same amount of feed corresponding to 3 % of body weight per day; twice daily at 9:00 and 16:00 h. Fish were fed with the same amount of feed corresponding to 3 % of body weight. At each feeding section, feed given and uneaten feed were recorded; in addition, uneaten pellets were collected and counted to determine the food intake accurately. In case of feed refusal occurred in on tank consistently, the feed given to all the tanks was reduced to prevent differences in realized feed intake between tanks.

The health status and welfare of the fish were visually checked daily. During weighing at the beginning and the end of experiment, the discomfort for the fish was reduced to a minimum through anesthetizing the fish by using of 2-phenoxy-ethanol (0.25 ml L^{-1},

Sigma). Fish with extremely abnormal behaviour and/or health problems indicated by e.g skin lesions and skin damage were removed from the experiment and euthanized by an overdose of 2-phenoxy-ethanol (1.0 ml L^{-1}, Sigma). Over all experimental treatments, the average mortality was below 5 % in this study, so the experiment was continued until the end.

4.2.4 Sampling and measurements

The entire experiment lasted for 8 weeks. To follow digestibility changes over time, faeces were collected twice a day, prior to feeding, at week 1, 4 and 8. Faeces were collected by using sedimentation columns described by Cho et al (1982), kept separately and stored at -20 ^{0}C.

During the growth period, fish were anesthetized of 2-phenoxy-ethanol (0.25 ml L^{-1}, Sigma) and group weighed every 2 weeks to calculate feed intake. At the end of week 1, week 4, and week 8, two fish were scooped gently in each tank (6 fish per treatment) using hand dip net and transferred to a plastic tub containing water with anaesthesia using an over dose of 2-phenoxy-ethanol (1.0 ml L^{-1}, Sigma), where after the entire intestinal tract was dissected for morphological analysis. The intestinal tract was divided into three regions: proximal (from the pyloric part of the stomach to the spiral part of the gut), middle (the spiral part of the gut) and distal (from end spiral part of the gut to 2 cm before anus) (Pirarat *et al.*, 2011). One-cm cuts of the three regions of the intestine were fixed by immersion in Bouin's fixative.

At the end of the experiment, the rest of fish in the tanks were anesthetized of 2-phenoxy-ethanol (0.25 ml L^{-1}, Sigma) weighed and subsequently euthanized by an overdose of 2-phenoxy-ethanol (1.0 ml L^{-1}, Sigma), according to the prescription of the Wageningen University Committee on Animal Experiments.

4.2.5 Light microscopy

After fixation, the intestinal samples were dehydrated and embedding in paraffin using standard procedures (Dimitroglou *et al.*, 2010). Transverse sections of 5 µm thickness were stained using Alcian blue periodic acid-Schiff staining technique (AB-PAS) (Grethen, 1962). The intestinal samples were photographed with an INFINITY 2 CCD digital camera (Lumenera®, Sony, Japan) connected to a Meiji microscope (Meiji Techno, Japan). Pictures were processed and analysed using the Infinitive Analyse software (Lumenera®, Sony, Japan).

The light microscopy regions were evaluated based on the quantitative method developed at Wageningen University for salmon (Urán, 2008), which was adapted for the response to SBM-induced enteritis in Nile tilapia (Tran-Ngoc *et al.*, 2016a). Briefly, the

measurements were done on four random villi selected per slide and per intestinal segment for each fish. The following intestinal morphology parameters were assessed: a) the number of goblet cells (GC, expressed in number per μm^2 of villi); b) the number of eosinophilic granulocytes (EG, expressed in number per μm^2 of villi); c) the thickness of the lamina propria (LP in µm) as the middle of the villi length; d) the thickness of the sub-epithelial mucosa (SM in µm), measured as the distance between the inner side of the loose connective tissue and the point where both enterocyte linings contact each other (Figure 4.1). Values of these four parameters were averaged per fish and per intestinal segment.

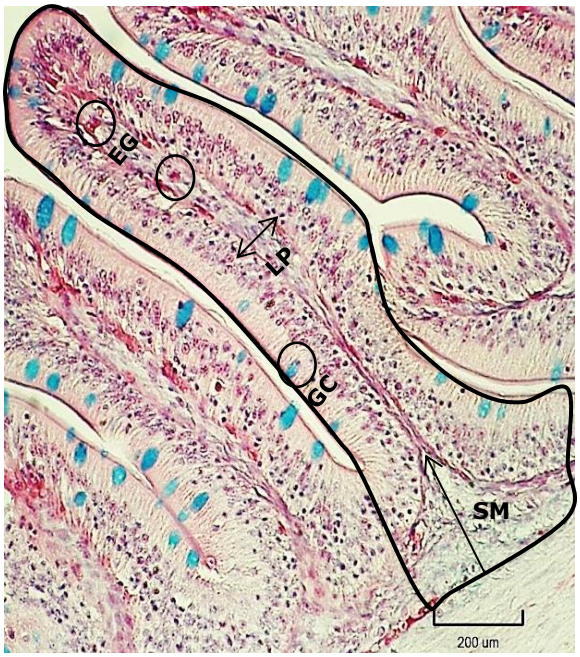


Figure 4.1 Intestinal proximal morphology of Nile tilapia cultured in normal condition. The submucosa (SM) is thin layer of connective tissue between base of folds and stratum compactum. The lamina propria (LP) is thin and delicate core of connective tissue in simple folds. The goblet cell (GC) is a type of mucus-secreting in the epithelium and scattered among the enterocytes. The eosinophilic granulocytes (EG) is inflammatory cell and occasionally migrated into LP. Staining: Haematoxyline/Eosin and Alcian blue, x40.

4.2.6 Chemical analysis

Chemical analysis of the feed, and faeces were done in triplicate for dry matter (EC 152/2009), crude protein (Nx6.25; Dumas, AOAC 99.03), crude fat (acid hydrolysis method), crude fiber (AOCS Ba-6a05), and chromic oxide (INRA M7-007-1). Total carbohydrate content was calculated as 1000 – (crude protein + crude fat + ash). Organic matter was calculated as 100 - % Ash (Thompson *et al.*, 2008).

4.2.7 Calculations

Specific growth rate was assessed as SGR (% bw d^{-1}) = [($\ln W_f$ - $\ln W_i$) / t] x 100, where W_f and W_i are the final and initial weight, respectively; t is the experimental duration in days. Feed intake (FI_{bw}) of fish was expressed as a percentage of body weight (in % bw d^{-1}) = FI / BW_{mean} x 100, where FI (g d^{-1}) is the average feed intake per fish per day and BW_{mean} is the mean body weight, which was calculated as BW_{mean} (g) = (W_f + W_i) / 2. Feed conversion ratio (FCR) was calculated as FCR (g g^{-1})= FI_{tot} / (W_f - W_i), where FI_{tot} (g) is the total feed intake per fish during the experimental period.

Apparent digestibility coefficients (ADC, in %) of dry matter, crude protein, crude fat, total carbohydrate, ash and phosphorus were determined as described by Cho *et al*. (1982):

$$ADC\ of\ nutrients\ (\%) = 100 - 100 \times (\%Cr_{feed} / \%Cr_{faeces}) \times (\%Nutrient_{faeces} / \%Nutrient_{feed})$$

Where Cr_{feed} and Cr_{faeces} are the dietary and faecal chromic oxide content and $Nutrient_{faeces}$ and $Nutrient_{feed}$ is the faecal and dietary nutrient content (all in % on dry matter basis).

4.2.8 Statistical analyses

Statistical analyses were performed using IBM SPSS 22 (SPSS Inc., Chicago, USA). A 2-way ANOVA was used to determine the effect of diet composition and salinity and their interaction on performance parameters. Data was tested for normality and homogeneity (Shapiro Wilk and Levene test, respectively). Survival rate data were not normally distributed, therefore values were arcsine transformed prior to ANOVA. The digestibility was repeatedly measured for each tank during week 1, 4 and 8. The effect of diet, salinity and time were determined by repeated measurement analysis. Histology were analysed for time, diet and salinity using 3-way ANOVA. For all tests, the level of significance was set at $P<0.05$, using Tukey's multiple range test.

4.3 Results

4.3.1 Fish performance

The survival of the fish during the experimental period was above 97 % and did not differ among the treatments (Table 4.2).

Salinity did not affect any of the growth parameters (Table 4.2). Diet composition affected final BW, SGR and FCR ($P<0.05$) due to a lower performance at the "Test" diet. This diet effect was unaffected by salinity, indicated by the absence of the interaction between salinity and diet composition for all growth parameters ($P>0.05$; Table 4.2).

Table 4.2 Growth performance of Nile tilapia fed the experimental diets for 56 days.

	Diet					*Main effect*		
	Control		Test					
	0 ‰	15 ‰	0 ‰	15 ‰	SEM	S	D	SxD
Experimental period (d)	56	56	56	56	-	-	-	-
Tanks (n)	3	3	3	3	-	-	-	-
Fish per tank (n)	35	35	35	35	-	-	-	-
$^{+}$Survival (%)	97	97	99	97	2.0	ns	ns	ns
Initial BW (g)	34.3	34.9	34.6	34.8	0.3	ns	ns	ns
Final BW (g)	120.4	120.1	102.1	99.7	3.0	ns	***	ns
Feed intake (% bw d^{-1})	2.37	2.43	2.44	2.39	0.04	ns	ns	ns
SGR (% bw d^{-1})	2.27	2.20	1.93	1.87	0.07	ns	**	ns
FCR (g g^{-1})	1.19	1.24	1.39	1.39	0.05	ns	*	ns

FW, freshwater; SW, saline water; SEM, standard error; S, salinity; D, diet; SxD, interaction effect between salinity and diet; SGR, specific growth rate; FCR, feed conversion rate; BW, body weight;
$^{+}$ Arcsine transform data
^{NS}P>0.05; *P<0.05; **P<0.01; ***P<0.001; 0.05<$^{\#}$P<0.1

4.3.2 Digestibility

Table 4.3 shows the average value of digestibility over the period of week 1, 4 and 8. The main effects of salinity as well as diet composition affected nutrient digestibility. Except for crude protein, the digestibility coefficients of all nutrients were higher at 15 ‰ salinity compared to 0 ‰ (P<0.01). Diet composition also influenced the nutrient digestibility, being higher at the "Test" diet (P<0.001). No significant interaction effect between diet and salinity on digestibility was presented during the experimental period (P_{SxD} >0.05).

Table 4.3 Effect of diet composition and salinity on apparent digestibility of the macronutrients.

	Diet					*Main effects*			*[1]Time effects*			
ADC	Control		Test		SEM							
(%)	0‰	15 ‰	0‰	15 ‰		S	D	SxD	W	WxS	WxD	WxS xD
Organic matter	86.7	88.9	88.2	90.2	0.4	***	***	ns	**	***	ns	ns
Crude Protein	95.2	95.0	97.0	96.8	0.2	ns	***	ns	***	***	ns	ns
Crude Fat	96.2	96.9	97.6	98.0	0.2	**	***	ns	ns	**	*	ns
Carbohydrates	80.5	83.9	81.3	84.3	0.6	***	ns	ns	**	***	ns	ns$^{\#}$

ADC; apparent digestibility coefficient; SEM, standard error; S, salinity; D, diet; SxD, interaction effect between salinity and diet; W, week; WxS, interaction effect between week and salinity; WxD, interaction effect between week and diet; WxSxD, interaction effect between week, salinity and diet; ^{NS}P>0.05; *P<0.05; **P<0.01; ***P<0.001; 0.05<$^{\#}$P<0.1
[1]Time effects are depicted in Figure 4.2.

Salinity affected digestibility, but those effects were not constant over time. The difference in digestibility of organic matter (Figure 4.2a), crude protein (Figure 4.2b) and carbohydrate (Figure 4.2c) between 0 ‰ and 15 ‰ salinity reduced with time during the experiment. Digestibility of these nutrients were already higher during week 1 of exposure to 15 ‰ salinity compared to 0 ‰. In contrast, the difference in digestibility of crude fat between fish at 0 ‰ versus 15 ‰ salinity increased over time and was comparable during the first week of the experiment (Figure 4.2d).

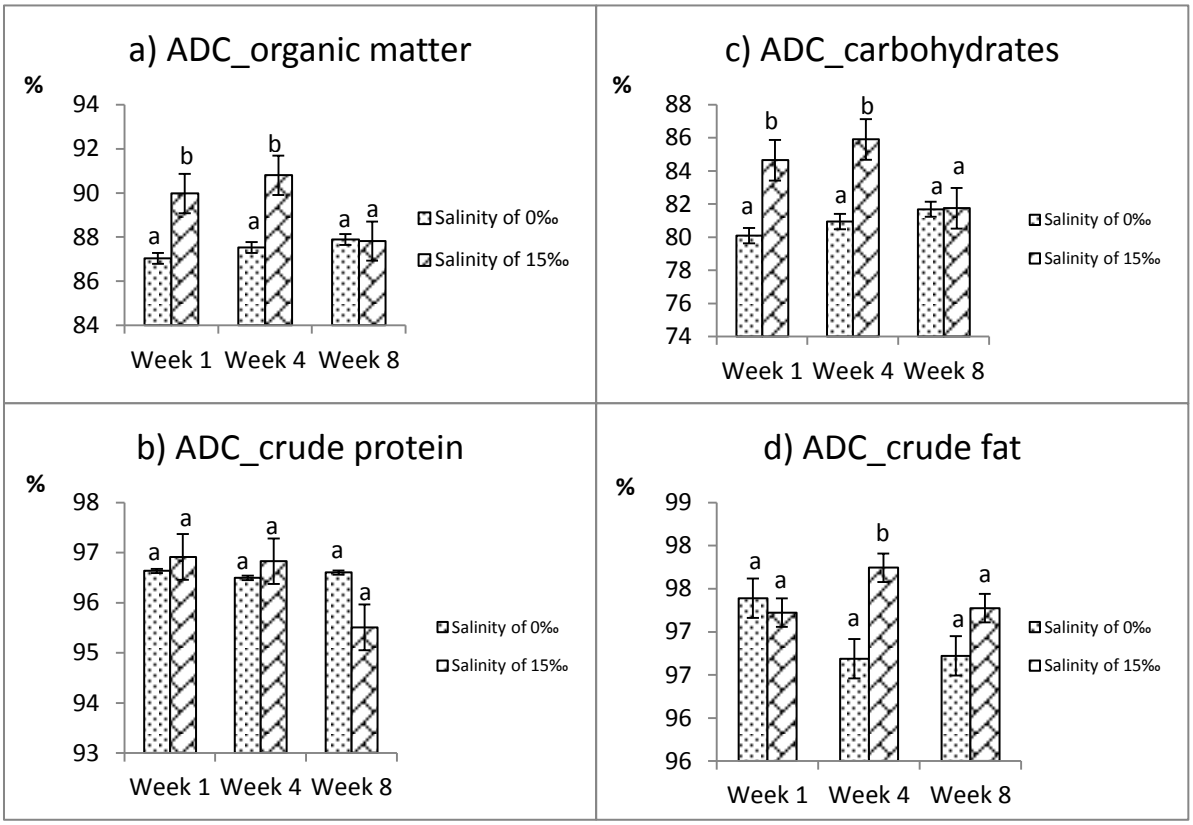


Figure 4.2 Effect of salinity on apparent digestibility coefficients (ADC) of organic matter (a), crude protein (b), carbohydrate (c) and crude fat (d) over time. Each bar shows overall mean the main effects between salinity and diet for each week with standard deviation represented by error bar. Bar labelled with different lower case letters are significantly different for each week (n=6; $p<0.05$). Statistical results (Table 3) shows the interaction on the digestibility of nutrient between week and salinity ($P<0.01$).

4.3.3 Intestinal morphology

Figure 4.3 shows the morphology of the proximal region of the intestine in Nile tilapia fed the "Control" diet and "Test" diet under salinity of 0 ‰ and 15 ‰ at week 1 and 8. There was a difference in intestinal structure in the fish exposed to salinity of 15 ‰ compared to 0 ‰ between week 1 and week 8. The same minor morphological changes were also found between fish fed "Control" and "Test" diet. At week 8, SBM enteritis-like symptoms were fully developed in the fish received the "Test" diet. The submucosa and lamina propria were increased in size and the number of eosinophilic granule cells and goblet cells increased.

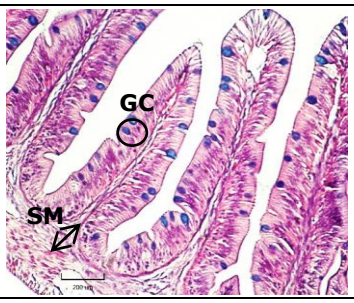
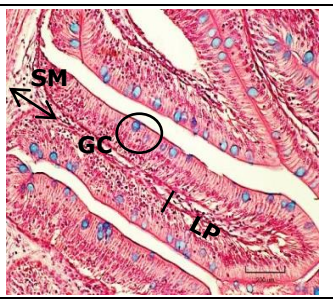
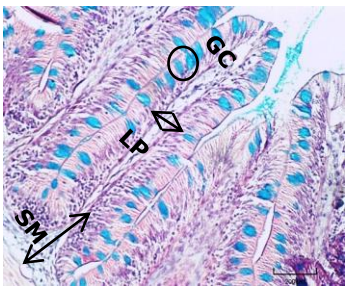
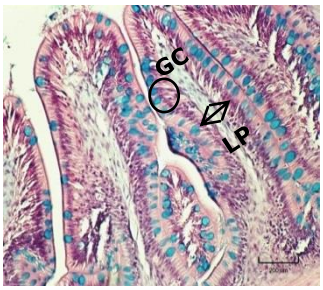
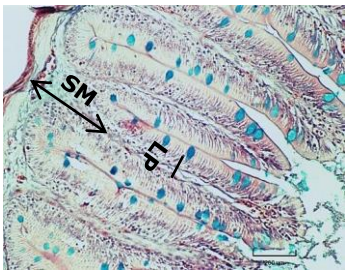
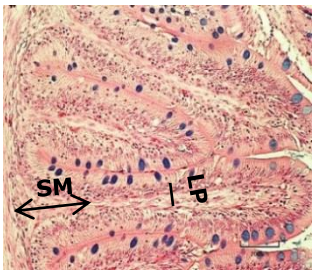
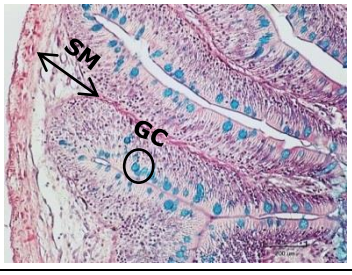
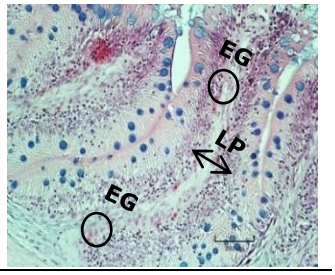
Treatment	Qualitative description	Week 1	Week 8
0‰ & Control diet	1. GC were scattered among the enterocytes 2. EG were hardly found. 3. LP was thin and delicate core of connective tissue in simple folds. 4. SM, between the basal part of the folds and muscularis mucosa, was not enlarged.		
15‰ & Control diet	At week 8, both SM and LP were enlarged and the number of GC widely spread. The number of EG migrated into LP (not shown).		
0‰ & Test diet	At week 8, the thickness of LP was enlarged.		
15‰ & Test diet	At week 8, the thickness of LP was enlarged. The number of EG increased in SM and some migrate into LP.		

Figure 4.3 The proximal morphology of the intestine in Nile tilapia fed "Control" and "Test" diet under 0 ‰ and 15 ‰ at week 1 and 8. Haematoxylin/Eosin and Alcian blue staining, X40. Bar is 200µm. SM, submucosa; LP, lamina propria; GC, goblet cell; EG, eosinophilic granulocyte.

4.3.3.1 Main effects of salinity, diet and their interaction

Table 4.4 shows the average value of intestinal parameters for week 1, 4 and 8. The results showed that salinity has a negative effect on intestinal morphology as shown by the increase of thickness of SM and LP and also by the number of GC and EG.

The effect of salinity was not apparent over whole gastrointestinal tract. The effect was stronger in the distal region than in the proximal region. The thickness of SM ($P_{salinity}$ <0.001; Table 4.4) and the number of GC were more strongly affected by salinity in the distal region ($P_{salinity}$ <0.01; Table 4.4).

Table 4.4 The effect of diet (Control vs. Test) and salinity level (0 ‰ vs 15 ‰) on morphological parameters at different intestinal sections, averaged over sampling moments.

	Diet					*Main effects*			*[1]Time effects*			
	Control		Test		SEM	S	D	SxD	W	WxS	WxD	WxSxD
	0 ‰	15 ‰	0 ‰	15 ‰								
Submucosa (µm)												
Proximal	236	287	280	304	32.5	*			***		*	
Middle	209	292	307	390	42	**	***		***	***	***	
Distal	253	352	322	371	35	***	*		***	***		
Lamina propria (µm)												
Proximal	45	51	54	75	7.2	**	***		***	***	**	*
Middle	51	57	68	92	13.1		**		***	*		
Distal	114	106	115	136	39.5				***	*		
Goblet cells (10^{-5} cells/µm^2)												
Proximal	3.6	4.6	4.5	5.1	0.6	*	*					
Middle	4.0	6.0	5.1	5.3	1.0	*						
Distal	3.4	5.1	4.8	6.8	0.9	**	**			**		
Eosinophilic granulocytes (10^{-5} cells/µm^2)												
Proximal	0.3	0.8	0.5	0.5	0.2	*		*	***	***	**	*
Middle	0.2	0.4	0.4	0.5	0.2				***	*		
Distal	0.3	0.6	0.3	0.5	0.2	*			*	*		

SEM, standard error; S, salinity; D, diet; SxD, interaction effect between salinity and diet; W, week; WxS, interaction effect between week and salinity; WxD, interaction effect between week and diet; WxSxD, interaction effect between week, salinity and diet; ; ^{NS}P>0.05; *P<0.05; **P<0.01;***P<0.001; 0.05<$^{\#}$P<0.1

[1]Time effects are related to Figure 4.4.

4.3.3.2 Time effects related to intestinal morphological changes

We found that salinity and diet composition had main effects on the intestinal morphology, but those effects were not constant over time. In general, the effect of salinity on the intestinal morphology aggravated over time. Figure 4a and 4b show the changes of LP and EG in the intestinal proximal section over time under the interaction between salinity and diet. In week 1, the changes of LP and EG were not clear, but in week 8, the highest value of LP and EG were found in the fish reared in the treatment combination "Test diet + salinity of 15 ‰" and "Control diet + salinity of 15 ‰", respectively (Figure 4a and 4b).

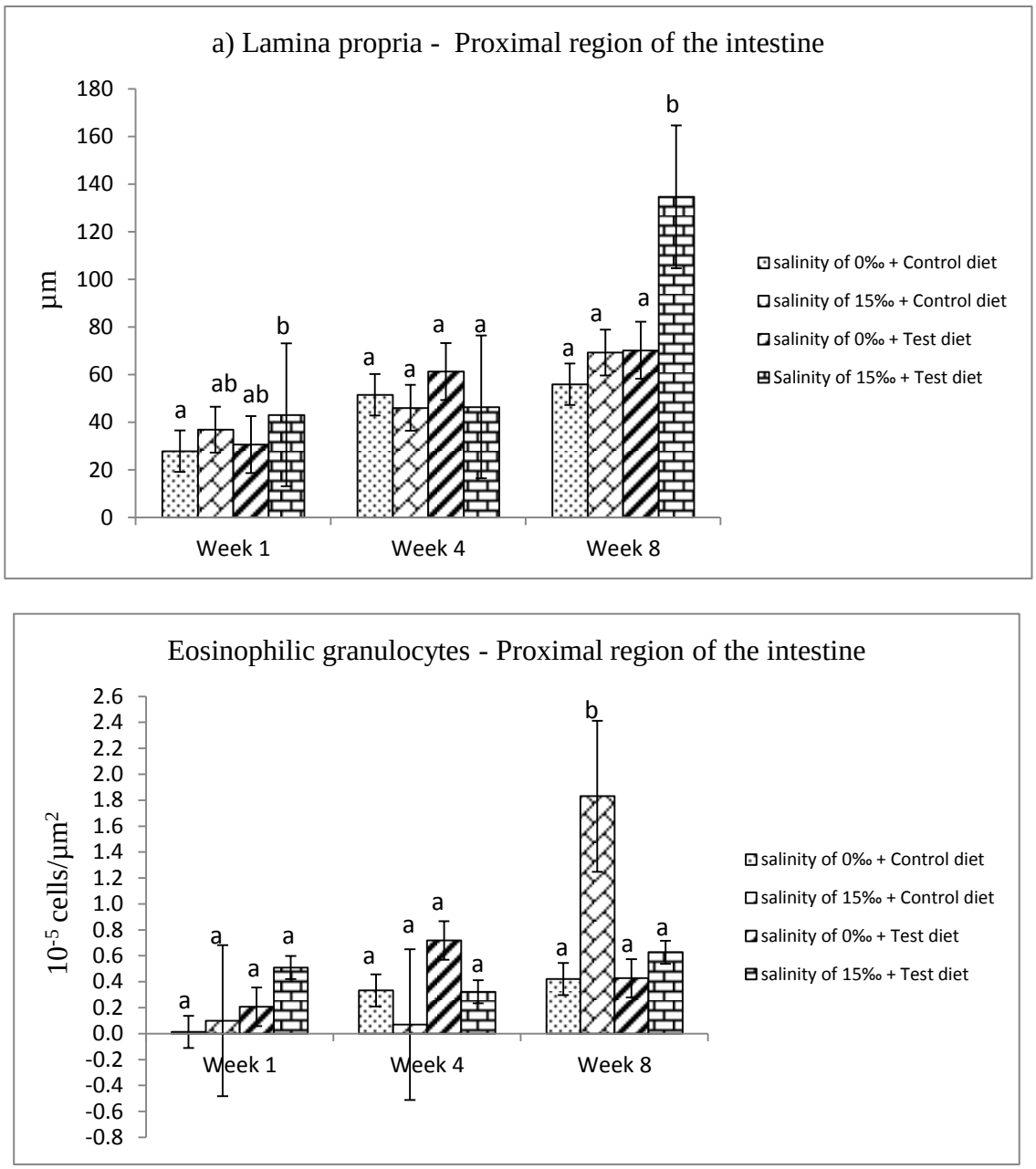


Figure 4.4 Time related alteration of the interaction effect between salinity and diet on (a) lamina propria and (b) eosinophilic granulocytes. Each bar shows mean of intestinal parameter at each combination of salinity (0 ‰ vs. 15 ‰) and diet effect ("Control" vs. "Test") during week 1, 4 and 8 of the experiment. Each bar shows overall mean the main effects between salinity and diet for each week with standard deviation represented by error bar. Bar labelled with different lower case letters are significantly different for each week (n=6; $p<0.05$). Statistical results (Table 4.4) shows the interaction on the intestinal morphology between, week salinity and diet ($P<0.05$).

4.4 Discussion

4.4.1 Salinity affects intestinal morphology and digestibility

4.4.1.1 Intestinal morphology

The current study showed that at a salinity of 15 ‰, the intestinal morphology of Nile tilapia was disturbed. This was exhibited in an increase in thickness of SM and LP, and an excessive production of GC and EG. The observed changes in the current study, in the intestinal morphology under stressful conditions are similar to those found by Tran-Ngoc et al., (2016a), which used low dissolved oxygen concentrations as a stressor. However, we found that the effect of salinity on the intestinal morphology occurs predominantly in the distal region of the intestine, whereas the effect of low oxygen concentration on intestine was more visible at the proximal region. We hypothesized that this stronger salinity effect might be related to the function of the distal region in osmoregulation (i.e., water absorption). We found a high number of GC in the distal region of the intestine. These cells most likely have an osmoregulatory function in saline water (Rodríguez *et al.*, 2005). Our results are in line with Li *et al.* (2014) who showed that, in Mozambique tilapia (*Oreochromis mossambicus*), the distal region of the intestine was highly responsive to salinity changes followed by proximal and middle region. Similar results, demonstrating that the intestinal distal morphology had been affected by the salinity, were also seen for the freshwater teleosts, Orange Chromide (*Etroplus maculatus)* (Virabhadrachari, 1961) and glass eel (*Anguilla Anguilla)* (Ciccotti *et al.*, 1993). Our current and previous study show that environmental stress conditions such as salinity (in the present study) and oxygen ((Tran-Ngoc *et al.*, 2016a) can weaken the epithelial barrier functions. The implications of those changes on the intestinal morphology may be used in future studies as a tool to evaluate the impact of environmental stress.

4.4.1.2 Digestibility

The impact of salinity on digestibility was also examined in this study. The higher digestibility coefficients among the proximate components (organic matter, crude fat, carbohydrate) were found at the salinity of 15 ‰. Those results seem to indicate that the exposure to slightly hypotonic osmotic conditions might have a positive impact on digestibility as the fish were able to absorb efficiently the nutrients present in both "Control" and "Test" diet. The digestibility of the protein was slightly lower at the 15 ‰ treatment in both the "Control" and "Test" diet, but this effect was not statistically significant. The reason for the higher values of digestibility under the salinity of 15 ‰ is not clear yet, but it is possible that salt affects the osmoregulation energy costs. Febry and Lutz (1987) noted that the osmoregulation energy costs for a hybrid tilapia (*Oreochromis mossambicus* ♀ x *O. hornorum* ♂) was higher in freshwater than in

seawater (35 ‰) and the lowest osmoregulation costs were found at an isosmotic salinity of 12 ‰. Suresh and Lin (1992) also found similar results in Tilapia species (*O.mossambicus*, *O. spilurus* and red hybrid tilapia *O.mossambicus* x *O.niloticus*), reared in an isosmotic salinity of 12 ‰. They found that the blood-medium osmotic gradient is minimized, and thus the osmoregulation of fish is also minimal. The two studies above seems to be in agreement with our study, showing that less energy is required to maintain the ion balance in the salinity of 15 ‰, with the energy be directed towards nutritional digestion activity.

Apart from the direct effect of water salinity on the gastrointestinal tract due to its osmoregulatory role, it is also involved in the nutrient supply to other osmotic-adjusting organs and tissues (Nitzan *et al.*, 2016). Ballantyne *et al.* (2001) showed that in some teleost fish, the amino acid levels in the tissues rise with an increase in water salinity. In contrast, Nordum *et al.* (2000) reported that salinity is independent uptake to total nutrient absorption. The influences of salinity on measured rates of amino acid absorption are more complex to understand because they can involve changes in the carrier-mediated and carrier-independent pathways of influx (Nordrum *et al.*, 2000). Several studies have investigated the impact of salinity on the apparent digestibility in fish, but results are not consistent in response. Protein digestibility decreased in milk fish (*Chanos chanos)* (Ferraris *et al.*, 1986) and Arctic charr (*Salvelinus alpinus)* (Ringø, 1991) when moved from seawater to freshwater. On the other hand, Dabrowski *et al.,* (1986) and De Silva and Perera (1984) noted that salinity had no significant effect on protein digestibility of rainbow trout (*Salmo gairdneri)* and Nile tilapia, respectively. These variability in impacts of salinity between studies on nutrient digestibility could be related to time exposure. In the current study, the temporal effect of salinity on digestibility was also investigated. We found that when fish were exposed to salinity of 15 ‰ up to 8 weeks, the digestibility first increased until week 4 starting to decrease after that until week 8. The changes of intestinal morphology in time when fish exposed to salinity of 15 ‰ could be a reason for a reducing digestibility. However, the interaction between salinity and diet composition on digestibility was not significant. The reason(s) why the digestibility is reduced when fish are exposed to brackish water remains unclear. The present study confirmed that an environmental stressors as salinity might increase digestibility for short term, but over time, this beneficial effect can disappear.

4.4.2 The effect of soybean meal on intestinal morphology

Soybean meal-induced enteritis is a well-described condition in the distal intestine of Atlantic salmon (van den Ingh *et al.*, 1991, van den Ingh *et al.*, 1996, Baeverfjord and Krogdahl, 1996, Urán *et al.*, 2008b), and of rainbow trout (Heikkinen *et al.*, 2006,

Venold *et al.*, 2012). The present study showed that SBM ("Test" diet) response, for Nile tilapia, is in line with these authors, with the fish fed with a SBM-based diet showing a thickening of both LP and SM. However, in salmon and trout, these studies demonstrated that the distal region of the intestine was most affected and with minor changes in the proximal region induced by SBM. In contrast, in Nile tilapia, the enteritis-like response is particularly present in the proximal region. The results suggest that the SBM response is species specific and might relate to the structure of the gastrointestinal tract. In Nile tilapia, the mucosa of the intestinal proximal section is longer and has more branched villi than the distal region (Gargiulo *et al.*, 1998) and therefore might be more vulnerable to intestinal disorder.

The gastrointestinal tract is functionally and morphologically divided into different regions, consequently the barrier function varies between the regions (Jutfelt, 2011). Our results showed that while a plant based diet affected more the proximal region of the intestine, salinity had a strong effect in the distal region.

4.4.3 The interaction between salinity and diet over time

We hypothesized that the impact of salinity on intestinal morphology might be enhanced by SBM. In this study, we confirm that as far as it concerns LP and EG, there was an interaction between salinity and diet composition over time, being stronger in the proximal section. Nile tilapia reared under brackish water tended to drink more water to compensate passively water lost (Bœuf and Payan, 2001). This leads to a notably increased epithelia paracelluar permeability in gastrointestinal tract due to the increase water transport (Jutfelt, 2006). However, the impact of salinity on the proximal region of the intestine was opposite between the thickness of LP and the number of EG. The number of EG were more dominant with salinity of 15 ‰ and the "Control" diet, where the thickness of LP was more challenged in the "Test" diet. In a previous study, together with high SBM-feeding level and under hypoxic condition, the number of EG were more affected at the "Test" diet in the distal intestine (Tran-Ngoc *et al.*, 2016a). Based on these results, the elevated salinity conditions could alter the effect of diet, but not consistent where it takes place in the gastrointestinal tract as well as the direction.

4.5 Conclusions

This study demonstrated that the salinity of 0 ‰ and 15 ‰ as well as the dietary SBM content influence the intestinal morphology in Nile tilapia. For SBM, the impact on morphology was larger in the proximal region of the intestine, whereas the salinity impact of 15 ‰ was largest in the distal region. The increased lamina propria thickness induced by SBM was enhanced at the salinity of 15 ‰. Salinity of 15 ‰ increased the nutrient digestion in Nile tilapia; however those impacts turned to affect negatively the

digestibility over time. Differences in nutrient digestibility induced by the salinity of 15 ‰ seems not to be related to alterations in the intestinal morphology.

Acknowledgement

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Chapter 5

Environmental conditions alter the effect of organic acids on digestibility and intestinal morphology in Nile tilapia (Oreochoromis niloticus)

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Abstract

Abstract

The impact of two dietary organic acids on nutrient digestibility and intestinal morphology was determined in Nile tilapia under optimal (normoxia) and suboptimal conditions (hypoxia). In the experiment, four diets with two organic acids were tested in a 2x2 factorial design. The control diet was formulated with 50 % of soybean meal as an ingredient to create soybean meal enteritis-like symptoms. Four diets designated as control treatment (0 % organic acid salt), KDF (0.2 % potassium diformate), CAB (0.2 % calcium butyrate) and their combination (0.4 % of a mixture of KDF and CAB, ration 1:1) were formulated. We hypothesized that fish under a hypoxic condition would respond better to the organic acids addition then under normoxic conditions. Therefore, the four diets were tested first under normoxic conditions (6 mg O_2L^{-1}) for a period of 5 weeks, followed by a test period under hypoxic conditions (3 mg O_2L^{-1}). The growth performance, digestibility, and morphology of intestine were assessed to evaluate the effectiveness of the organic acids. The results showed that although organic acids did not significantly improve growth performance and nutrient digestibility under normoxic conditions, they did under hypoxic conditions. Fish fed organic-acid-supplemented diets all showed improvements in morphology of the intestine under the normoxic conditions, and these effects were more enhanced under the hypoxic conditions. This indicates that environmental conditions can alter the effect of organic acids on nutrient digestibility and intestinal morphology in tilapia. A synergistic effect by the combination of formic and butyric acid on growth, digestibility and intestinal morphology was not found.

5.1 Introduction

Fish in commercial farming can be exposed to suboptimal environmental conditions. In general, unfavourable environmental conditions, which threaten the animal's homeostasis are defined as stressors (Moberg and Mench, 2000). As an example, a low level of dissolved oxygen in the water is a potential stressor for many fish species. In Nile tilapia low oxygen levels can disturb the feeding behaviour and growth rate (Tsadik and Kutty, 1987); alter the energy metabolism (Ishibashi *et al.*, 2002); hamper innate immunity by a decreased lysozyme activity (Abdel-Tawwab *et al.*, 2015). Moreover, dissolved oxygen can influence nutrient digestibility and alter the intestinal morphology (Tran-Ngoc *et al.*, 2016a). Exposure to unbalanced diets can weaken the intestinal barrier function (Jutfelt, 2011) or modulate the lysozyme activity of fish (Saurabh and Sahoo, 2008). To counteract disturbances of the intestinal barrier function, feed additives such as acidifiers have been tested as growth promoters (Lückstädt, 2008, Ng and Koh, 2011) or to control bacteria and fungi in the feed and in the intestine of the fish (Park *et al.*, 2013). The possible mode of action of organic acids in the intestinal tract involves two different mechanisms: 1) it reduces the pH level in the stomach and in the small intestine, through the delivery of H^+ ions, 2) it inhibits growth of Gram-negative bacteria through the dissociation of the acids and the production of anions inside bacterial cells (Lückstädt, 2007). Studies on the use of organic acids in tilapia diets are numerous, however, between studies results are often highly variable, both in type of response and significance (Zhou *et al.*, 2009, Ng *et al.*, 2009, Hassaan *et al.*, 2014, Elala and Ragaa, 2015, Ramli *et al.*, 2005).

Recently, Tran-Ngoc *et al.* (2016a) showed that both dietary and environmental conditions affect intestinal morphology, and that their negative impacts amplify each other. Based on this observation, we hypothesize that the variability in responses to using organic acids as found in literature may be related to variations in the culture conditions, more specifically regarding water and diet quality. This study assessed if the impact of supplementation of organic acids to a "sub-optimal" diet on performance, nutrient digestibility and intestinal morphology in Nile tilapia is dependent on the environmental conditions. Formic acid and butyric acid were added to a diet with more than 50% soybean meal. This soybean meal diet induces alterations in intestinal morphology. The efficacy of these organic acids were tested at normoxia (i.e., optimal) and at hypoxia (i.e., suboptimal) conditions.

5.2 Materials and Methods

5.2.1 General design

The present study was conducted according to the Dutch legal requirements for doing animal experiments, approved by the Committee on Ethics of Animal Experiments of the Netherlands. In this experiment, four diets were used, in which two organic acids (OA) were tested according to 2x2 factorial design. The tested OA were formic acid and butyric acid. These OA were added to the diet as salt: potassium diformate and calcium butyrate. It was hypothesized that the impact of OA depends on environmental conditions, being more pronounced under harsh conditions (i.e. hypoxia). Therefore, two different environmental conditions, normoxia and hypoxia, were created sequentially in time within this study. The four diets were studied first under normoxic conditions for a period of 5 weeks, followed by a 5 weeks period at hypoxic conditions. The experiment was conducted at the Fisheries Faculty of Nong Lam University, Ho Chi Minh city, Vietnam. The all-male Nile tilapia juveniles used during this experiment were obtained from a local commercial farm.

5.2.2 Diets and feeding

The control diet was formulated with the aim to challenge the intestinal morphology of Nile tilapia. The control diet contained 52 % of soybean meal (SBM) (Table 5.1) and was identical to Tran-Ngoc *et al.* (2016a), who found that this diet induces enteritis-like symptoms in Nile tilapia.

The 2 types of studied OA were: 1) a commercial potassium diformate product (KDF) (ADDCON, NordicAS, Porsgrunn, Noway); and 2) a commercial calcium butyrate product (CAB) (Kemin Industries, Inc., U.S.A). We wanted to test the effect of 2 different OA on the intestinal morphology and digestibility in tilapia. Our main questions were: (a) do each of these acids have an effect and (b) do they have a synergistic or additive effect when both are added to the diet together. To test these questions, we designed a 2 x 2 factorial experiment (Figure 5.1) and analysed them accordingly in a two-way ANOVA. This led to four experimental diets that were formulated by supplementing 0 % (NO), 0.2 % (YES) of each organic acid in the basal diet. The use of a two-way ANOVA analysis allowed us to test the interaction effect between the two organic acids.

		Acid A	
		YES	NO
Acid B	YES	Diet 1	Diet 2
	NO	Diet 3	Diet 4

Figure 5.1 The 2 x 2 factorial design of the experiment

Table 5.1 Ingredient composition of the control diet

Ingredients	Inclusion (%)
Soybean meal	52
Rice bran	12
Cassava	10
Corn gluten meal	15
Fish oil	3
Soybean oil	3
DL-Methionine	1
Dicalcium phosphate	1
Vitamin and mineral Premix*	2
Chromic oxide	1

* Vitamin and minerals premix (per kg of feed): contain vitamin A 40,000 IU; D_3 9600 IU; E 300 mg; C 700 mg; K_3 60 mg; B_1 54 mg; B_2 64 mg; B_6 64 mg; Niacin 96 mg; Pantothenic acid 132 mg; Choline 800 mg; Fe 298 mg; Cu 48 mg; Zn 1300 mg; Mn 240mg; Co 0.48 mg; I 19.2 mg; Se 2.4 mg; Folic acid 20 mg; Biotin 1 mg; Inositol 192 mg; Carrier.

CAB was chosen in its microencapsulated form to be more effective at the distal part of the intestine. The 0.2 % inclusion levels of the KDF and CAB products should result in a concentration of 0.14 % formic acid and 0.08 % butyric acid in the final feed product.

The feed ingredients were mixed to produce a basal mash diet. KDF and CAB were then added to this basal diet at the mentioned concentrations and then thoroughly mixed. The Cr_2O_3 marker was added to the diet at 1 % to allow the determination of nutrient digestibility. Water (+/-50 % volume/weight) was added to the dry mash and 2 mm pellets were produced using a meat grinder (Hobbart 4822-36 22C/E, Germany). The pellets were then dried in an air flow oven at 70^0C until the water moisture content was lower than 10 %. The dry pellets were kept in feed containers and stored until further used. Analysed proximate composition and organic acid content of the four experimental diets are shown in Table 5.2.

Table 5.2 Analysed proximate composition and organic acid content of the experimental diets (on dry matter basis %).

	[1]CT	[2]KDF	[3]CAB	KDF+CAB
Dry matter	91.8	91.8	91.6	91.7
Crude protein	38.2	38.4	38.4	38.6
Ash	7.7	7.6	7.6	7.5
Gross energy (MJ kg^{-1})	20.5	20.8	20.8	20.6
Chromic oxide	1.3	1.3	1.2	1.4
Formic acid	nd	0.18	nd	0.15
Butyric acid	nd	nd	0.03	0.02

[1]CT, control diet; [2]KDF: potassium diformate; [3]CAB, calcium butyrate; nd: not detectable

At each feeding moment, feed given and uneaten feed were recorded. In addition, uneaten pellets were collected and counted to determine the food intake accurately. Fish were fed restrictively at a level of 3 % of the body weight. Within each period, it was aimed to have equal feed intakes (in g $fish^{-1}$) for all tanks. In case feed refusals occurred in one tank consistently, also the feed given to all the other tanks was reduced to prevent differences in realized feed intake between tanks.

5.2.3 Experimental conditions

Twelve cylinder-conic tanks (150 L) were stocked with 40 Nile tilapia with an initial mean body weight of 30 ± 0.5 gram. In the first period (normoxia), the dissolved oxygen (DO) content was aimed to be close to 100 % saturation. DO saturation was achieved by aerating the water in the water storage tank with air stones before supplying it to the fish tanks and adding additional air stones inside each fish tank. In the second period (hypoxia), the aeration was reduced to reach approximately 50 % of DO saturation in the storage tank by adjusting the output of air stones, and the fish-tanks were not aerated. DO concentration was measured daily using oxygen meter (VWR® DO 220 model, VWR International Ltd., USA). The measured DO concentrations inside the fish tanks were 82 % (6.2 ± 0.3 mg L^{-1}) and 39 % (2.9 ± 0.4 mg L^{-1}) during respectively the normoxic and hypoxic period, respectively. During the trial, the water temperature was maintained at 30 ^{0}C, and pH at 6.7. Total ammonia nitrogen (TAN) was kept below 2 mg L^{-1} in both periods (given 0.008 mg L^{-1} unionised ammonia). Therefore, ammonia was not unintended physiological stressor in the experiment.

5.2.4 Sampling and measurements

During the growth period, fish were anesthetized with 2-phenoxyethanole (0.25 ml L^{-1}, Sigma, USA) and group weighed every 2 weeks to calculate feed intake. During the last week of each period, faeces were collected twice a day prior to feeding in order to measure nutrient digestibility. During faecal collection, the faecal collecting bottles were placed in a thermostatic box with ice to avoid the bacterial degradation of nutrients in faeces. Faeces were collected using sedimentation columns as described by Cho *et al.* (1982), kept separately for each tank and stored at -20 ^{0}C until further analysis.

Fish were simultaneously sampled for lysozyme activity analysis, gastro-intestinal pH and intestinal morphology in order to minimize the use of experimental animals. At the end of week 5 and 10, four hours postprandially, three fish from each tank (9 fish per treatment) were sampled. First, fish were scooped gently from each tank using a hand dip net, transferred to a plastic cub containing water with the anaesthesia, 2-phenoxyethanol (0.25 ml L^{-1}, Sigma), weighed and finally blood was collected. The blood was taken from the caudal vein using a 1 ml syringe then transferred into an Eppendorf and centrifuged at 5800 rpm at 4 ^{0}C for 10 min. The supernatant, serum, was stored at -20^{0}C for posterior lysozyme activity analysis.

After blood collection, fish were subsequently euthanized by an over dose of 2-phenoxyethanol (1 ml L^{-1}, Sigma), and then fish were dissected and the intestinal tract were sampled for gastrointestinal pH measurement and histological studies. The intestine was divided in three regions: proximal (from the pyloric part of the stomach to the spiral

part of the intestine), middle (the spiral part of the intestine), and distal (from end spiral part of the intestine to 2 cm before the anus) (Pirarat *et al.*, 2011). The gastro-intestinal pH was measured by pooling the digestive liquid of each section for all three fishes within each replicate of treatment, using a digital pH meter (Trans pH Model HP4030, DTK Water) as described by Amirkolaie *et al.,* (2006) and Saravanan *et al.,* (2013). For intestinal histology, one-cm portion of each of the three intestinal segments were fixed by immersion in Bouin's fixative solution.

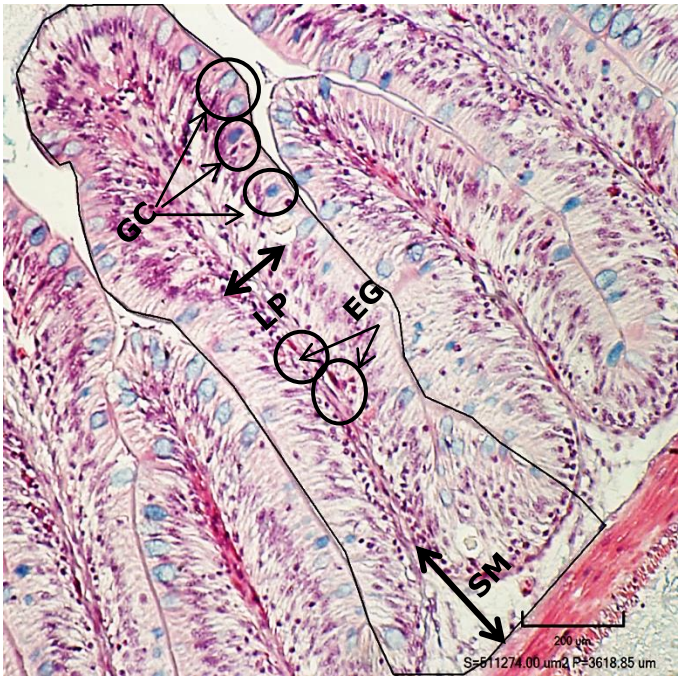


Figure 5.2 Intestinal proximal morphology of Nile tilapia cultured in normal condition. The submucosa (SM) is the thin layer of connective tissue between the base of folds and stratum compactum. The lamina propria (LP) is the thin and delicate core of connective tissue in simple folds. The goblet cell (GC) is a type of mucus-secreting cells in the epithelium and scattered among the enterocytes. The eosinophilic granulocytes (EG) are inflammatory cell which occasionally migrate into LP. Staining: Haematoxyline/Eosin and Alcian blue, x40.

5.2.5 Light microscopy

After fixation, the intestinal samples were dehydrated and embedded in paraffin using standard procedures (Dimitroglou *et al.*, 2010). Transverse sections of 5 µm thickness were stained using Alcian blue periodic acid-Schiff staining technique (AB-PAS) (Grethen, 1962). The intestinal samples were photographed with an INFINITY 2 CCD digital camera (Lumenera®, Sony, Japan) connected to a Meiji microscope (Meiji Techno, Japan). Pictures were processed and analysed using the Infinitive Analyse software (Lumenera®, Sony, Japan). The light microscopy regions were evaluated based on the quantitative method developed at Wageningen University for salmon (Urán, 2008), which was adapted for the response to SBM-induced enteritis in Nile tilapia (Tran-Ngoc *et al.*, 2016a). Briefly, the measurements were done on four random villi selected per slide and

per intestinal segment for each fish. The following intestinal morphology parameters were assessed: a) the number of goblet cells (GC, expressed in number per µm of villi – the length of the villi); b) the number of eosinophilic granulocytes (EG, expressed in number per µm of villi – the length of the villi); c) the thickness of the lamina propria (LP in µm) as the middle of the villi length; d) the thickness of the sub-epithelial mucosa (SM in µm), measured as the distance between the inner side of the loose connective tissue and the point where both enterocyte linings contact each other (Figure 5.2). Values of these four parameters were averaged per fish and per intestinal segment.

5.2.6 Chemical analysis

Chemical analysis of the feed and faeces was done in triplicate for dry matter (EC 152/2009), crude protein (Nx6.25; Dumas, AOAC 99.03), gross energy (adiabatic bomb calorimetry), ash (EC 152/2009) and chromic oxide (INRA M7-007-1). Organic matter was calculated as 100 - % Ash (Thompson *et al.*, 2008).

Lysozyme activity was measured by using a turbidometric assay (Sigma-Aldrich Co. LLC, St. Louis, MO 63103, USA), following the directions of the manufacturer in which the activity of lysozyme is determined from a standard curve indicating the degree of lysis of *Micrococcus lysodeikticus* by a known concentrations of hen egg white lysozyme.

Formic and butyric acid analyses were conducted at CASE, Ho Chi Minh city, Vietnam, following the method described by Gao *et al.* (2011). The samples were prepared by milling, homogenization, dilution and mixing with water. The prepared sample was filtered at 4 µm and analysed by HPLC on a cation exchange column designed for separation of organic acids at 50^0C. Formate was detected at 205 nm and butyrate by a RI-detector.

5.2.7 Calculations

Specific growth rate was calculated as SGR (% bw d^{-1}) = $[(\ln W_f - \ln W_i) / t] \times 100$, where W_f and W_i are the final and initial weight, respectively; t is the experimental duration in days. Feed intake (FI_{bw}) of fish was expressed as a percentage of body weight (in % bw d^{-1}) = $FI / BW_{mean} \times 100$, where FI (g/d) is the average feed intake per fish per day and BW_{mean} is the mean body weight, which was calculated as BW_{mean} (g) = $(W_f + W_i) / 2$. Feed conversion ratio (FCR) was calculated as FCR (g/g)= $FI_{tot} / (W_f - W_i)$, where FI_{tot} (g) is the total feed intake per fish during the experimental period.

Apparent digestibility coefficients (ADC, in %) of dry matter, crude protein, ash, organic matter, and energy were determined as described by Cho *et al*. (1982)

ADC of nutrients (%) = 100 – 100 x (%Cr_{feed} / %Cr_{faeces}) x (%$Nutrient_{faeces}$ /%$Nutrient_{feed}$)

Where Cr_{feed} and Cr_{faeces} are the dietary and faecal chromic oxide content and $Nutrient_{faeces}$ and $Nutrient_{feed}$ is the faecal and dietary nutrient content (all in % on dry matter basis).

5.2.8 Statistical analyses

Statistical analyses were performed using IBM SPSS 22 (SPSS Inc., Chicago, USA) software. Data were analysed separately for period 1 (normoxia condition) and period 2 (hypoxia condition). For each period, a 2-way ANOVA was used to determine the effect of the two types of organic acid (potassium diformate and calcium butyrate) and their interaction effect on growth parameters, digestibility, pH in the intestine, lysozyme activity and intestinal morphology. A post-hoc Tukey test followed when the interaction effect was significant ($P<0.05$).

5.3 Results

5.3.1 Fish growth performance

The effects of the dietary supplementation of OA on the growth performance of Nile tilapia are summarised in Table 5.3. The mean survival rate during the total experimental period was 95 % and was unaffected by dietary treatments.

No significant differences were detected for growth performance parameters ($P>0.05$) among the experimental groups during normoxia. During hypoxia, there was an interaction effect between both OA on all growth performance parameters (final body weight, SGR and FCR). Supplementation with a single dose of KDF or CAB significantly improve growth performance, however the combination of two OA did not further enhance growth performance.

5.3.2 Digestibility

In general, the digestibility for all nutrients at normoxia (Period 1) was higher than at hypoxia (Period 2) (Table 5.4). The effects of KDF and CAB supplementation on digestibility, if present, predominantly appeared at hypoxia. During the normoxic period, the digestibility of dry matter, organic matter, ash and energy were unaffected by diet, whereas the digestibility of protein showed an interaction effect between KDF and CAB. During hypoxic condition, the difference in protein digestibility between dietary treatments was larger ($P<0.05$), and the OA supplementation effects on energy digestibility appeared ($P<0.01$). Protein and energy digestibility were both affected by the interaction effect between KDF and CAB. All diets supplemented with OA had higher digestibility values, but there was no synergy between KDF and CAB on protein and energy digestibility during the hypoxic period.

Table 5.3 Effect of dietary organic acid supplementation on growth performance of Nile tilapia during normoixa (Period 1) and hypoxia (Period 2).

	Diets					*Effects*		
Normoxia								
	CT	KDF	CAB	KDF +CAB	SEM	KDF	CAB	Interaction
Initial BW (g)	30.21	30.16	29.97	30.68	0.30	ns	ns	ns
Week 5 BW(g)	71.39	73.54	71.15	73.12	1.30	ns	ns	ns
Feed intake (% bw d^{-1})	2.93	2.95	2.97	2.93	0.03	ns	ns	ns
SGR (% bw d^{-1})	2.43	2.50	2.53	2.47	0.07	ns	ns	ns
FCR (g g^{-1})	1.23	1.17	1.23	1.20	0.03	ns	ns	ns
Hypoxia								
	CT	KDF	CAB	KDF +CAB	SEM	KDF	CAB	Interaction
Initial BW (g)	72.72	71.88	71.81	73.45	0.80	ns	ns	ns
Week 10 BW(g)	131.50	143.67	139.60	136.10	0.90	**	ns	***
Feed intake (% bw d^{-1})	2.21	2.22	2.19	2.19	0.02	ns	ns	ns
SGR (% bw d^{-1})	1.67	1.97	1.90	1.77	0.04	#ns	ns	**
FCR (g g^{-1})	1.43	1.27	1.30	1.37	0.03	ns	ns	*

CT, control diet; KDF, potassium diformate; CAB, calcium butyrate; SEM, standard error mean; bw, body weight; d, day; SGR, specific growth rate; FCR, feed conversion ratio; $^{ns}p>0.05$; $0.05<{}^{\#}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

Table 5.4 Effect of dietary organic acid supplementation on nutrient digestibility (%) of Nile tilapia during normoxia (Period 1) and hypoxia (Period 2).

	Diets					Effects		
	Control	KDF	CAB	KDF+CAB	SEM	KDF	CAB	Interaction
Normoxia								
Dry matter	78.8	77.5	78.8	76.0	1.5	ns	ns	ns
Organic matter	82.8	81.8	82.7	80.6	1.2	ns	ns	ns
Crude protein	94.8	95.2	95.1	94.6	0.3	ns	ns	*
Crude ash	43.5	38.4	41.7	30.9	4.4	ns	ns	ns
Energy	83.9	85.1	85.3	84.7	0.5	ns	ns	ns
Hypoxia								
Dry matter	74.2	76.1	78.2	75.7	1.2	ns	ns	ns
Organic matter	77.9	79.4	80.9	78.7	1.1	ns	ns	ns
Crude protein	91.3	93.7	93.4	92.6	0.3	*	ns	**
Crude ash	36.2	36.3	46.2	39.4	3.6	ns	ns	ns
Energy	79.7	83.6	84.3	81.8	0.4	ns	**	***

KDF, potassium diformate; CAB, calcium butyrate; SEM, standard error mean; $^{ns}p>0.05$; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

5.3.3 Chyme pH

Numerically, chyme pH as well as dietary effects on pH were quite similar during normoxia and hypoxia (Table 5.5). In the middle and distal part of the intestine, chyme pH was neutral and showed minor differences (physiological irrelevant), but some statistical effects appeared at hypoxia. Stomach pH was influenced by the interaction effect of both types of OA (P<0.001) during normoxia and hypoxia. Especially with the KDF diet, stomach pH was reduced compared to the control diets. The other diets (CAB

and KAD+CAB) had an intermediate stomach pH. Also in the proximal intestine during both periods, pH was affected by the interaction effect. During normoxia, only the pH of the KDF diet was reduced compared to the control (from 6.8 to 6.2), whereas at hypoxia all diets supplemented with OA had a reduced pH in the proximal intestine (7.4 versus 7.0-7.1; Table 5.5).

Table 5.5 Effect of dietary organic acid supplementation on chyme pH of Nile tilapia during normoixa (Period 1) and hypoxia (Period 2).

	Diets					*Effects*		
	Control	KDF	CAB	KDF+CAB	SEM	KDF	CAB	Interaction
Normoxia								
Stomach	2.7	1.8	2.5	2.5	0.08	***	*	***
Proximal intestine	6.8	6.2	7.0	6.9	0.04	***	***	***
Middle intestine	7.3	7.5	7.5	7.4	0.08	ns	ns	ns$^{\#}$
Distal intestine	7.4	7.5	7.4	7.5	0.04	ns#	ns	ns
Hypoxia								
Stomach	2.6	2.0	2.4	2.3	0.04	***	ns	***
Proximal intestine	7.4	7.1	7.0	7.1	0.05	ns	**	**
Middle intestine	7.6	7.6	7.4	7.2	0.04	ns	*	ns
Distal intestine	7.8	7.8	7.9	7.7	0.04	ns	ns	*

KDF, potassium diformate; CAB, calcium butyrate; SEM, standard error mean; $^{ns}p>0.05$; $0.05 <^{ns\#}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

5.3.4 Lysozyme activity

Lysozyme activity was significantly increased (P<0.01) in fish fed diets supplemented with CAB versus none supplemented diets during both the normoxic and hypoxic period (Table 5.6). The effect of KDF supplementation on the lysozyme activity became more evident during the hypoxic period (P<0.01). All diets supplemented with OA had higher lysozyme activity values, but there was no synergy between KDF and CAB on the lysozyme activity during both periods, as indicated by the absence of the interaction effect between CAB and KDF (Table 5.6).

Table 5.6 Effect of dietary organic acid supplementation on serum lysozyme activity of Nile tilapia during normoxia (Period 1) and hypoxia (Period 2).

	Diets					***Effects***		
Serum lysozyme activity (U ml^{-1})	Control	KDF	CAB	KDF+CAB	SEM	KDF	CAB	Interaction
Normoxia	498	604	636	640	29	ns#	**	ns#
Hypoxia	467	579	584	614	23	**	**	ns#

KDF, potassium diformate; CAB, calcium butyrate; SEM, standard error mean; $^{ns}p>0.05$; $0.05 <^{ns\#}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

5.3.5 Intestinal morphology

The control diet was formulated with the intention to induce SBM enteritis. During normoxic conditions, minor morphological changes were observed in fish fed the control diet. These symptoms were more obvious during the hypoxic period where an increase of the LP and of the number of GC was observed. However, these SBM enteritis-like symptoms were less evident in fish fed the OA supplemented diets. The OA effects on the thickness of LP and SM seemed to be stronger during hypoxia than during normoxia (Figure 5.3).

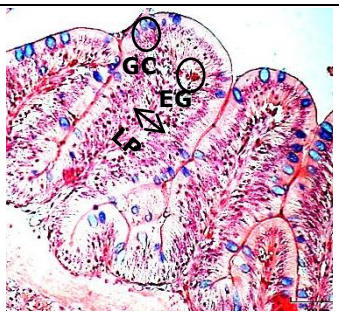
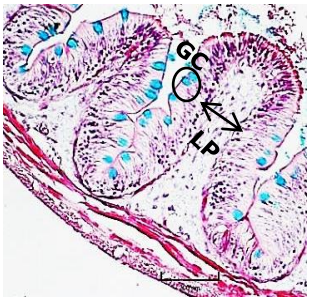
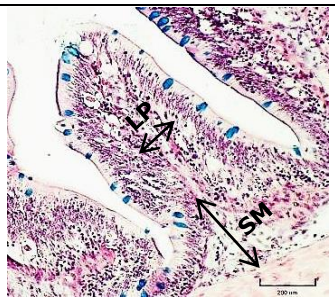
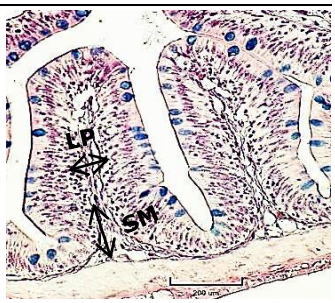
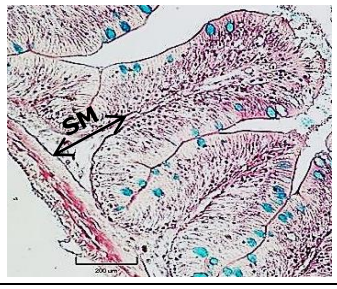
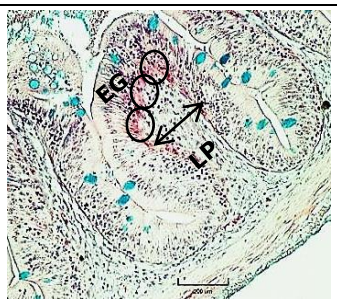
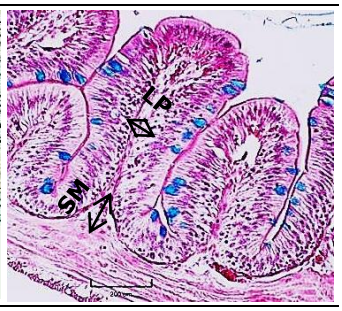
Treatment	Qualitative description	Normoxia period	Hypoxia period
Control diets	1. The number of GC were scattered among the enterocytes during normoxia and increased during hypoxia 2. The number of EG were distributed in LP 3. The thickness of LP increased from normoxia to hypoxia period.		
0.18% KDF supplemented diets	The thickness of SM and LP reduced in the hypoxia period.		
0.03% CAB supplemented diets	The thickness of SM was smaller compared to the control diets in the normoxia period. This effect was stronger in the hypoxia period.		
0.15% KDF : 0.02% CAB supplemented diets	In the normoxia period, the thickness of LP was enlarged and the EG were infiltrated in LP. The thickness of SM and LP reduced in the hypoxia period.		

Figure 5.3 The morphology of the distal intestine of Nile tilapia fed the control diets (0% organic acid) and KDF supplemented diets, CAB supplemented diets and the combination of KDF+CAB during normoxia (Period 1) and hypoxia (Period 2). Haematoxyline/Eosine and Alcian blue staining, x40. SM, submucosa; LP, lamina propria; GC, goblet cells; and EG, eosinophilic granulocytes; KDF, potassium diformate; CAB, calcium butyrate.

Table 5.7 Effect of dietary organic acid supplementation on intestinal morphology of Nile tilapia during normoxia (Period 1) and hypoxia (Period 2).

	Diets					Effects		
	Control	KDF	CAB	KDF+CAB	SEM	KDF	CAB	Interaction
Normoxia								
SM (µm)								
Proximal	480	430	315	430	34	ns	*	*
Middle	493	405	384	430	21	ns	ns#	**
Distal	501	498	518	508	15	ns	ns	ns
LP (µm)								
Proximal	56	48	43	51	2	ns	ns#	**
Middle	80	60	81	65	4	***	ns	ns
Distal	211	143	110	85	10	***	***	*
GC (10^{-3} cells µm^{-1})								
Proximal	12	11	10	9	1	ns	**	ns
Middle	17	14	16	17	1	ns	ns	*
Distal	13	12	10	11	1	ns	ns#	ns
EG (10^{-3} cells µm^{-1})								
Proximal	03	0.0	0.3	0.1	0.1	*	ns	ns
Middle	0.3	0.3	0.7	0.3	0.2	ns	ns	ns
Distal	1.0	1.0	0.3	0.3	0.3	ns	ns#	ns
Hypoxia								
SM (µm)								
Proximal	445	408	389	423	22	ns	ns	ns
Middle	461	340	397	418	20	*	ns	**
Distal	666	489	478	519	17	***	***	***
LP (µm)								
Proximal	68	51	44	58	3	ns	*	***
Middle	99	87	92	85	6	ns	ns	ns
Distal	172	119	134	159	12	ns	ns	**
GC (10^{-3} cells µm^{-1})								
Proximal	20	14	13	11	1	**	***	ns#
Middle	20	13	17	17	1	**	ns	**
Distal	11	10	8	5	1	**	***	*
EG (10^{-3} cells µm^{-1})								
Proximal	0.9	0.4	0.3	0.3	0.2	ns	ns	ns
Middle	0.2	0.3	0.05	0.3	0.1	ns#	ns	ns
Distal	1.2	1.1	1.1	1.2	0.3	ns	ns	ns

SEM, standard error mean; KDF, potassium diformate; CAB, calcium butyrate; $^{ns}p>0.05$; $0.05 <^{ns\#}p<0.1$; $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$.

The average scoring values per diet of intestinal parameters, which quantified the SBM enteritis response for each diet and each period (normoxia and hypoxia) are described in Table 5.7. The results show that the OA supplementation had a positive effect on all four intestinal parameters by decreasing the thickness of SM and LP and the number of GC and EG under the normoxic conditions. During hypoxic conditions, the effects of OA supplementation on intestinal morphology were more pronounced, especially on SM and GC. Although supplementation with a single dose of OA improved the intestinal morphology, the combination of KDF and CAB has no benefit for the intestinal morphology.

5.4 Discussion

The response of OA supplementation on fish performance in literature is highly variable. Some studies found no effect of OA on growth rate (Owen *et al.*, 2006, Zhou *et al.*, 2009, Gao *et al.*, 2011, Zhu *et al.*, 2014) whereas others did find an effect (Ramli *et al.*, 2005, Liebert *et al.*, 2010, Castillo *et al.*, 2014). Lim *et al.* (2010) stated that the variability in OA effects on growth between studies depends on fish species, age, and the types and levels of organic acids used. Other factors being related to this variability between studies are diet composition, culture management and water quality. The current study also demonstrated that the impact of OA on growth performance, digestibility and intestinal morphology depends on water DO levels (hypoxia versus normoxia).

The inclusion, YES or NO, of formic acid and butyric acid was examined in a 2x2 factorial design. This led to four experimental diets that were formulated by supplementing 0 % (NO) and 0.2 % (YES) of each organic acid to the basal diet. The 0 % organic acid supplementation is considered as the control diet.

5.4.1 Growth performance

KDF did not improve growth performance under normoxic conditions, but it had beneficial impacts on fish culture under more harsh conditions. Under hypoxia, fish fed on KDF showed a significant increase in the body weight compared to the control. These results corroborate those of other authors who could not find growth improvement due to OA supplementation under normoxic conditions (Owen *et al.*, 2006, Gao *et al.*, 2011). For example, Owen *et al.* (2006) tested 0.02% of sodium butyrate for African catfish (*Clarias gariepinus*) in two diets, differing only in their major protein source: fishmeal *vs* full-fat soya, and the SGR and FCR between treatments showed non-significant differences compared to the control.

5.4.2 Digestibility

Although the accumulation of faeces in the water can create a different source of error if the faeces are not removed rapidly from the water, in our experiment, we observed that faeces sank and settled in a short period of time. Floating faecal pellets, when observed, were rapidly removed, to avoid any possible leaching caused by a long exposure time to the water. In addition, tilapia produces string-type of faeces that are involved in a mucus envelope, which makes them more resistant to leaching. Therefore, the estimations of protein digestibility in our study is valid.

Testing OA under optimal conditions (good water quality and balanced diets) have probably contributed to the lack of significant effects between treatments (Petkam *et al.*, 2008). In the current study, we found that environmental conditions altered the response

of OA on digestibility. OA supplementation numerically improved the digestibility of protein and energy in the normoxic periods, however these effects were more pronounced in the hypoxic periods. Although the effect of single dose OA improved the digestibility in hypoxia, the effect of the combination together was not more effective. In a previous study, Tran-Ngoc *et al.* (2016a) showed that the combination of hypoxia and a SBM-based diet reduced protein digestibility in Nile tilapia. This might explain the increasing difference in protein digestibility of the control and OA diets between normoxic to hypoxic period. Tran-Duy *et al.* (2008a) suggested that the reduced nutrient digestibility at hypoxic conditions is caused by hampering the energy expenditure and consequently reducing the nutrients absorption.

In the current study, we found a significant decrease in the stomach and proximal intestinal pH of the KDF groups in both periods. Lower gastric pH is associated with a higher pepsin activity that contributes in this way to improve the protein digestibility and nitrogen retention (Liebert *et al.*, 2010). This may also be the reason for the improvement of final body weight and protein digestibility in the KDF supplemented diet groups. Similarly, Ng *et al.* (2009) demonstrated that 60 % soya bean meal-based diets supplemented with 0.2 % KDF tended to improve the nutrient digestibility on red hybrid tilapia, *Oreochromis* sp. Elala and Ragaa (2015) also reported a better apparent protein digestibility in Nile tilapia fed 0.2 and 0.3 % KDF. The beneficial effects of formic acid and acid salts result mainly from their strong antimicrobial activity and growth promotion effect (Ng *et al.*, 2009, Zhou *et al.*, 2009, Elala and Ragaa, 2015, Koh *et al.*, 2016). Dietary KDF also resulted in higher final body weight and digestibility of protein compared to other treatments in hypoxia.

The CAB effect on digestibility was also more pronounced in the hypoxic periods. The energy digestibility in the CAB treatments was higher (1.4 % and 4.6 %) compared to control diets during normoxic and hypoxic periods respectively. The improvement in energy digestibility might be associated with the beneficial effect of butyric acid on the proliferation of the intestinal epithelium (Topping and Clifton, 2001) and with the improvement in villi development which increases the absorption surface area, leading to a better nutrient utilization (Robles *et al.*, 2013).

5.4.3 Intestinal morphology

In a previous paper, Tran-Ngoc *et al.*, (2016a) has proven that intestinal morphology is dependent of the environmental conditions. In the current study, fish fed with OA supplemented diets all showed improvements in the intestinal morphology under the normoxic conditions, and these effects were even more enhanced in the hypoxic conditions. Fish fed KDF and CAB during hypoxia appeared to have a thinner thickness of submucosa and lamina propria and a lower number of goblet cells in the distal intestine

compared to the fish fed control diets. Gao *et al.* (2011) observed that the 1 % of Na-formate and Na-butyrate (ration 2:1 on acid moiety weight basis) added in plant protein-based diet did not affect the morphological appearance of the distal intestine of rainbow trout. Rainbow trout fed high levels of soybean meal were slower to develop distal intestinal enteritis compared to Atlantic salmon (Refstie *et al.*, 2000). This might explain the trend we found. From our results, and looking at the results from other authors, we believe that the ability of organic acids to strengthen the intestinal morphology may not be very noticeable when fish are cultured without being subjected to stressful (i.e. suboptimal) conditions. Therefore, the effects of formic and butyric acid were negligible in fish fed KDF and CAB during the normoxic period.

The results regarding the changes of intestinal morphology could have been induced by a carry-over/longer exposure effect rather than the effect of normoxia and hypoxia. However, we expected that the effect of the second period was predominately driven by oxygen since for slower metabolism species, such as salmonids, the SBM enteritis are normally stable 21-28 days after being fed a SBM diet (Urán *et al.*, 2009). Furthermore, observations in Nile tilapia showed that the development of SBM enteritis predominately occurred between week 1 and week 4, and the chances for alteration of intestinal morphology were minor between week 4 and 8 (Tran-Ngoc *et al.*, 2016a). Therefore, and in order to minimize any possible effects of statistical dependence, we analysed the end-point results of the two periods (end of week 5 – normoxia period and end of week 10 – hypoxia period) separately. We strongly believe that the difference in intestinal morphology between these 2 endpoints reflect the effect of hypoxia, although we cannot exclude that also the exposure duration to the OA have influenced these results.

Butyric acid and butyrate are efficient in providing energy for epithelia growth which leads to increased nutrient absorptive capacity (Topping and Clifton, 2001). Moreover, butyric acid and butyrate also influence a variety of cellular functions relevant to intestinal health such as mitigating mucosal inflammation and oxidative stress, and improving the intestinal epithelia barrier function (Hamer *et al.*, 2008). Given that butyric acid is efficient in enhancing the intestinal morphology, CAB supplemented groups significantly improved the intestinal morphology in both periods. Liu *et al.* (2014) reported that dietary supplementation of Na-butyrate increased microvillus density and helped to prevent or repair the intestinal mucosal damage in common carp (*Cyprinus carpio*) fed a diet prepared with oxidised soybean oil. Sodium butyrate as a fat-soluble substance can be quickly absorbed and utilised as an energy source by enteric epithelial cells (Roediger, 1980). In our study, the use of the encapsulated form of calcium butyrate could have sustained the release along the whole intestine of tilapia. This assumption seems to be correct, since we found that the effect of CAB on intestinal

morphology parameters, especially SM and GC, was more pronounced in the distal intestine over time from normoxia to hypoxia.

The enhanced lysozyme activity might have contributed to the observed improvement of the intestinal morphology. Lysozyme activity is an important defence molecule of the innate immune system, mediating protection against microbial invasion (Saurabh and Sahoo, 2008), and an important index of innate immunity of fish (Dominguez *et al.*, 2005). In the present study, we found significantly higher lysozyme activity under hypoxic conditions in fish fed OA as either CAB or KDF. The difference in the lysozyme concentration between treatments could also be triggered by inflammation or bacterial translocation. However, this is unlikely in the present study because of the noted alteration in intestinal morphology response. Fish fed the OA supplemented diets showed improvements of the intestinal morphology, whereas fish fed the control diets showed enteritis symptoms in both periods. The reasons why the lysozyme concentration was high in the OA supplementation groups could be either one or more interdependent factors: Elala and Ragaa (2015) showed that formic acid was able to modify microbial communities in tilapia guts, helping the ability of the fish to initiate an immune response. Butyric acid contains 4 carbon atoms with pKa 4.82 and enhances the synthesis of ATP (Uribe and Jagendorf, 1967), thereby improving the body's non-specific immunity and stress resistance ability.

Each organic acid has its own spectrum of effects due to their specific physical and chemical properties (Dibner and Buttin, 2002). In this case, formic acid aims to induce a low pH in the stomach, whereas butyric acid is related to improving the intestinal epithelia barrier function. Therefore, we expected an advantage of using the combination of formic and butyric acid. However, in our results, we could not find any proof of the positive synergistic effects of using a combination of OA either in terms of growth, digestibility and intestinal morphology. The ineffectiveness of using a blend of OA was shown before in other works. For instance, Gao *et al.*(2011) reported that including 1% of sodium formate and butyrate in a plant protein-based diet did not improve the growth rate or feed utilization of rainbow trout. Bjerkeng *et al.* (1999) also reported that no significant effects on growth or digestibility in Atlantic salmon were found when fish were fed diets supplemented with 0, 0.5, or 2 % of an organic acid blend containing sodium salts of acetic, propionic and butyric acid (5:5:2 w/w/w). A review of current literature on using the combination of OA indicates that in most of the cases, researchers have not reported the recovery of OA in the diets. In the current study, the lower effectiveness of the combination of OA may be due to the low inclusion of butyric acid recovery in the combination. The reason for this still remains unclear. It could be either the chemical interaction between the two compounds, e.g., formic and butyric acid or it could be due to other issues in the diet making. However, the low inclusion of butyric acid in the

combination does not have a strong impact on the outcome of this study because the significant differences between OA supplemented groups and control groups on growth, digestibility and intestinal morphology were also found during the hypoxic period. Therefore, the lower dose of OA in the combination does not change the message of our study, e.g., that the environmental conditions alter the response of OA on growth performance, digestibility and intestinal morphology in Nile tilapia.

5.5 Conclusion

The current study shows that although OA did not significantly improve growth performance and digestibility under normoxic conditions, it has a beneficial impact on fish under hypoxic conditions. It demonstrates that the impact of OA is dependent on the environmental conditions, being more pronounced under suboptimal conditions (such as hypoxia). Under hypoxic conditions, the OA improve the growth performance, digestibility and intestinal morphology of Nile tilapia. Therefore, the OA supplementation in diets is more relevant when the fish are cultured under stressful (e.g., suboptimal) conditions. While the beneficial effects of formic acid are mainly on growth and improving of the protein digestibility, butyric acid is more efficient in enhancing the intestinal morphology. A synergistic or additive effect was not found in the combination of formic and butyric acid. It demonstrates that the mode of action of OA still needs further study.

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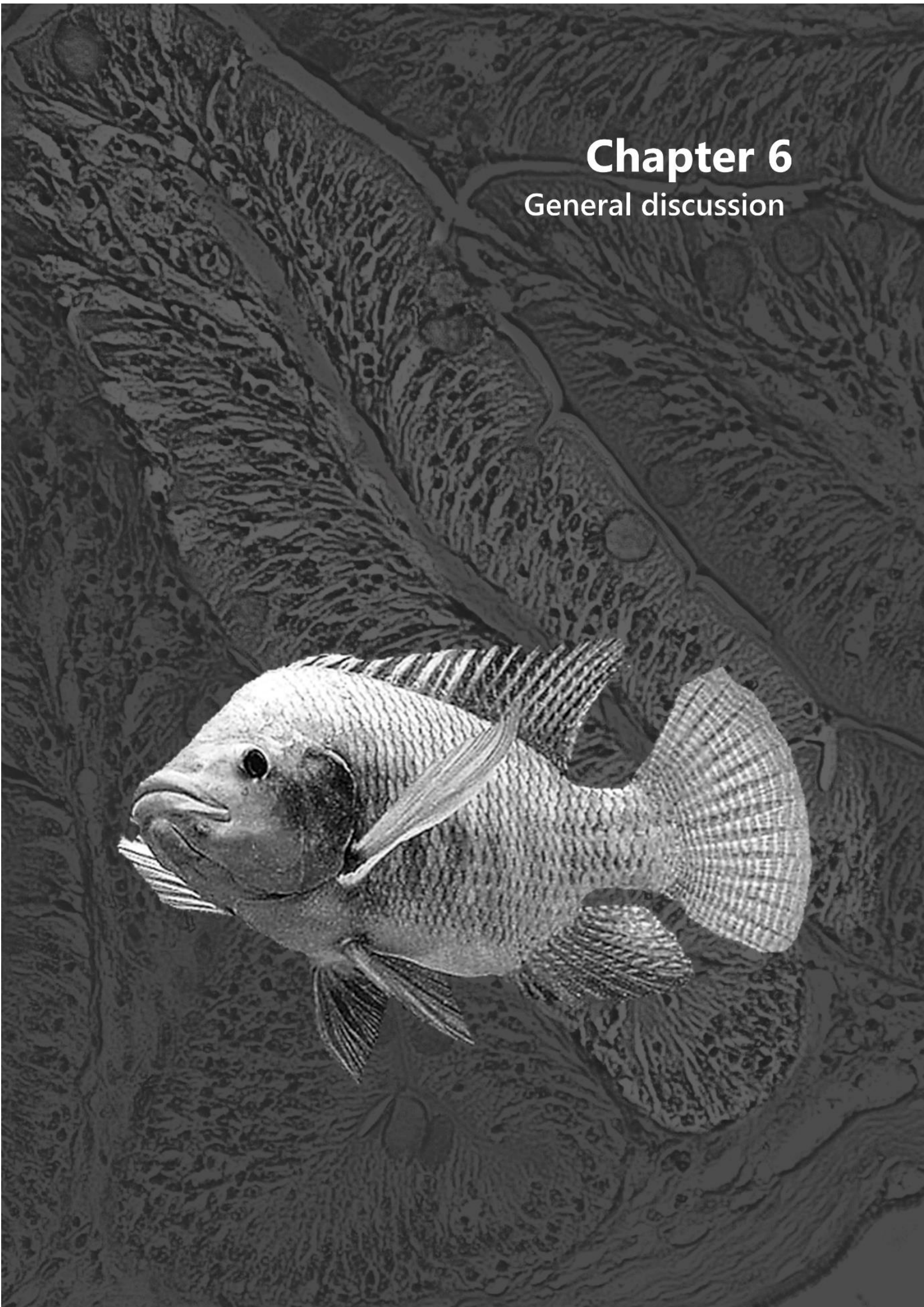
Conflict of interest

There are no contractual agreements for the presented data, which might cause conflicts of interest.

Chapter 6
General discussion



6.1 Synergistic effects of diet and environmental conditions on the intestinal functions

In commercial farming, fish can be exposed to suboptimal environmental conditions that might disturb the feeding behaviour and growth rate, alter energy metabolism (Ishibashi *et al.*, 2002) even hamper intestinal homeostasis (Cain and Swan, 2010). Exposure to unbalanced diets can also weaken the intestinal barrier functions (Jutfelt, 2011). A single stressor might have a minor impact on the intestinal functions but when combined with others stress factors, the negative impacts can strongly increase. Therefore, the central hypothesis of this thesis was that adverse environmental conditions may aggravate the negative effects of diet on the intestinal functions to an extend that mild effects become severe and perceptible.

There are various studies in fish focusing on the effect of one single stress factor on the intestinal functions. As examples of diet related stress factor, Atlantic salmon (Urán *et al.*, 2009), rainbow trout (Nordrum *et al.*, 2000), and gilthead sea bream (Bonaldo *et al.*, 2008) showed enteritis symptoms when fish were fed soybean meal (SBM) based diets. To illustrate the effect of an environmental stress factor, Sundh *et al.* (2010) and Niklasson *et al.,* (2011) showed that paracellular permeability increased in both the proximal and distal intestine of Atlantic salmon when subjected to 50 % of dissolved oxygen (DO) saturation conditions. However, the question whether dietary induced enteritis is influenced and/or aggravated by coinciding environmental stress, has been poorly studied thus far. In this thesis, the interaction between diet composition and environmental conditions on intestinal functions was presented in chapters 3, 4 and 5.

Chapter 1 showed that although soybean meal caused alterations in the intestinal morphology, it was highly digestible ingredient, even at an inclusion level on the diet of 30 %. Therefore, we believe that the difference between zone A and zone B (Figure 6.1) will be minor, certainly in the case of soybean meal. This goes in agreement with the hypothesis is that the effect of dietary changes may not be very noticeable when fish are cultured in optimal conditions.

Chapter 3 and 4 showed there was an interactive effect of dissolved oxygen (chapter 3) or salinity (chapter 4) and diet composition on the intestinal functions. The negative effects of diets with high inclusion level of SBM (50 %) on the intestinal morphology (widening the thickness of lamina propria and submucosa, increasing the number of goblet cells and eosinophilic granulocytes) were enhanced by hypoxic conditions (chapter 3) and when salinity was elevated (chapter 4). A schematic overview of these results can be seen in figure 6.1 The combination of a soybean meal based diet at zone B and the suboptimal environmental conditions (zone C/D) significantly decreased the intestinal functions as indicated by zone E and F. Also this confirms the central hypothesis that the

intestinal functions accumulated dietary damage more rapidly or more severely when fish are expose to adverse environmental conditions.

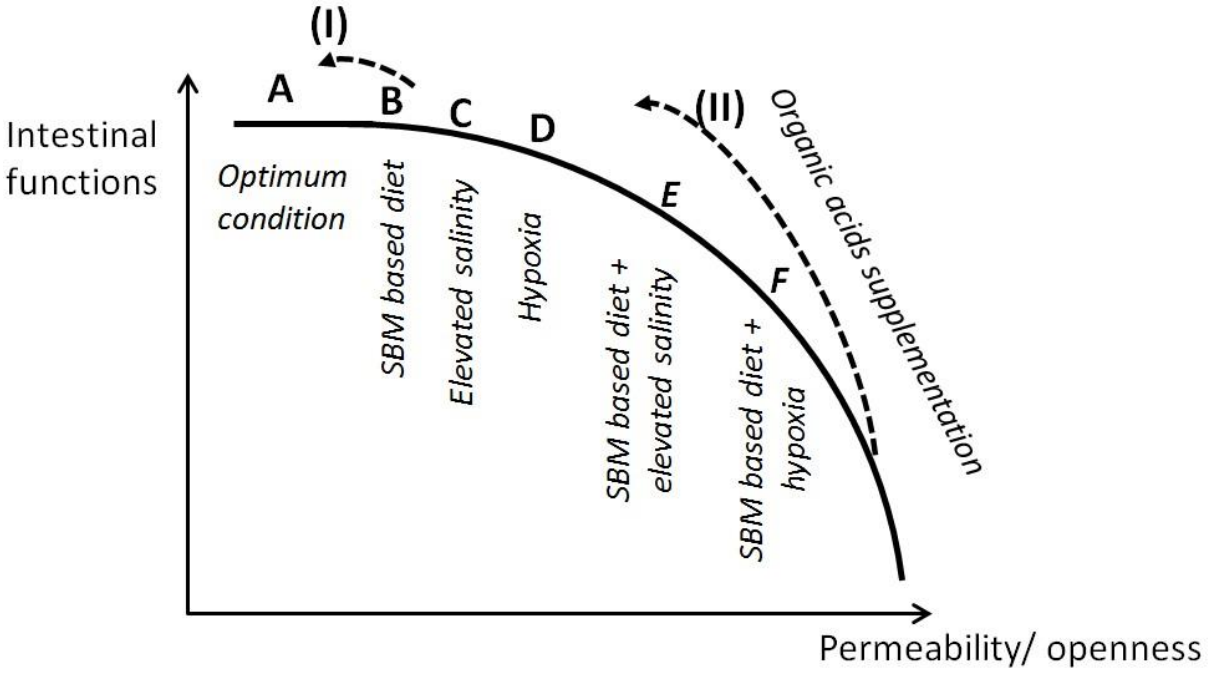


Figure 6.1 Schematic overview of the main results of the PhD thesis. Zone A represents the optimum condition where environmental conditions and dietary conditions were optimal (control). Zone B indicates the area where fish were fed a soybean meal based diet under optimal environmental condition (Chapter 2). Zone D and C indicates the area where fish were fed an optimal diet but under a suboptimal environmental conditions, such as hypoxic (Chapter 3) and elevated salinity (Chapter 4) conditions, respectively. Zone E and F indicates the area where soybean meal based diet at zone B and a suboptimal environmental condition at zone C and D were combined, respectively. The impact of using organic acids supplementation at zone F (Chapter 5) was more significantly than at zone B indicated by arrow I and II.

These effects of adverse environmental conditions on the intestinal functions were not homogenously dispersed over the whole intestinal length. The effect of salinity on the intestinal morphology occurred predominantly in the distal intestine, while the effect of oxygen was more visible in the proximal region. This regional effect of salinity might be related to the role of the distal part of the intestine in osmoregulation process (i.e. water absorption). As shown in Chapter 3, fish cultured at an elevated salinity had high count of goblet cells in the distal part of their intestine. Most likely, in saline water, these cells have an osmoregulatory function (Rodríguez *et al.*, 2005). Li *et al.,* (2014) showed that in Mozambique tilapia (*Oreochromis mossambicus*), the distal region of the intestine was highly responsive to salinity changes, more than the proximal and middle region. However, in combination with a soybean meal diet, effects were primarily observed the proximal intestine. Therefore, elevated salinities can aggravate the effect of an adverse diet, but where it takes place in the intestine, is not consistent.

On the other hand, hypoxic conditions tended to clear exert their effects on intestinal morphology in the proximal region (Chapter 3). Niklasson *et al.* (2011) found that at a temperature of 16^0C, low DO levels created an increased expression of interleukin-10 (IL-10) in the proximal intestine of Atlantic salmon, resulting in the inflammation of this region. In the same line of results, the combination of a soybean meal diet and hypoxic condition also altered the intestinal morphology in the proximal region.

Alterations in the intestinal morphology, as found in this study, can have wider effects on the performance of the affected fish. For example, Mahmoud (2009) and Raskovic *et al.* (2011) suggested that differences in nutrient digestibility among diets could be attributed to the alteration of the intestinal morphology. The evidences for these effects were also found in this study. In Chapter 3, the protein digestibility decreased under hypoxic conditions at week 8, which parallels with the time related alteration in intestinal morphology. Chapter 4 showed that when fish were raised at 15 ‰ of salinity, nutrient digestibility increased; however, this positive effect decreased when the intestinal morphology has changed.

Chapter 5 intended to investigate if the impact of the challenging conditions could be mitigated by the use of organic acids. As shown in Chapter 3 and 4, the combined effect of a soybean meal based diet and hypoxia was stronger compared to the combination with elevated salinity. The possible adaptation of Nile tilapia water of 15 ‰ might be a reason for this. Indeed, in Chapter 4, the fish was acclimate to brackish water for 8 weeks, therefore, it is believed that the difference in response between Chapter 3 and 4 can be explained by the fish having adapted to the brackish water conditions in Chapter 4. Subsequently, the combination of hypoxic conditions and a soybean meal based diet was chosen to test the hypothesis that the effect of organic acids can be more perceptible under stressful culture conditions. The present results explained why in literature, the observed impacts of a diets on the growth performance and nutrient digestibility are often highly variable (Ramli *et al.*, 2005, Zhou *et al.*, 2009, Ng *et al.*, 2009, Hassaan *et al.*, 2014, Elala and Ragaa, 2015). Most data from literature are obtained from studies where feed additives were tested under optimal conditions instead of stressful conditions. In the current thesis, it demonstrated that the organic acids supplementation did not significantly improve growth performance and nutrient digestibility under normoxic conditions, but it had a beneficial impact on fish under hypoxic conditions. Figure 6.1 showed that if organic acids were supplemented at zone B (without suboptimal environmental conditions), a small impact was found indicated by arrow I. When organic acids were supplemented at zone F (soybean meal diet and hypoxic conditions combined), the impact was more pronounced as indicated by arrow II.

6.2 Relevance of this study to aquaculture

In the majority of the fish groups, the intestinal tract is functionally and morphologically divided into distinct regions, and the barrier function varies between these intestinal regions (Jutfelt, 2011). The typical signs of SBM-induced enteritis was described in the distal intestine of Atlantic salmon which might be related to the complex structure of the distal intestine compared to the proximal intestine of the carnivorous fish. In Nile tilapia, however, the mucosa of the proximal intestine is longer and has more branched villi than the distal intestine (Gargiulo *et al.*, 1998), and is therefore more vulnerable for intestinal disorders. As a result, in Nile tilapia, the enteritis-like changes seem to be particularly present in the proximal segment whereas in Atlantic salmon, these changes occurred in the distal intestinal segment. Furthermore, in tilapia, changes in the intestinal morphology occurs later with less severe symptoms when compared with salmonids (Chapter 2). Being a herbivorous species, it was expected that tilapia were more adapted to the SBM-based diets, but the alteration in the intestinal morphology seems to be persistent, showing no signals of recovery (Chapter 2, 3,4 and 5). On the contrary, in common carp, the effects of SBM-based diets on the intestinal morphology was only temporary and the species recovered from these alterations after a certain period of time (Urán *et al.*, 2008a). The reason why tilapia is not capable of recovering from the changes in intestinal morphology while common carp does, remains unclear. The difference between the recovery process on these two species might be related to the different structure of the digestive system. Tilapia is notice to be a more herbivorous species than common carp (omnivorous) and with a clearly longer digestive tract. Furthermore, tilapia also has a clear stomach with very low pH that is lacking in carp (Sklan *et al.*, 2004b, Raskovic *et al.*, 2009). Therefore, the SBM-induced enteritis symptom is clearly species-dependent.

Several aspects involved in the production process of different soybean products may affect the quality and the composition of these products and may also be responsible for different responses when fed to fish. Chapter 2 illustrates an experiment in which the inclusion levels of dehulled solved-extracted SBM was 30 %, while the inclusion levels in Chapter 3, 4 and 5 was about 50 %. It could be noticed that the lower inclusion level resulted in a mild response in contrast to the response at the higher level which was a stronger response. The alteration of intestinal morphology was clearly dose-dependent.

In traditional tilapia pond culture, proper environmental conditions are maintained by balancing the inputs of feed with the natural assimilative capacity of the pond environment. The pond's natural biological productivity (algae, higher plants, zooplankton and bacteria) serve as an additional food source; therefore, the problem with soybean meal in diets is probably less pronounced in these systems. In the current

work, the enteritis developed only with the high soybean meal inclusion level (50 %). On the other hand, as proven in this study, environmental conditions can alter the effect of diet on intestinal functions. Therefore, all aquaculture production systems should provide a suitable environment to promote fish health and fish welfare. This should be taken in consideration either the fish are cultured in ponds, cages, or in tanks or raceways.

Histopathology together with other parameters such as growth performance, disease occurrence, nutrient digestibility can be used as biomarkers tools assessing effects of both internal (feed used) and external (aquatic) environmental conditions (reviewed by Rašković *et al.*, 2011). Too many nutritional papers focus only on the growth response of fish. In this study, the interaction of diet composition and environmental conditions on intestinal functions was assessed. Modern aquaculture can benefit from this approach. Furthermore, histological analysis of the digestive system is considered as a good indicator of the nutritional status of fish, therefore monitoring of the intestine functions is considered necessary for nutrient studies.

6.3 Methodological aspects

The intestinal morphology was evaluated based on a semi-quantitative method developed at Wageningen University for salmon (Urán, 2008), which was adapted for the response to SBM-induced changes in the intestine in Nile tilapia. The following intestinal morphology parameters were assessed: a) the number of goblet cells (GC), b) the number of eosinophilic granulocytes (EG), c) the thickness of the lamina propria (LP) and d) the thickness of the sub-epithelial mucosa (SM). An increase in the numbers of GC and EG, or the thickness of SM and LP are markers for SBM-induced enteritis symptoms in Nile tilapia, which leads subsequently to a decline in nutrient digestibility as shown in this study. However, in Chapter 2, the nutrient digestibility was positively related with the thickness of SM in the proximal and mid intestine. The thicker the SM in the proximal and mid intestine, the better the digestibility of protein, lipid and energy. This was an unexpected result. Most probably, the thickness of SM may be larger due to the nutrient absorption in the proximal part in the intestine, resulting in the proliferation of the intestinal cells. Furthermore, the presence of EG was not clearly observed, probably because in Nile tilapia the SBM-based diets had less severe effects than in salmonids. On the other hand, the thickness of LP and the number of GC are stronger indicators for the alterations in intestinal morphology. According to this findings, care should be taken in interpreting changes in the intestinal morphology using these intestinal parameters only. Other parameters such as enterocyte height, mucosal folds and supranuclear vacuoles should be also considered as useful histological parameters that could be monitored when evaluating different types of ingredients (not only as a replacement of fish meal).

The interaction between a challenging diet and suboptimal condition was expected to amplify the change in the intestinal morphology to such an extent that the effect of organic acids may become measurable in reducing those changes. While the beneficial effects of formic acid is mainly for growth promotion and improving the protein digestibility, butyric acid is more efficient in enhancing the intestinal morphology. This concept could be applied for studies of the impact of nutrients on intestinal functions. Testing other feed additives (i.e. probiotic, prebiotic) or other feed ingredients should be investigated under the suboptimal conditions rather than optimal conditions. However, the effects of suboptimal conditions might be species specific. Therefore, the results found in tilapia under an elevated salinity or hypoxic condition could be different compared to other species.

6.4 Final conclusions

The following main conclusions are derived from this thesis:

1. In Nile tilapia, soybean meal alters intestinal morphology. Differences in intestinal morphology between plant ingredients do not correlate with nutrients digestibility (Chapter 2).
2. The negative effects of plant ingredients on the intestinal functions can be amplified if fish are cultured under suboptimal environmental conditions. The negative effect on intestinal morphology of soybean meal in the diet is enhanced under low oxygen level and elevated salinity. The effect of salinity on the intestinal morphology occurs predominantly in the distal intestine, whereas the effect of low oxygen concentration is more visible at the proximal intestine (Chapter 3 and 4).
3. The impact of organic acids on intestinal functions is dependent on environmental conditions, being more pronounced under challenging conditions (e.g. hypoxia) (Chapter 5). Therefore, studies on both positive (using feed additives) and negative effects (using high inclusion of plant ingredients) of dietary factor should be done under suboptimal conditions.

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Summary

Summary

Diet composition, ingredient and nutrients, are important to consider for maintaining intestinal functions. Studies on both positive (using feed additives) and negative effects (using high inclusion of plant ingredients) of fish feeds are numerous, however, between studies results are often highly variable, both in type of response and in significance. The central hypothesis of this study was that adverse environmental conditions may aggravate negative effects of plant ingredients on the intestinal functions to the extent that mild effects become severe and perceptible. To do so, dietary factors and environmental conditions were evaluated and the interaction between diet composition and environmental conditions were studied in Nile tilapia.

In **Chapter 2**, six common raw materials including hydrolysed feather meal (HFM), soybean meal (SBM), rice bran (RB), rapeseed meal (RM), sunflower meal (SFM) and dried distiller grains with solubles (DDGS) were chosen to determine the effect of nutrient digestibility, nitrogen/energy balance and changes in intestinal morphology. The study demonstrated that feed ingredients do have an impact on the alteration in intestinal parameters, but also on the nutrient digestibility and the nitrogen/energy balance. Although being well digested, soybean meal caused the most obvious alteration in the intestinal morphology. These alterations were not related to the nutrient digestibility nor to nitrogen/energy balance parameters. Soybean meal, causing the most alterations in the intestinal morphology, was further used in all subsequent chapters of this thesis.

Chapter 3 and 4 described the interaction between diet composition and environmental conditions on the intestinal functions. This was studied with two different environmental conditions, dissolved oxygen (Chapter 3) and salinity (Chapter 4). These two chapters evaluated whether suboptimal environmental conditions (low dissolved oxygen or elevated salinity in water) may interact with a soybean meal based diet in nutrient digestion and intestinal morphology of tilapia. The study demonstrated that environmental stressors can aggravate/reveal the negative intestinal morphology changes induced by a soybean meal based diet. However, these effects of adverse environmental conditions on the intestinal functions were not homogenously dispersed over the whole intestinal length. The effect of salinity on the intestinal morphology occurred predominantly in the distal intestine, whereas the effect of low oxygen concentration was more visible at the proximal intestine. Alterations in the intestinal morphology, as found in this study, have wider effects on the performance of the affected fish. In Chapter 3, the protein digestibility decreased under hypoxic conditions at week 8, which parallels with the time related alteration in intestinal morphology. Chapter 4 showed that when fish were raised at 15 ‰ salinity, nutrient digestibility increased; however, this positive effect decreased when the intestinal morphology changed. The

study also found that the combined effect of a soybean meal based diet and hypoxia was stronger compared to the combination with elevated salinity. Therefore, the combination with hypoxia was further used in the next study of this thesis.

In **Chapter 5**, the combination of hypoxic condition and a soybean meal based diet was chosen to test the hypothesis that only under stressful conditions, the effects of feed additives can be noticed. The impact of two dietary organic acids, formic acid and butyric acid, on nutrient digestibility and intestinal morphology was determined under optimal (normoxia) and suboptimal conditions (hypoxia). The results showed that although organic acids did not significantly improve growth performance and nutrient digestibility under normoxic condition, they did so under hypoxic conditions. Fish fed organic acid supplemented diets all showed improvements in the morphology of intestine under normoxic conditions, and these effects were more enhanced under hypoxic conditions. This indicates that environmental conditions can alter the effect of organic acid on nutrient digestibility and intestinal morphology in tilapia.

Finally, **Chapter 6** provided a synthesis of the main findings, and a reflection on the methodologies used in Chapters 2-5 as well as a discussion on the relevance of this study to aquaculture. It is concluded that although being well digested, soybean meal caused the most obvious alteration in intestinal morphology. The adverse environmental conditions aggravated negative effects of soybean meal based diets on the intestinal functions to the extent that mild effects become severe and visible. The negative effect on intestinal morphology of soybean meal in the diet is enhanced at low oxygen level and at elevated salinity. The effect of salinity on the intestinal morphology occurs predominantly in the distal intestine, whereas the effect of low oxygen concentration is more visible at the proximal intestine. The thesis indicated that the impact of organic acids on intestinal functions is dependent on environmental conditions, being more pronounced under challenging conditions (e.g. hypoxia). Therefore, studies on both positive (using feed additives) and negative effects (using high inclusion of plant ingredients) of dietary factors should be done under suboptimal conditions.

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Kim Tran

About the author

Trần Ngọc Thiên Kim was born on August 10th, 1982, in Ho Chi Minh City in Vietnam. After high school, she started her studies in Aquaculture Engineering at the Faculty of Fisheries at Nong Lam University, Ho Chi Minh City, Vietnam. She graduated in 2005 completing a BSc thesis on "Effect of phytase on growth performance and nutrient digestibility of Pangasius (*Pangasinodon hypothalamus*) fingerlings" which was carried out at the Faculty of Fisheries facilities. After she finished her BSc, she became a lecturer of the Faculty of Fisheries at the Department of Aquatic Animal Pathology, a position that she still maintains.

In 2011, she obtained her MSc degree in Sustainable Aquaculture under an AusAID scholarship from Curtin University, Perth, Australia. Her MSc thesis was entitled "Effect of enrichment of live food with immunostimulants on the performance of Yellowtail Kingfish larvae (*Seriola lalandi*)". With a strong desire to help the fish farmers, after returning home from Australia, she always thought of research to develop scientific methods for the treatment of fish disease, considering it an important tool to help people have more affluent life from fish farming. Her motivation and enthusiasm for research in fish nutrition directed her towards applying for a scholarship from The Netherlands Fellowship Program (NUFFIC) to carry out her PhD studies at the Aquaculture and Fisheries Group of Wageningen University, Wageningen, The Netherlands in 2012. She was awarded this scholarship. The PhD program and research was also sponsored by Skretting Aquaculture Research Centre, Stavanger, Norway, a Nutreco company. Believing that as in humans, the overall health of a fish started with a healthy gut, she decided to explore this subject more and undertake a PhD in Nile Tilapia gut health. Four years of her research work led to the submission of this thesis entitled "Feeds, water quality, gut morphology and digestion in Nile Tilapia (*Oreochromis niloticus*)".

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List of publications

Tran-Ngoc, Kim T., Haidar, M.N., Roem, A.J., Verreth, J.A.J. & Schrama, J.W. (2016b) Effects of feed ingredients on nutrient digestibility, nitrogen/energy balance and morphology changes in the intestine of Nile tilapia (*Oreochromis niloticus*). Aquaculture submitted.

Tran-Ngoc, Kim T., Dinh, T.N, Nguyen, H.T., Roem, A.J., Schrama, J.W. & Verreth, J.A.J. (2016a) Interaction between dissolved oxygen concentration and diet composition on growth, digestibility and intestinal health of Nile tilapia (*Oreochromis niloticus*). Aquaculture, 462, 101-108.

Tran-Ngoc, Kim T., Schrama, J.W., Le, M.T.T., Nguyen, T.H., Roem, A.J. & Verreth, J.A.J. (2017b) Salinity and diet composition affect digestibility and intestinal morphology in Nile tilapia (*Oreochromis niloticus*). Aquaculture, 469, 36-43.

Tran-Ngoc, Kim T., Huynh, T.S., Nguyen, H.T., Roem, A.J., Verreth, J.A.J. & Schrama, J.W. (2017a) Environmental conditions alter the effect of organic acids on digestibility and intestinal morphology in Nile tilapia (*Oreochromis niloticus*). British Journal of Nutrition, under revision to re-submission.

Contributions to conferences and seminars

Tran-Ngoc, Kim T., Roem, A.J., Jacklofsky, M., Verreth, J.A.J. & Schrama, J.W. (2013) Effects of plant feedstuffs on nutritional physiology and gut histology of tilapia – Preliminary results. *Asia-Pacific Aquaculture*, *Ho Chi Minh, Vietnam*, 10-13th December 2013.

Tran-Ngoc, Kim T., Nguyen, H.T., Verreth, J.A.J, Roem, A.J. (2014) Effects of immunostimulants on survival of Nile tilapia (*Oreochromis niloticus*). *Aquaculture Europe 14, San Sebastian, Spain*, 14-17th October 2014.

Tran-Ngoc, Kim T., Dinh, T.N., Nguyen, H.T., Roem, A.J., Schrama, J.W., Verreth, J.A.J, (2015). Effects of oxygen level and dietary composition on growth performance and intestinal morphology in Nile tilapia (*Oreochromis niloticus*). *Aquaculture Europe 15, Rotterdam, The Netherlands*, 20-23th October 2015.

Tran-Ngoc, Kim T., Huynh, T.S., Nguyen, H.T., Roem, A.J., Verreth, J.A.J, Schrama, J.W. (2016) Organic acids work bets in Nile tilapia (*Oreochromis niloticus*) under suboptimal conditions. *Aquaculture Europe 16, Edinburgh, Scotland*, 20-23th September 2015.

Training and Supervision Plan

Education and Training		
The Basic Package	year	credits
WIAS introduction course, October 23 – 26	2012	1.5
Ethics and Philosophy in Life Sciences, April 10-12	2013	1.5
Introduction interview with WIAS scientific director and secretary, October 4	2012	
Introduction interview with WIAS educator coordinator, October 18	2012	
Introduction interview with WIAS PhD students confidant, October 18	2012	
Subtotal Basic Package		**3.0**

Scientific Exposure		
International conference	year	credits
Asian-Pacific Aquaculture, Ho Chi Minh city, Vietnam, December 10-13	2013	1.2
Aquaculture Europe 14, Donostia – San Sebastian, Spain, October 14-17	2014	1.2
Aquaculture Europe 15, Rotterdam, The Netherlands, October 20-23	2015	1.2
Aquaculture Europe 16, Edinburgh, Scotland, September 20-23	2016	1.2
Seminars and workshops		
WIAS Science Day	2013	0.3
WIAS Science Day	2016	0.3
Presentations		
Oral presentation in Asian-Pacific Aquaculture, Ho Chi Minh, Vietnam, December 10-13	2013	1
Poster presentation in Aquaculture Europe 14, Donostia-San Sebastian, Spain, October 14-17	2014	1
Oral presentation in Aquaculture Europe 15, Rotterdam, The Netherlands, October 20-23	2015	1
Oral presentation in Aquaculture Europe 16, Edinburgh, Scotland, September 20-23	2016	1
Poster presentation in WIAS Science Day	2013	1
Oral presentation in WIAS Science Day	2016	1
Subtotal Basic Package		**11.4**

Statutory Courses	year	credits
Use of Laboratory Animals, Utrecht, February 4-15	2013	3
Subtotal Basic Package		**3.0**

In-Depth Studies		
Disciplinary and interdisciplinary courses	year	credits
Fish immunology Workshop, April 21-25	2013	1.5
Real-Time PCR techniques: GMO and SNP detection, Ho Chi Minh, Vietnam, October 28 – November 1	2013	1.5
Long term effects of diets low in fish oil and fish meal, Gran Canaria, Spain, January 13-15	2016	0.9
Advanced statistics courses		
Basic statistics, December 12,13,18,19,20	2012	1.5
Advanced statistics: Course design of experiment, October 10-12	2012	1.0
Statistic for the Life Sciences, May 23-30	2013	2.0
Subtotal Basic Package		**8.4**

Professional Skills Support Courses	year	credits
Academic writing, level C1, February	2013	2.4
Project and Time Management, November, 1, 4 and December 12	2012	1.5
PhD Competence assessment	2013	0.3
Information Literacy PhD + EndNote, June 11-12	2013	0.6
Teaching and Supervising MSc Thesis, June	2013	1.0
Techniques for writing and presenting a scientific paper, April 16-19	2013	1.2
Modern leadership and teamwork skills, Australia Awards Alumni Program, Vung Tau, Vietnam, June 6-7	2015	0.6
Subtotal Basic Package		**7.6**

Research Skills Training	year	credits
Repairing own PhD research proposal	2013	6
External training period		
Japan PhD knowledge exchange program	2016	1.5
Interpreter for Visit delegation from Vietnam: Adaptive Delta Management, October 9-14	2016	1.2
Subtotal Basic Package		**8.7**

Didactic Skills Training	year	credits
Supervising and practical and excursions		
Teaching: Fish disease, Ho Chi Minh, Vietnam	2013	1.6
Sustainable in Fish and Seafood excursion, Urk, 26 Feb	2016	0.3
Supervising theses		
MSc Student (major), Ngu T. Dinh	2013	2
MSc Student (major), Mai T.T. Le	2013	2
MSc student (major), Son T. Huynh	2015	2
Subtotal Basic Package		**7.9**

Education and Training Total (minimum 30, maximum 60 credits)	**50.0**

Colophon

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