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Modulation of intestinal health and hepatic vacuolation in gilthead sea bream (*Sparus aurata*) juveniles by a mixture of dietary esterified butyrins, emulsifiers from plants and yeast extracts at low and high fish meal inclusion

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Abstract

This study examined the modulatory effect of two commercial feed additives, Lumance® (0.2% and 0.5%) and Novigest® (0.4%), on the growth and microscopic structure of the intestine and liver of juvenile gilthead sea bream (*Sparus aurata*), when added to high (HFM-0) and low fish meal (LFM-0) diets. Lumance® was added only in the HFM-0 diet (HFM-0.2 and HFM-0.5), while a mixture of the two additives was used in the LFM-0 diet (LFM-0.6: 0.2% Lumance® + 0.4% Novigest® and LFM.0.9: 0.5% Lumance® + 0.4% Novigest®). ~~(*Sparus aurata*), when added to high (HFM) and low fish meal (LFM) diets. Lumance® was added only in the HFM diet, while a mixture of the two additives was used in the LFM diet.~~ Fish fed the HFM diets exhibited the highest overall growth, and significant differences were recorded in the specific growth rate (SGR), daily growth index (DGI), feed conversion ratio (FCR) and thermal growth coefficient (TGC), between the HFM and LFM dietary treatment groups. Supplementation of the additives had no effect on the growth performance in either of these groups. The analysis of the intestinal histomorphometric measurements showed signs of intestinal inflammation in the fish fed the LFM-0 diet. The addition of the two additives exhibited some modulatory effects, particularly increased intestinal villi length and lamina propria width in the mid-intestine. An increased number of intraepithelial cells and mucus production was also observed, as well as a decrease in hepatic vacuolation in the LFM-0.6 and LFM-0.9 groups, but not at a statistically significant level.~~An increased number of intraepithelial cells and mucus production was also observed, as well as a decrease in hepatic vacuolation when the combination of the two additives was added to the LFM diet but not at a statistically significant level.~~

Keywords: gilthead sea bream, low fish meal diets, esterified butyrins, emulsifiers, histology, intestine, liver

Abbreviations: HFM, high fish meal; LFM, low fish meal.

1. Introduction

Gilthead sea bream (*Sparus aurata*) is one of the most commercially important farmed fish species in the Mediterranean area. As a carnivorous species, gilthead sea bream requires substantial levels of high-quality protein. Traditionally, fish meal (FM) and fish oil have been used as major constituents in aquafeeds, due to their ideal amino acid and micronutrient profile and fatty acid profile, respectively. However, due to economic and sustainability incentives (Malcorps et al., 2019) there is an increased interest and intensive efforts in substituting FM with alternative raw materials, mainly of plant origin.

Numerous studies in different fish species have demonstrated that dietary inclusion of plant raw materials may induce several negative effects, especially to carnivorous species. Reduced growth performance is often observed for various reasons, such as low digestibility and absorption of nutrients (Santigosa et al., 2011), presence of indigestible components with binding activity (Vahouny et al., 1981), ~~lack~~ reduced levels of several essential amino acids, and poor palatability (Peres et al., 2003). Another risk that plant ingredients pose in aquatic organisms is the presence of antinutritional factors, e.g., lectins, protease inhibitors, saponins, phytic acid, phytoestrogens, which may elevate levels of oxidative stress and lead to inflammation in several organs, ~~and~~ particularly ~~in~~ the gastrointestinal tract and liver (Francis et al., 2001). Supplementation with functional feed additives can ameliorate some of the negative impacts of plant ingredients and disease risks through improved feed utilization and/or gut health.

The aim of this preliminary research ~~was~~ is to study the modulatory effect of two commercial additives, Lumance® and Novigest® (Innovad NV, Belgium) on the growth performance and the intestinal and liver health of juvenile gilthead sea bream (*Sparus aurata*) fed on HFM and LFM diets. Lumance® contains a blend of esterified butyrins, medium chain fatty acids (mainly lauric, capric and caprylic acids), essential oils, plant extracts, and antioxidants rich in polyphenols. Novigest® (Innovad NV, Belgium) is an emulsifier premixture that combines primarily taurine with yeast and plant extracts, carriers and anticaking agents. Taurine is mainly used to increase the synthesis and excretion of taurine-conjugated bile salts and stimulate the catabolism of cholesterol to bile acids (Xu et al., 2020; Murakami et al., 2016) ~~primarily taurine (which increases the synthesis and excretion of taurine-conjugated bile salts and stimulates the catabolism of cholesterol to bile acids) (Xu et al., 2020; Murakami et al., 2016) with yeast and plant extracts, carriers and anticaking agents.~~ Novigest®, was added only in the LFM diets, to examine whether it had any additional hepatoprotective properties.

2. Materials and Methods

2.1 Fish rearing and samplings

All procedures were carried out according to the EU Directive 2010/63/EU for animal experimentation and following the ARRIVE 2.0 guidelines. For this experiment, approximately 1,200 juvenile gilthead sea bream were transferred to the Hellenic Center for Marine Research (HCMR) facility in Agios Kosmas, Athens. The sex of the fish was not considered relevant in the present study. Once acclimated for one week, ~~all~~ 630 fish with an initial average body weight (BW) of 7.19 ± 2.44 g were randomly distributed among 18 cylindroconical 100 L tanks, 35 fish per tank, 3 tanks per dietary group. At the beginning of the experiment, the initial fish population was individually weighed. Before weighing, the fish were anaesthetized using 2-phenoxyethanol (~~0.25~~300 ~~m~~g/L). The tanks were continuously supplied with filtered seawater (salinity 35 ppt) in a flow-through system with a dissolved oxygen level of 6 ppm or higher. The water temperature followed the ambient temperature throughout the experiment with an average of 26.8 ± 1.9 ° C. The photoperiod followed the natural cycle of the season. The fish were hand-fed at apparent satiation, three meals per day (8:30, 11:30 and 15:00) and the daily consumption was recorded. The trial started on 28 May 2018 and the experimental period was 82 days (about 3 months). After 42 days, all fish were weighed individually. The fish were then transferred to larger 1000 l tanks to avoid high fish density issues due to their rapid growth. At the same time, the diets were adjusted according to the nutritional requirements of the increase in fish body size (Table 1). After an additional 40 days of feeding, the experiment was terminated. At the end of the trial, all fish were starved for 24h for digestive tract evacuation and upon collection, they were weighed individually. Three fish from each tank were sampled for histological examination (9 fish per dietary treatment) and ~~killed~~ euthanized with an overdose of 2-phenoxyethanol (1 ~~m~~g/L).

2.2. Experimental diets

Six ~~isoproteic~~ isonitrogenous and isoenergetic diets (1.5 mm pellets) were designed and produced by cooking-extrusion employing a lab scale twin-screw extruder (CLEXTRAL, Firminy, France) with an extrusion temperature less than 100 °C tofeed juvenile sea bream in the experimental installations of HCMR in Ag. Kosmas, Athens, Greece. Two main dietary groups were formulated (Table 1): the first (HFM) incorporated fish meal as its main protein source at the 54% inclusion level, along with a mixture of plant proteins that included ingredients such as soybean meal, ~~soy cake,~~ wheat flour, and corn gluten. The second dietary group (LFM) incorporated lower fish meal

concentration (35%), while the dietary inclusion of plant ingredients was increased, and soy protein concentrate was added to the mixture, to achieve partial fish meal replacement of almost 20%. After the intermediate weighing, the diet was adjusted and the soybean meal level in the HFM and LFM groups was 20% and 35%, respectively. In addition, varying concentrations of dietary feeding additives provided by INNOVAD NV (Belgium) were included in the feeds as follows. In the HFM group, only Lumance® was used to examine any additional beneficial effect. The subgroups were: 0% additive (HFM-0), 0.2% additive (HFM-0.2) and 0.5% additive (HFM-0.5). LFM diets contained a combination of the two additives, Lumance® and Novigest®, in order to examine their synergistic effects, The subgroups in the LFM group were: 0% additive (LFM-0), 0.2 + 0.4% Lumance® and Novigest® (LFM-0.6) and 0.5 + 0.4% Lumance® and Novigest® (LFM-0.9), respectively. The levels used were chosen based on the manufacturer's recommendations. The proximate composition of the experimental diets is presented in Table 1.

2.3. Chemical analyses

Samples of the formulated diets were analyzed according to AOAC (Horwitz and Latimer, 2005) for dry matter (method 934.01), crude protein (method 990.03), crude fat (Method 920.39), and ash (method 942.05) (AOAC International, 2016). The crude protein content was analyzed using the Kjeldahl method (N × 6.25) (Kjeltec 8100, FOSS, Denmark) and the total fat was estimated gravimetrically using Soxtec™ (FOSS, 2050 automated analyzer 2050, Denmark) and extraction of petroleum ether after acid hydrolysis SoxCap™ (FOSS, Denmark).

2.4. Growth performance and survival rate

Fish growth performance and feed consumption indices were calculated according to the following equations:

- Survival rate (%) = (Final number of fish/Initial number of fish) × 100 ~~Survival %~~
-
- Specific growth rate, (SGR) (%/d) = 100 × [(ln FBW – ln IBW)/feeding days], where IBW and FBW are the initial and final body weight, respectively
- Weight gain (WG) = final weight - initial weight
- Total feed intake (TFI) per fish = g DM feed/fish, where DM is the dry matter of the mean feed consumption per fish
- Daily growth index, DGI (%) = ($FBW^{1/3}$ - $IBW^{1/3}$) / number of feeding days x 100
- Thermal growth coefficient, (TGC)= ($FBW^{1/3}$ - $IBW^{1/3}$) × $(\Sigma D0)^{-1}$, where ΣD0 is the thermal sum (feeding days × average temperature, ºC)
- Feed conversion ratio (FCR) = feed consumed / weight gain
- Protein efficiency ratio (PER) = weight gain / protein intake

2.5. Histomorphometry

For the histomorphometric assessment, 9 fish per dietary treatment were sampled (3 per replicate). From each fish, tissue samples from the anterior (about 0.5 cm posterior to the stomach), mid-intestine (about 0.5 cm anterior to the point that the diameter of the intestine increased) and liver were collected and fixed immediately in 4% buffered formalin and then processed using standard methods (Bancroft and Gamble, 2007). Finally, two 5 µm thick sections were cut from each tissue and stained with hematoxylin/eosin (H&E). The sections from the intestine were cross and thus they appeared as rings, where all layers were visible. Tissue sections were observed using light microscopy. Initially, the sections were examined for the presence of any abnormal alterations. Subsequently, an independent observer contacted a blind semiquantitative assessment to detect any differences between the experimental groups, using the criteria described by ~~(~~Urán et al., (2009) with small modifications (Table S1 - Supplementary files). The histomorphometric indices that were assessed were: goblet cells frequency (GC), mucosal fold height (MFH), lamina propria width (LPW), submucosa width (SBW), intraepithelial lymphocytes (IL) and hepatic vacuolation (HV). Example images with different scores are provided in the supplementary images Figures S1-S5.

2.6. Statistical analyses

For the growth performance parameters, tanks were considered as the experimental units and fish represented the sampling units. All data from individual observations were tested for normality and homogeneity of variance using the Kolmogorov–Smirnov and Levene tests, respectively, prior to further analysis. One-way ANOVA, was employed to ~~observed~~identify differences between treatments, since absence of tank effect within treatment groups was verified also by one-way ANOVA. ~~with one-way ANOVA.~~ Significant differences between

means were determined using Tukey's test (Statistica version 12.0). The level of significance was set at $P < 0.05$. Absence of tank effect within treatment groups was verified by one-way ANOVA.

For the histomorphometric indices, as no tank effects were detected in any of the parameters with the Kruskal-Wallis one-way test, fish were considered as the experimental units and ordinal logistic regression was applied using the 'ordinal package' (Christensen, 2019) in R (the proportional odds assumption was met using the 'brant' package (Steenbergen, 2020) due to the ordinal nature of the response variables (that is, scale from 1 to 5 where 1 is optimal and 5 is the poorest). For two of the response parameters (i.e., anterior mucosal fold height and anterior submucosa width) in which the levels of the outcome were only two (score 1 and score 2) binomial logistic regression was implemented. As independent variables, the levels of fish meal, as well as the two additives, Lumance® and Novigest® were used. When a coefficient was significant, pairwise comparisons were performed by least-squares means with the Dunn-Sidak method using the 'emmeans' package (Lenth, 2021). This part of the analysis was performed in the open-source environment R version 3.6.2 (R. Core Team, 2018).

3. Results and discussion

3.1. Growth performance

The growth performance indices evaluated in the present study are shown in Table 3. The survival rate of fish in all treatments ranged from about 93% (LFM-0) to 99% (HFM-0). In general, fish fed the HFM diets exhibited a higher overall growth performance compared to the LFM diets. Significant differences in the specific growth rate (SGR), daily growth index (DGI), feed conversion rate (FCR), and thermal growth coefficient (TGC) were recorded between the HFM and LFM diets. A decrease in TFI was observed in LFM diets compared to the HFM diets, particularly in HFM-0 and HFM0.2 diets, ~~(6-8 g differences),~~ although it was not found to be statistically significant ($P > 0.05$).

Nutrient supply and utilization are among the main factors influencing growth performance especially for organisms of the same age and breed while housed under same conditions (Moloney and McGee, 2017). Furthermore, the tolerance to different plant dietary ingredients and the ability to be utilized, depends on the fish species and its dietary preferences (Bonaldo et al., 2008). Main factors that affect the supply and utilization of the nutrients in fish are feed palatability and digestibility and bioavailability of its nutrients (Glencross et al., 2007). The TFI in the current trial was not significantly affected by ~~FM~~ the reduction of FM ($P > 0.05$) despite the reduction observed in LFM diets, while the addition of additives did not have a significant effect on it. Feed intake in fish is tightly connected to the palatability of the feed and the feeling of satiation, which are both related to the feed composition. It should be noted that the digestibility of the feeds was not measured directly in this study, due to the size of the fish. However, based on the observation of some differences in the assessed histomorphometric indices, and especially the quality of the mucosal folds (see below), which are critically involved in the digestion of the feeds and the absorption of the nutrients, it can be hypothesized that the absorption of nutrients was influenced by the addition of plant ingredients to some extent.

Although in the current study supplementation of diets with the aforementioned additives had no effect on overall growth performance, previous studies have shown that the addition of organic acids and particularly butyric acid can improve feed intake and growth performance, as these acids can act as feed attractants, but also as modulators of the gut microbiota (Abdel-Latif et al., 2020). However, careful dosing is necessary for practical applications, since some authors have observed in broilers a decrease in feed intake at high doses, while mixtures perform better than single acids (i.e. synergistic effect) (Polycarpo et al., 2017). Additionally, dietary emulsifiers, such as those included in Novigest®, can facilitate fat digestion and enhance lipase activity (Al-Marzooqi and Leeson, 1999). Therefore, the slight improvement in the growth observed in the supplemented with Novigest® LFM diets, could be potentially attributed to the combined presence of Lumance®, as no such trend was observed in the HFM diet, which was supplemented only with Lumance®. However, further research that employs additional plant-based dietary treatments with just Novigest® and a combination of both additives would also be needed in a future trial.

3.2. Histomorphometry

Various dietary ingredients can induce detrimental structural changes in the digestive tract and liver of fish, thus affecting the digestion, absorption, and metabolism of the nutrients and ultimately the growth performance (Kokou et al., 2015). In the present study, the transition from HFM to LFM diets and the inclusion of additive mixtures, had significant effects on some histomorphometric indices in the intestine and liver of gilthead sea bream (Figure 1; Figure S6).

Shortening of intestinal folds, often accompanied by thickening of the folds and loss of mucosal indentation, is a usual finding in fish studies, when ~~increasing~~ high level dietary soybean meal is used (Urán et al., 2009). Consequently, the absorptive area of the intestine is reduced (Dimitroglou et al., 2010; Kumar et al., 2020). Interestingly, in the present study, the height of the intestinal folds appeared similar (anterior intestine) or slightly

increased (mid intestine) in the LFM-0, compared to the HFM-0. A possible explanation for the lack of a significant difference between the HFM-0 and the LFM-0 diet is believed to be the relatively low inclusion level of soybean meal used in the first period, or the short second period, where a higher level of soybean level was used, but the exposure time was not enough to induce significant changes. When both HFM-0 and LFM-0 diets were supplemented with additives, the height of the intestinal folds increased, particularly in the mid intestine. ~~Furthermore, when both HFM and LFM diets were supplemented with additives, the height of the intestinal fold increased further, particularly in the mid intestine.~~ In particular, the difference ~~in~~ between the HFM-0 ~~with~~ and the LFM-0.5 treatments was statistically significant. Studies in broilers have shown that butyric acid, being an energy source for enterocytes, has a positive effect on mucosal recovery, following intestinal damage, as for example Abdelqader and Al-Fataftah (2016) demonstrated. In that study, the authors suggested that the effect occurred through a direct stimulation of the epithelial cell proliferation and/or inhibition of the enterocyte apoptosis. However, they also noted that the form of delivery is important (e.g., encapsulated or not) along with the exposure period. Similar findings have also been reported in fish (Abdel-Latif et al. 2020), including g~~G~~ilthead sea bream (Estensoro et al., 2016). As no significant effects within the two fish meal groups were noted in our study, further investigation of the effects of short-chain fatty acids like butyric acid, in this fish species, should be performed, with different forms or feeding periods. Here, it should also be noted that various probiotics, including yeasts, can also increase the height of the intestinal folds in fish (Cerezuela et al., 2012; Abdel-Aziz et al., 2020). Therefore, an additional synergistic effect on the intestinal fold height induced by the yeast extracts present in the Novigest® is also possible.

Lamina propria and submucosa are layers of the intestinal wall that mainly contain connective tissue, within which, many types of cells can be found, including various immune cells. They appear as relatively thin layers at the core of the intestinal folds and just below the intestinal folds respectively (Ferguson et al., 2006). They are tissues that play an important role in local immune responses, and increased thickening of these layers is usually associated with increased infiltration by many immune cells, following ~~irritation~~ inflammation by various feed ingredients (like soybean meal), or infection by pathogens (Hunyady et al., 2000). In the present study, the LFM-0 diet, compared to HFM-0, showed increased LPW and SMW in both the anterior and mid intestine, but the effect ~~but~~ appeared more pronounced in the mid intestine. This effect was related to the experimental diet and is believed to be associated with an increased presence of various immune cells. These immune cells are normally found in all layers of the intestinal mucosa and submucosa, as part of the fish gut-associated lymphoid tissue (GALT), and an increase in their number is one of the early signs of intestinal inflammation (Urán et al., 2009). Previous studies have demonstrated increased immune cell infiltration, induced by various plant ingredients and particularly soybean meal (Bonaldo et al., 2008~~);~~ ~~(~~Kokou et al., 2015). This is, ~~associated~~ with increased levels of pro-inflammatory cytokines and / or decreased levels of anti-inflammatory cytokines (Wang et al., 2017). In the present study, supplementation of the diets with the additives and particularly Lumance® at 0.5% resulted in a slight increase of SBW and a statistically increased LPW in the mid intestine. This is believed to be related to increased infiltration of immune cells, as the assessment of intraepithelial lymphocytes indicates. The more pronounced effects in the mid intestine that were observed, are believed to be related to the increased role of this segment in the immune responses of the intestine, compared to the anterior segment, which is more involved in the digestion and absorption of nutrients (Bjørgen et al., 2020).

Intraepithelial lymphocytes (IL) are part of the fish GALT and are normally present in the intestinal epithelium, and they increase in response to the presence of chemical or biological agents. In our study, increased levels of plant ingredients in the LFM group did increase the number of IL especially in the mid part ~~and~~ in the LFM-0 dietary group, yet the pairwise comparisons did not detect a significant difference between the HFM-0 and LFM-0 ($P = 0.19$). Various substances found in plant ingredients, like saponins, can have a direct effect on these cells, probably due to the damage on the epithelial cells (Urán et al., 2009; Couto et al., 2014). The addition of the two additives to the LFM diet at the highest level appeared to slightly decrease the IL index. On the other hand, addition of only Lumance® in the HFM-0 diet had the opposite effect. The anti-inflammatory role of short-chain fatty acids and particularly that of butyric acid is known in both mammals and fish (Venegas et al., 2019; Cholan et al., 2020). However, it appears that the response is dose-related, and increased concentrations can result in increased infiltration of immune cells. For example, ~~(~~Estensoro et al., (2016) demonstrated increased infiltration of IL in gilthead sea bream, when sodium butyrate (Gustor BP-70 ®Norel) was added at 0.8%. The results of the present study were in line with ~~those~~ the results of that ~~one~~study, and apparently the level of inclusion that can elicit such a change depends on the form of the added butyrate and the overall composition of the diet. However, more research is needed to confirm whether the observed effect is beneficial or not, as various probiotics can also increase the number of various immune cells, and this effect is considered positive, as it improves defense against potential pathogens (Abdel-Aziz et al., 2020). For example, Piazzon et al., (2017) observed that addition of 0.8% sodium butyrate (Gustor BP-70 ®Norel) in the diet enhanced the resistance of gilthead sea bream against *Photobacterium damselae* subsp. *piscicida*. The authors speculated that this could have been related to the lowering of the pH, or the modulation of the gut microbiota. However, as the same concentration of the same commercial product increased immune cell infiltration in the study by ~~(~~Estensoro et al., (2016), the contribution of this infiltration in protection against potential pathogens cannot be excluded.

Goblet cells produce mucus that covers the intestinal epithelium. The main functions of the mucus are: a)

lubrication, b) protection of the epithelium against mechanical and chemical injury, c) participation in the formation of a protective barrier against potential pathogens (mainly through the continuous removal of potential pathogens, but also because it contains many antimicrobial substances), d) enhancement of the digestion and absorption of nutrients and e) buffer the intestinal fluids. In general terms, increased mucus production is considered a defense mechanism and it has been observed in many fish species, including gilthead sea bream, when increased levels of plant ingredients are used in aquafeeds (Monge-Ortiz et al., 2016). In the present study, no differences between the HFM-0 diet and the LFM-0 diets were noted in both the anterior and mid intestine, probably due to the reason mentioned previously, for the intestinal fold height. ~~In the present study, no differences between the HFM-0 diet and the LFM-0 diets were noted in both the anterior and mid intestine, probably due to the low inclusion level of soya bean meal in the first period or the short second period.~~ However, when the HFM diet was supplemented with 0.5% Lumance®, a slight increase in the GC index was observed in the anterior intestine. Similarly, the addition of the two additives to the LFM-0 diet resulted in a slight increase in the index in the mid intestine (both results were not statistically significant). This result was not surprising, as short chain fatty acids and specifically butyric acid, tend to upregulate many mucin genes (Jiminez et al., 2017). Furthermore, it is known that various yeast extracts, like β glucans, can increase mucus production in the intestinal tract (Selim and Reda, 2015). Novigest® includes such ingredients and, therefore, could have contributed to this result. It should be noted that intense stimulation of the goblet cells, often results in their depletion, particularly when the stimulation is prolonged (Chen et al., 2020). Therefore, the interpretation of this index should always be done with caution.

Hepatic vacuolation is one of the main indices used in the evaluation of the liver and is mainly associated with the accumulation of lipids or glycogen in the cytoplasm (Wolf and Wolfe, 2005). No significant effect on the HV index was observed in the present study between the HFM-0 and LFM-0 group, although it is known that increased levels of plant ingredients inclusion can cause increased vacuolation of hepatocytes in fish, mainly due to increased accumulation of intracellular lipids. In gilthead sea bream, increased vacuolation associated with the inclusion of plant ingredients, such as soybean meal, in aquafeeds has also been shown, but only when the inclusion levels were greater than 20% (Kokou et al., 2015; Baeza-Ario et al., 2016). Although the mechanisms of this accumulation are not fully understood, the increased lipid accumulation could be related to *de novo* fatty acid synthesis in the liver, although other mechanisms might also be involved (Dias et al., 2005). In the present study, the effect of the additives depended on their combination. Thus, when only Lumance® was added to the HFM diet, a slight (though not statistically significant) increase in the vacuolation was observed, while the supplementation of the LFM-0 diet with the combination of the two additives reduced the vacuolation. Although butyric acid appears to reduce hepatic steatosis in many animals (Baumann et al., 2020) through various mechanisms, increased levels have the opposite effect, as (El-Sayed Ali et al., (2018) have observed in Nile tilapia (*Oreochromis niloticus*). In that study, an increase in lipid accumulation was observed when sodium butyrate was added to the diet at 2% or more. ~~Its~~ The ~~toxic~~ threshold concentration of butyric acid that can induce hepatic vacuolation in fish is probably species-related and~~,~~ based on our observations, probably the tolerance of Gg~~i~~lthead sea bream is lower. Interestingly, the addition of Novigest® appeared to ameliorate this effect. This protective effect could be attributed to the presence of emulsifiers, such as bile salts, which can decrease steatosis in fish at low concentrations (Jiang et al., 2018).

4. Conclusion

In this preliminary study, the effects of Lumance® and Novigest® on growth performance and intestinal architecture of gilthead sea bream juveniles were examined. The observations have been in line with some previous studies but also raised questions for future research. As expected, reducing fish meal in LFM diets overall had a significant negative effect on the FBW and SGR parameters. The observed decreased growth can probably be attributed to a) the presence of antinutritional substances in plant feedstuffs that impacted the digestibility and bioavailability of nutrients, b) concomitant absence of valuable bionutrients intrinsic to fish meal, c) palatability issues and d) histological alterations in the intestine that affected its function.

Transitioning from high to low fish meal without any of the tested additives displayed some negative effects regarding the intestinal health, but addition of both Lumance® and Novigest® at specific levels exhibited some modulatory effects and particularly increased intestinal villi length, number of intraepithelial cells and mucus production. Furthermore, decreased hepatic vacuolation was also observed when the combination of the two additives was added to the LFM diet, although it was not statistically significant. However, these findings need to be confirmed in long-term trials, with different fish sizes and particular focus should be placed on the effects of these additives on the fish gut microbiota. ~~However, the results demonstrate that inclusion of such functional ingredients can partly ameliorate the negative effects of some antinutrient factors and could affect resistance against potential fish pathogens.~~

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Conflict of interest

None.

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Table 1. Ingredients and chemical composition (as fed) of the experimental diets (%).

	HFM-0	HFM-0.2	HFM-0.5	LFM-0	LFM-0.6	LFM -0.9
Raw materials			**Period 1**			
Fishmeal[a]	54.40	54.40	54.40	35.25	35.21	34.97
Soybean meal (non-GM)	12.00	12,00	12,00	17.00	17.00	17.00
Wheat Flour	8.21	8.00	7.68	1.22	0.62	0.93
Wheat Gluten	7.15	7.17	7.19	4.60	4.61	4.55
Soya protein concentrate	0.00	0.00	0.00	9.21	9.22	9.11
Fish oil	10.00	9.97	9.93	12.57	12.56	12.55
Corn Gluten	7.15	7.17	7.19	18.42	18.45	18.21
Lumance®	**0.00**	**0.20**	**0.50**	**0.00**	**0.20**	**0.50**
Novigest®	**0.00**	**0.00**	**0.00**	**0.00**	**0.40**	**0.40**
DL-Methionine[d]	0.00	0.00	0.00	0.04	0.04	0.06
L-Tryptophane[e]	0.00	0.00	0.00	0.02	0.02	0.02
Novinat FF*	0.10	0.10	0.10	0.10	0.10	0.10
L-Lysine[f]	0.00	0.00	0.00	0.56	0.56	0.59
Premix	1.00	1.00	1.00	1.00	1.00	1.00
Raw materials			**Period 2**			
Fishmeal	30.00	30.00	30.00	16.50	16.50	16.50
Soybean meal (non-GM)	20.00	20.00	20.00	35.00	35.00	35.00
Wheat Flour	12.63	12.41	12.11	2.69	2.00	1.70
Wheat Gluten	7.15	7.17	7.19	3.00	3.00	3.00
Soy protein concentrate	0.00	0.00	0.00	9.21	9.22	9.11
Fish oil	13.16	13.14	13.10	14.96	14.94	14.90
Corn Gluten	15.96	15.98	16.00	17.46	17.56	17.71
Lumance®	**0.00**	**0.20**	**0.50**	**0.00**	**0.20**	**0.50**
Novigest®	**0.00**	**0.00**	**0.00**	**0.00**	**0.40**	**0.40**
DL-Methionine	0.00	0.00	0.00	0.08	0.08	0.08
Novinat FF	0.10	0.10	0.10	0.10	0.10	0.10
Premix Sea bream	1.00	1.00	1.00	1.00	1.00	1.00

[a]Fishmeal was supplied by Norsildmel Innovation AS, [b]Soya protein concentrate was supplied by Bankom, [c]L-Threonine was supplied by Ningxia Eppen Biotech Co., Ltd, [d]DL- Methionine was supplied by Adisseo, [e]L-Tryptophane was supplied by CJ CheilJedang Corp. and [f]L-Lysine was supplied by Daesang.
*Novinat FF is an additive of INNOVAD for protection against ectoparasites, acting especially on the fish gills.

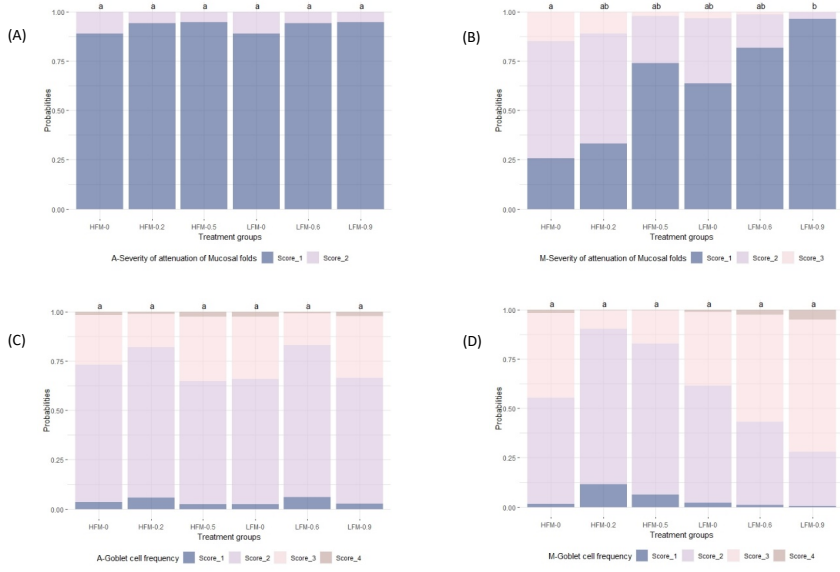
Table 1. Proximate analysis of the experimental diets over the two periods (% as is).

	HFM-0	HFM-0.2	HFM-0.5	LFM-0	LFM-0.6	LFM -0.9
Proximate analysis			**Period 1**			
Crude Protein	53.58	54.89	53.86	53.48	53.08	52.86
Crude Fat	18.56	19.45	18.42	19.22	18.75	18.56
Crude Fiber + N-free extract	14.03	12.17	13.81	15.28	15.30	16.61
Crude Ash	8.25	8.37	8.32	6.69	7.05	7.00
Moisture	5.58	5.12	5.59	5.33	5.82	4.97
Proximate analysis			**Period 2**			
Crude Protein	47.17	47.16	47.29	46.8	47.19	47.57
Crude Fat	17.73	17.91	17.08	17.8	17.35	17.18
Crude Fiber + N-free extract	23.71	22.93	24.24	24.92	24.78	22.89
Crude Ash	6.68	6.75	6.95	6.25	6.58	6.63
Moisture	4.71	5.25	4.44	4.23	4.1	5.73

Table 3. Growth performance indices of the gilthead sea bream over the entire feeding period.

	HFM-0	HFM-0.2	HFM-0.5	LFM-0	LFM-0.6	LFM-0.9
IBW	7.46±0.14	7.37±0.37	7.39±0.24	7.33±0.29	7.53±0.08	7.51±0.40
FBW	99.61±0.39^{a}	100.31±1.76^{a}	97.61±1.71^{ab}	84.55±6.75^{c}	86.77±3.68^{c}	87.99±2.96^{bc}
WG	92.15±0.38^{a}	92.94±1.45^{a}	90.21±1.59^{a}	77.22±6.47^{b}	79.25±3.61^{b}	80.48±2.65^{b}
FCR	1.12±0.02^{ab}	1.08±0.02^{a}	1.08±0.02^{a}	1.19±0.03^{b}	1.18±0.05^{b}	1.18±0.03^{b}
SGR	3.16±0.02^{a}	3.19±0.04^{a}	3.15±0.03^{a}	2.98±0.06^{b}	2.98±0.04^{b}	3.00±0.04^{b}
DGI	3.27±0.02^{a}	3.29±0.02^{a}	3.24±0.03^{a}	2.98±0.11^{b}	3.01±0.07^{b}	3.04±0.04^{b}
TGC	0.12±0.00^{a}	0.12±0.00^{a}	0.12±0.00^{a}	0.11±0.00^{b}	0.11±0.00^{b}	0.11±0.00^{b}
Survival (%)	99.05±1.65	95.24±3.30	97.14±2.86	93.33±4.36	95.24±1.65^{a}	94.29±2.86^{a}
PER	1.82±0.04^{ab}	1.82±0.03^{ab}	1.86±0.09^{a}	1.67±0.08^{b}	1.71±0.08^{ab}	1.72±0.06^{ab}
TFI	97.20±2.41	95.53±1.17	92.53±2.04	89.02±10.76	89.31±0.82	91.61±5.27

Data are presented as mean ± SD (n = 3). Values sharing the same superscript letter showed no significant differences (P > 0.05). Initial Body Weight (IBW), Final Body Weight (FBW), Weight Gain (WG), Feed Conversion Ratio (FCR), Specific Growth Rate (SGR), Daily Growth Index (DGI), Thermal Growth Coefficient (TGC), Survival (%), Protein Efficiency Ratio (PER), Total Feed Intake (TFI).



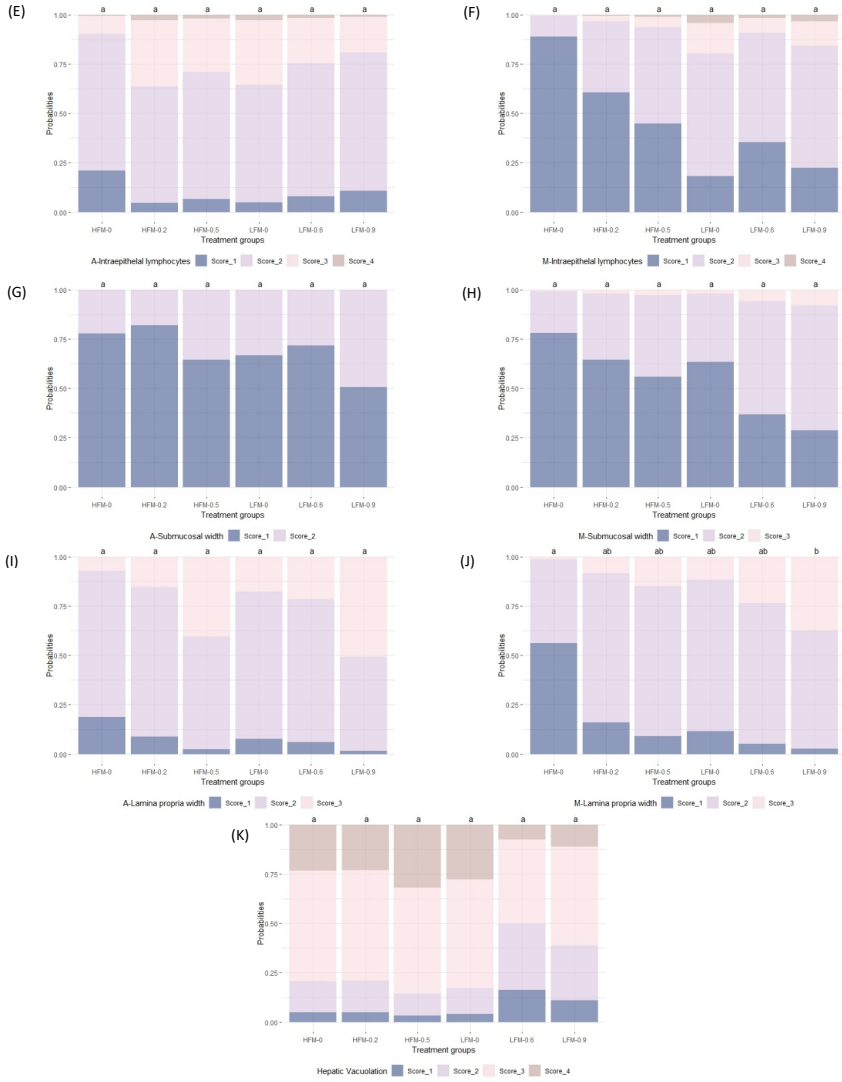


Figure 1 (A, B, C, D, E, F, G, H, I, J & K). Results of the semi-quantitative histomorphometric analysis with ordinal logistic regression of the anterior (A), mid (M) intestine and liver. In the anterior mucosal fold height and anterior submucosa width, where the levels of the outcome were only 2 (scores of 1 and 2), binomial logistic regression was used. The Y-axis shows the probability of being one of the scores for each dietary treatment, and statistical differences are indicated with small letters. HFM-0: High fishmeal with 0% additives; HFM-0.2: High fishmeal with 0.2% Lumance®; HFM-0.5: High fishmeal with 0.5% Lumance®; LFM-0: Low fishmeal with 0% additive, LFM-0.6: Low fishmeal with 0.2% Lumance® + 0.4% Novigest®; LFM-0.9: Low fish meal with 0.5% Lumance® + 0.4% Novigest®; (A & B): Severity of attenuation of mucosal folds in the anterior and mid intestinal part respectively, (C & D): goblet cells frequency in the anterior and mid intestinal part respectively, (E & F): intraepithelial lymphocytes in the anterior and mid intestinal part respectively, (G & H) SMW: submucosa width in the anterior and mid intestinal part respectively, (I & J): lamina propria width in the anterior and mid intestinal part respectively, (K): hepatic vacuolation.