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Dietary supplementation of a combination of formic acid and sodium formate in practical diets promotes gilthead sea bream (*Sparus aurata*) gut morphology and disease resistance

Silvia Torrecillas [a,c,*], Ehab Aboelsaadat [b], Marta Carvalho [a], Félix Acosta [a], Luis Monzón-Atienza [a], Álvaro Gordillo [b], Daniel Montero [a]

[a] Grupo de Investigación en Acuicultura (GIA), IU-ECOAQUA, Universidad de Las Palmas de Gran Canaria, Las Palmas, Spain
[b] BASF, FZE, Dubai. United Arab Emirates
[c] Aquaculture Program, Institut de Recerca i Tecnología Agroalimentaries (IRTA), La Rápita, Spain

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ABSTRACT

Optimizing growth and feed conversion ratios by improving gut function and health is critial to ensuring cost-effective production in aquaculture, especially in the current context of low fishmeal and low fish oil-based diets. However, the use of practical diets based on high levels of plant-based raw materials as an alternative to traditional marine ingredients has been associated with negative effects on fish performance, feed utilization, and health. Organic acids and their salts have been widely used as functional ingredients not only for their antimicrobial and immunomodulatory properties, but also for their potential to promote animal digestive capacity and gut health. The aim of the present study was to evaluate the effects of a mixture of formic acid and sodium formate (Amasil NA®, BASF, Germany- AMA diet) at a dietary level of 0.3%, on key performance indicators, gut morphology and disease resistance to *Vibrio anguillarum* in juvenile gilthead sea bream (*Sparus aurata*). The results of the present study showed that fish fed the AMA diet for 8 weeks, performed similar to fish fed the control diet in terms of growth but presented an optimization of 8% in the utilization of a low in fishmeal and high in plant proteins based diet. The AMA-diet also increased the folds length of gilthead sea bream anterior gut, increasing intestinal absorption area, and decreasing the submucosa width and goblet cell size in the posterior gut when included in a high dietary plant protein content diet. Furthermore, dietary supplementation with Amasil NA® at 0.3% increased gilthead sea bream disease resistance against *V. anguillarum* compared with fish fed the unsupplemented diet. These results highlight the potential of this combination of formic acid and sodium formate based product as a feed efficiency enhancer, and as a gut health promoter in gilthead sea bream plant protein-based diets.

1. Introduction

In the current scenario, the livestock production sector is challenged to cope with an increasing global demand for animal protein in a context where the European Union (EU) and the European Food Safety Authority (EFSA) are promoting policies to reduce the use of antibiotics in animal production. Aquatic food is currently contributing more than ever before to the global nutrition. The production of aquatic animals in 2020 has increased by 20% in relation to the 2000 s and by 60% compared to the 90 s (FAO, 2022). However, an effective development of the aquafeed sector implies the incorporation of new/emerging sustainable ingredients into aquafeed formulas. Changes in feed composition, especially those aimed at reducing the use of traditional ingredients, have in some cases been associated with negative effects on fish performance, feed utilization, and health (Montero and Izquierdo, 2010; Oliva-Teles, 2012; Torrecillas et al., 2017; Aragão et al., 2022). In particular, the integrity and functionality of the fish gut have been shown to be directly affected by changes in aquafeed, particularly in relation to reduced nutrient absorption, alterations in intestinal permeability and gut microbiota, and impaired gut-associated lymphoid tissue (GALT) immunological response (Øverland et al., 2009; Merrifield et al., 2011; Król et al., 2016; Torrecillas et al., 2017), which together

* Corresponding author at: Grupo de Investigación en Acuicultura (GIA), IU-ECOAQUA, Universidad de Las Palmas de Gran Canaria, Las Palmas, Spain.
E-mail address: silvia.torrecillas@irta.cat (S. Torrecillas).

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faciliates the occurrence of inflammatory processes and disease outbreaks (Torrecillas et al., 2017; Aragão et al., 2022).

In this sense, functional ingredients seem to be an accredited solution not only to reduce the above-mentioned health effects but also to facilitate the establishment of new/emerging ingredients in practical aquafeeds formulations and to help reduce the use of therapeutics from a proactive approach. Among these, short-chain organic acids (OA) and their salts have been widely used in animal nutrition as supplements for their effects in optimizing animal digestive capacity and maintaining gut health (Nguyen et al., 2020; Fabay et al., 2022). For example, acidifiers and their salts are commonly used as preservatives due to their antimicrobial and fungicidal properties (Ng and Koh, 2016; Ricke et al., 2020). The beneficial effects of OA on the fish gut include the stabilization of the pH after meal ingestion, the triggering of digestive enzymes activities and fish digestive capacity (Fabay et al., 2022), and the modulation of gut microbiota and GALT function (Ng and Koh, 2016).

In particular, formic acid (FA) is a short-chain fatty acid (SCFA) that favours upper intestinal acidification due to its low pKa and high density compared to other OA, profiling FA as an acid-dependent enhancer of digestive enzyme activity or inhibitor of digesta complexes assembly. For example, Vielma and Lall (1997) demonstrated the ability of dietary FA to optimize intestinal pH and, consequently phosphorous, magnesium and calcium availability in rainbow trout (*Oncorhynchus mykiss*). Studies in shrimp post larvae (*Litopenaeus vannamei*) reported increased mineral retention, improved disease resistance and reduced gut bacterial counts after FA supplementation (Chuchird et al., 2015). Salts of formic acid, such as sodium and potassium formate (NaF and KF) or sodium diformate (NaDF), have also been associated with animal production and health promotion. For instance, NaDF has been demonstrated to improve macronutrients and amino acids digestibility in rainbow trout (Morken et al., 2011) and growth, feed efficiency, and nutrient retention in Nile Tilapia (*Oreochromis niloticus*) (Liebert et al., 2010). In red hybrid tilapia (*Oreochromis* sp) the supplementation of soybean meal-based diets with potassium diformate (KDF) promoted growth performance, feed efficiency, and diet digestibility (Ng et al., 2009). Atlantic Salmon (*Salmo salar*) fed with KDF presented a trend towards better protein, dry matter and gross energy digestibility coefficients (Lückstädt et al., 2008) and red tilapia (*Oreochromis sp.*) supplemented with FA and NaF (Amasil NA®, BASF, Germany) had better growth and feed efficiency, as well as reduced total intestinal aerobic bacterial population (Kore et al., 2019).

Conversely, little is known about how FA supplementation in low fishmeal (FM) and fish oil (FO)-based diets for marine warm-water fish species may affect fish growth performance and disease resistance, as most of the previous studies have been focused on the supplementation of other OAs, such as propionate and butyrate. For example, butyrate supplementation for 8 months on a very low FM/FO diet content (3% /2.5%) in gilthead sea bream (*Sparus aurata*) was able to reverse the upregulation of pro-inflammatory cytokines and muscle markers of cellmorphogenesis and protein degradation (Benedito-Palos et al., 2016). However, in this study, butyrate supplementation was unable to reverse the detrimental effects on fish growth performance associated with low FM/FO diets. Similarly, other authors have reported the ability of sodium butyrate to reduce the effects associated with a similar dietary FM/FO content in gilthead sea bream in terms of inflammatory markers, antioxidant defenses, epithelial permeability, and mucus production (Estensoro et al., 2016).

Thus, the present study aimed to determine the effects of a commercial mixture of formic acid and sodium formate (Amasil NA®, BASF, Germany) on key performance indicators of gilthead sea bream juveniles, including also gut health, and disease resistance against *Vibrio anguillarum* when supplemented in commercial formula-based diets.

Table 1
Ingredients composition of the experimental diets. Control diet. AMA: Amasil® NA functional diet.

	Experimental diets	
Raw material (%)	**Control**	**AMA**
Fishmeal Super Prime[a]	15.00	15.00
Poultry meal[2]	5.00	5.00
Soy protein concentrate[3]	15.00	15.00
Wheat gluten[4]	12.50	12.50
Corn gluten meal[5]	9.60	9.60
Soybean meal 44[6]	5.00	5.00
Sunflower meal 40[7]	6.00	6.00
Wheat meal	10.08	9.78
Faba beans (low tannins)[8]	6.00	6.00
Vitamin and mineral premix[9]	1.00	1.00
Antioxidant[10]	0.10	0.10
Monocalcium phosphate	1.30	1.30
L-Lysine HCl 99%[11]	0.20	0.20
Yttrium oxide[12]	0.02	0.02
Fish oil[13]	6.00	6.00
Rapeseed oil	7.20	7.20
Amasil NA®[14]		0.30

[a] Fishmeal Super Prime: Pequera Diamante (Peru). [2] Poultry meal: SAVINOR UTS (Portugal).[3] Soya protein concentrate: ADM (The Netherlands).[4] Wheat gluten: Roquette (France).[5] Corn gluten meal: COPAM (Portugal). [6] Soybean meal 44, solvent extracted: Ribero &Sousa Lda (Portugal). [7] Sunflower meal 40 (HiPro), Dehulled solvent extracted: AGP Slovakia s.r.o. (Slovakia).[8] Faba beans: Ribero &Sousa Lda (Portugal). [9] Mineral and Vitamin premix: Premix Lda (Portugal). Vitamins (IU or mg/Kg diet): DL-alpha tocopherol acetate, 100 mg; sodium menadione bisulfate, 25 mg; retinyl acetate, 20000 IU; DL-cholecalciferol, 2000 IU; thiamine, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotidin acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium panthotenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 0.65 mg; copper sulfate, 9 mg; ferric sulfate, 6 mg; potassium iodide, 0.5 mg; manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulfate. 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middlings. [10]Verdilox (Kemin Europe NV, Belgium). [11] L-Lysine HCl 99%: Ajinomoto EUROLYSINE S.A.S (France).[12] Yttrium oxide: Sigma Aldrich (USA). [13] Fish oil: Sopropêche (France).[14] Amasil NA® BASF (Germany).

2. Materials and methods

2.1. Diets

Two experimental diets were formulated to meet the nutritional requirements of gilthead sea bream. One of them was supplemented with AMASIL®NA at 0.3% (AMA), replacing standard carbohydrates and the control diet was devoid of the functional ingredient (Table 1). AMASIL®NA is a commercial mixture of formic acid and sodium formate consisting in 61% formic acid and 20.5% sodium formate of active substances (pH=2.6–3.2) (BASF, Germany). Diets (46% CP, 16% CL, 21.4 MJ/kg feed), with a pellet size of 3.0 mm, were manufactured by SPAROS Lda (Olhão, Portugal). For diet production, all powder ingredients were mixed in a double-helix mixer (TGC Extrusion model 500 L, France) and ground (<400 mm) before being manufactured with a twin-screw extruder (Clextral model BC45, France). The extrusion conditions were: feeder rate (80–85 kg/h), screw speed (247–266 rpm), water addition in barrel 1 (345 mL/min), temperature in barrel 1 (32–34 ºC), temperature in barrel 2 (59–62 ºC) and temperature in barrel 3 (111–114 ºC). After production, pellets were dried and cooled and oils added by vacuum coating (Dinnissen model PG-10VCLAB, Netherlands).

2.2. Experimental conditions

2.2.1. Feeding trial

The present study was run at the facilities of the Parque Científico-Tecnológico Marino (PCTM), University of Las Palmas de Gran Canaria (ULPGC) (Telde, Canary Island, Spain). Gilthead sea bream (*Sparus*

aurata) juveniles of own production were acclimatized for two weeks to the experimental conditions (6.0 ± 1.0 ppm dissolved O_2, 22.9–18.9 °C). Afterwards, fish were randomly distributed into nine 500 L open flow-through water system tanks at an initial density of 3.7 kg·m^3 (30 fish/tank). Fish average initial weight and length were 62.41 ± 1.39 g and 14.23 ± 0.07 cm, respectively (mean ± SD). The fish were exposed to natural photoperiod (12 L:12D) and fed to apparent satiation three times a day, six days a week for 8 weeks. Two sets of three tanks were fed with the control diet and used as negative and positive controls for the challenge test (see Section 2.2.2), and a third set of 3 tanks were fed with the AMA-supplemented diet. Fish were individually weighted and measured after 4 and 8 weeks of feeding for estimating fish growth performance. Before sampling, each fish was anesthetized in accordance with the regulations of the European Union Directive (2010/63/EU) and Spanish legislation (RD 53/2013) for animal experiments. Fish handling was performed under natural clove oil anesthesia (0.02 mL/L; Guinama S.L; Spain, Ref. Mg83168), and for sampling, fish were euthanized using an overdose of natural clove oil. At the end of the feeding trial, the fish gut was dissected out (n = 5 fish/tank) and stored in buffered formaldehyde until processed. Samples of blood from 5 fish per tank were taken by caudal sinus puncture, allowed to clot overnight and serum was separated by centrifugation and stored at − 80 °C for immune parameters activities determination. The Bioethical Committee of the ULPGC approved all the protocols used in the present study (approval no. OEBA-ULPCG 18/2021).

2.2.2. Experimental infection against V. anguillarum

After 8 weeks of feeding, 25 fish per tank were transported to the Marine Biosecurity (MBS) facility situated in the same PCTM-ULPGC and exposed to an experimental bacterial challenge by intraperitoneal injection against *Vibrio anguillarum* (10^7 cfu/fish, strain 507, isolated from a clinical outbreak in the Canary Islands) for 10 days. For the duration of the challenge test, fish were fed with their respective diets two times a day. The negative control fish group was intraperitoneally injected with sterile inoculation media (placebo), mimicking the same conditions of the infection groups. Fish survival was recorded daily during the challenge experiment and described by Kaplan-Meier curves for each dietary treatment. Naturally dead fish were collected and necropsied, and *V. anguillarum* was confirmed as the causative agent of the death recorded by standard biochemical procedures.

2.3. Morphological studies

Fish anterior, posterior gut and rectum were dissected as previously detailed by Torrecillas et al. (2019). From each segment, three to six transverse sections (n = 15 fish per diet) were taken and fixed in buffered formaldehyde. Afterward, samples were embedded in paraffin, serially sectioned (4 μm) and stained with Alcian Blue (pH = 2.5) (Martoja and Martoja-Pierson, 1970) to differentiate goblet cell secreting acid mucins. Slides were digitally scanned in a digital scanner Olympus VS120 (Optic system BX61VS, Tokyo, Japan) equipped with VC50 and VS-XM10 cameras and acquired with Olympus VS software (VS-NIS-SQL-V2.6, Tokyo, Japan). Digitalized images of intestinal transverse sections (Alcian Blue pH=2.5) were used to determine the morphometric characteristics of intestinal folds, submucosa layer and goblet cells. In the case of goblet cells, CellSens Dimension Desktop 1.16 (Olympus Iberia, Spain) was calibrated to determine the cell area (μm^2), minimum diameter (μm) and minimum perimeter (μm) for each transverse section of the gut.

2.4. Immune parameters

Alternative complement pathway was performed as described by Sunyer and Tort (1995) for gilthead sea bream using rabbit RBC. The reciprocal of the serum dilution causing 50% lysis of RBC is designed as ACH50 and the results are presented as ACH50 units/mL. Lysozyme level in blood serum was determined by turbidimetric assay according to the method described by Anderson and Siwicki (1994) using hens' egg white lysozyme as a standard. Serum bacteriolytic and bacteriostatic activity was measured according to Sunyer and Tort (1995).

Table 2
Growth performance and feed utilization along the feeding trial of gilthead sea bream (*Sparus aurata*) juveniles fed with the experimental diets.

		Experimental diets	
		Control	AMA
Initial	**Standard length (cm)**	14.23 ± 0.03	14.30 ± 0.10
	Body weight (g)	62.72 ± 1.70	63.03 ± 1.31
4 weeks	**Body weight (g)**	81.32 ± 2.24	79.59 ± 1.93
	Standard length (cm)	15.46 ± 0.20	15.30 ± 0.02
	Feed intake 0-4 weeks (g)	1038.53 ± 1.01	1008.98 ± 52.64
	Condition factor (K)[1]	2.20 ± 0.04	2.22 ± 0.04
	DGI[2] 0-45 (% day)	1.28 ± 0.06	1.15 ± 0.07
	FCR[3]	1.96 ± 0.15	2.04 ± 2.24
8 weeks	**Body weight (g)**	88.81 ± 2.04	89.33 ± 2.91
	Standard length (cm)	18.07 ± 0.52	18.14 ± 0.29
	Feed intake 0-8 weeks (g)	1669.79 ± 26.06	1609.48 ± 99.56
	Condition factor (K)	1.51 ± 0.09	1.50 ± 0.04
	DGI[2] 0-8 weeks (% day)	0.87 ± 0.01	0.88 ± 0.05
	FCR[3]	2.23 ± 0.20	2.05 ± 0.25
	Survival (%)	98.9 ± 1.92	100 ± 0.00

Values expressed in mean ± SD. (n = 3 tanks/diet). 1Condition factor (K) = [(weight)/(length) 3]; 2 Daily Growth Index (DGI) = [(final weight1/3- initial weight1/3)/number of days] x 100; 3FCR (Food conversion ratio)= (ingested feed/gain weight); Different superscript letters are significantly different ($P < 0.05$) based on Mann-Whitney U test. Control= control diet; AMA= Amasil®NA at 0.3%.

2.5. Statistical analyses

All data were tested for normality and homogeneity of variance. Means and Standard Deviations (SD) were calculated for each parameter measured and statistical differences were considered significant at $p < 0.05$. Statistical analyses followed methods described by Sokal and Rolf (1995). Differences between dietary treatments were determined using a parametric and non-parametric pair comparison (t-test and Mann-Whitney U test). When required, data transformation was performed (Fowler et al., 1998). Analyses were performed using the SPSS Statistical Software System v21.0 (SPSS, Chicago, IL, USA) and GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

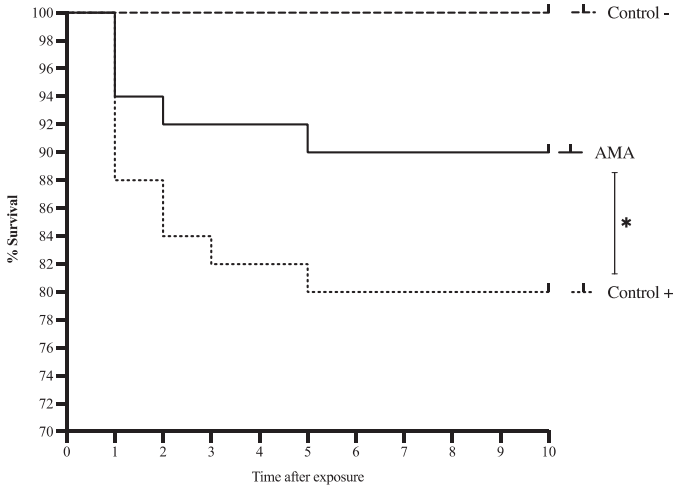

Fig. 1. Cumulative survival (%) of gilthead sea bream (*Sparus aurata*) along the challenge test against *V. anguillarum* fed the experimental diets. Symbols denote statistical differences (p £ 0.05; Kaplan-Meier survival). Control= control diet; AMA= Amasil®NA at 3 g/kg.

Table 3
Serum immune parameters measured at the end of the feeding trial (8 weeks) of gilthead sea bream (*Sparus aurata*) juveniles fed with the experimental diets.

	Experimental diets	
Immune parameters activity	**Control**	**AMA**
ACH50 (U/mL)	33.34 ± 14.69	38.57 ± 10.52
Lysozyme (IU/mL)	570.78 ± 39.27	568.96 ± 49.07
Bacteriolytic activity	61.96 ± 24.51	64.33 ± 27.15
Bacteriostatic activity	77.30 ± 15.39	78.77 ± 15.6

Values expressed in mean ± SD. (n = 3 tanks/diet). ACH50, alternative complement pathway activity. Different superscript letters are significantly different ($P < 0.05$) based on Mann-Whitney U test. Control= control diet; AMA= Amasil®NA at 0.3%.

3. Results

3.1. Feeding trial

The experimental diets were well accepted by the fish and did not affect fish survival or condition factor during the feeding trial. At the end of the experiment, feeding AMA diet did not affect ($p > 0.05$) final fish weight and daily growth index (DGI) compared to fish fed control diet. Although total feed intake and FCR remained statistically unaffected ($p > 0.05$), fish fed the AMA diet showed a trend toward optimized FCR (+8%) compared to fish fed the control diet at the end of the feeding trial (Table 2).

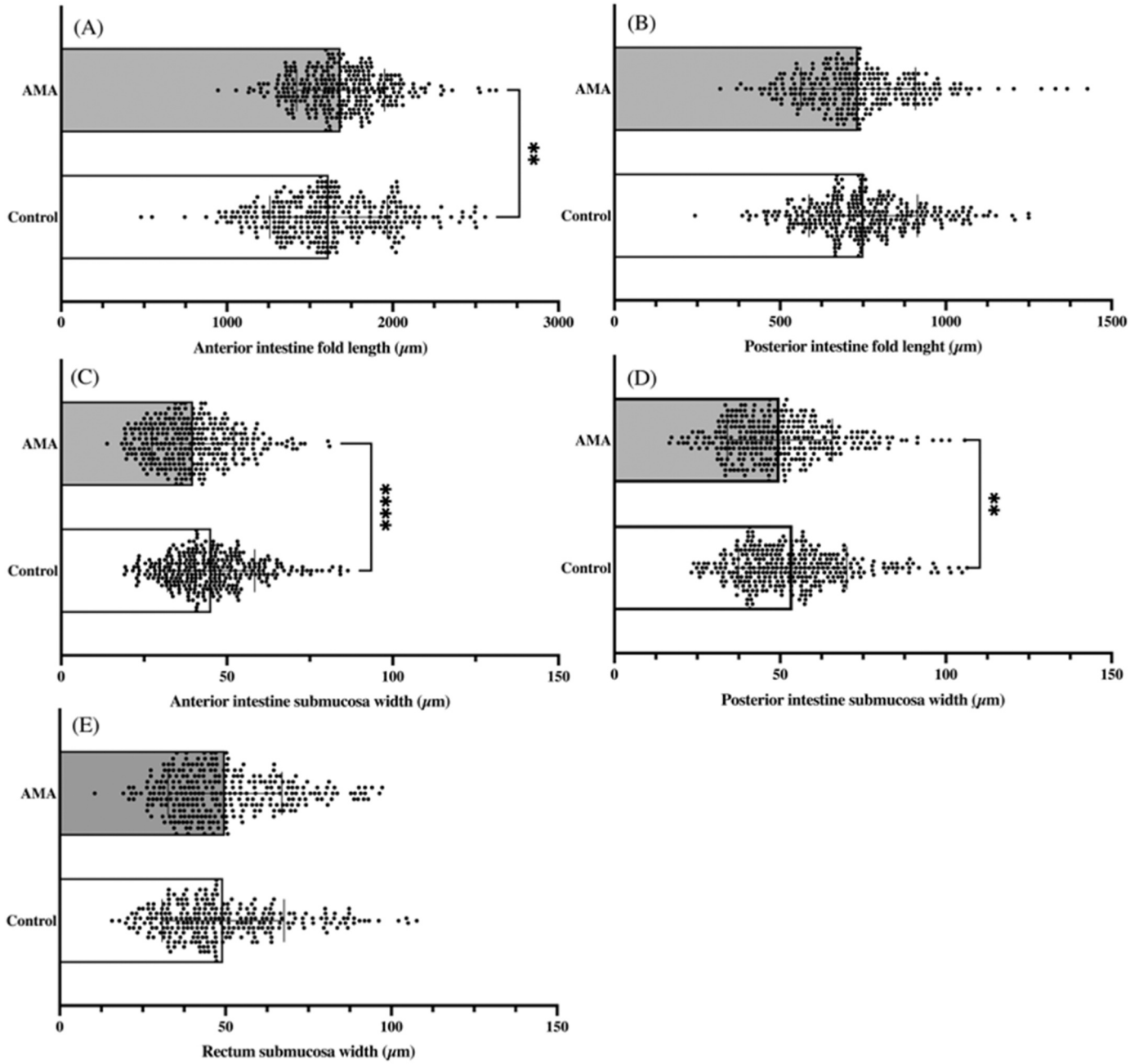

Fig. 2. Gut morphometric parameters of fish fed the experimental diets at the end of the feeding trial (8 weeks). (A) Anterior gut fold length, (B) posterior gut fold length, (C) anterior gut submucosa width, (D) posterior gut submucosa width and (D) rectum submucosa width (μm). Different symbols indicate significant differences (**$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$) between supplemented fish and control fish. Control= control diet; AMA= Amasil®NA at 3 g/kg.

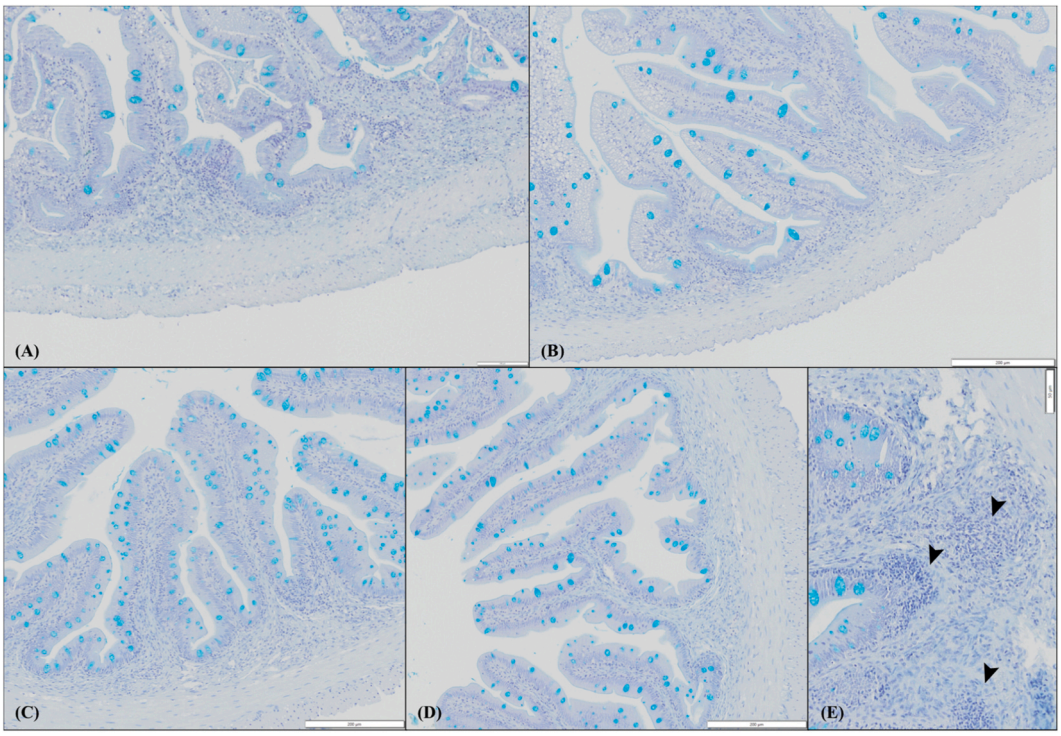

Fig. 3. Gilthead sea bream (*Sparus aurata*) anterior and posterior gut detailed micrographs after 8 weeks of feeding stained with Alcian-Blue (pH=2.5). Anterior gut general morphological pattern observed in fish fed fed control diet (A) and AMA diet (B). Scale bar 100 μm – 200 μm. Posterior gut general morphological pattern observed in fish fed fed control diet (C) and AMA diet (D). Scale bar 100 μm. Observe the thinner submucosa and the lower level of intraepithelial lymphocytes in the mucosa of supplemented fish as confirmed my morphometric analyses. (E) Detailed micrograph of a submucosa area with several lymphocytic focuses (➤). Scale bar 50 μm.Control= control diet; AMA= Amasil®NA at 3 g/kg.

3.1.1. Challenge test

At the end of the *V. anguillarum* challenge (10 days), a comparison of survival percentages between the two experimental groups showed that fish fed the AMA diet for 8 weeks presented reduced ($p \leq 0.05$) mortality after the experimental infection with *V. anguillarum* compared to fish fed the control diet (Fig. 1). The pattern of mortality observed in both treatments, highlights the higher mortality of fish fed the control diet during the first 48 h after pathogen exposure compared to fish fed the AMA diet.

3.1.2. Immune parameters

Fish fed the AMA diet for 8 weeks did not present ($p > 0.05$) significant effects on ACH50, lysozyme, bacteriolytic and bacteriostatic activities. In absolute values, fish fed the AMA diet had 10% higher ACH50 activity than fish fed the control diet (Table 3).

3.1.3. Gut morphometry

The fish intestine sections evaluated showed a well-organised folding pattern, lack of cellular debris and a preserved intestinal epithelial barrier in both experimental groups. In general, the anterior gut showed longer folds than the posterior gut, and the posterior intestine and rectum tended to have a wider submucosa layer than the anterior intestine (Fig. 2). Evaluation of morphometric characteristics related to dietary treatment revealed that fish fed the AMA diet for 8 weeks had longer ($p < 0.05$) anterior gut folds (Fig. 2) compared to fish fed the control diet, but no effects were observed on posterior gut fold length. Fish fed the AMA diet presented reduced ($p < 0.05$) submucosa width in the anterior (Fig. 2; Fig. 3A-B) and posterior (Fig. 2; Fig. 3C-D) intestinal segments, whereas no effect was observed in the post-ileorectal valve intestinal section (Fig. 2), indicating a reduced inflammatory status after AMA supplementation, concentrated in the proximal intestinal segments. Several areas of inflammation with lymphocytic foci were observed in the mucosa and/or submucosa of the experimental fish (Fig. 3E), irrespective of the dietary treatment fed.

3.1.4. Gut goblet cells morphometry

In terms of goblet cells morphological characteristics, the anterior gut had larger goblet cells than the posterior gut with its subsequent reduction in goblet cell perimeter. A similar goblet cell distribution pattern along the fold was observed in both intestinal sections, however, the functional additive affected some of the morphological features of goblet cells, particularly in relation to their size. The anterior gut of sea bream fed the AMA diet for 8 weeks showed a trend towards smaller ($p < 0.1$) goblet cells compared to fish fed the reference diet (Fig. 4; Fig. 5A-5B). Similarly, the posterior intestine of fish fed the AMA diet showed reduced ($p < 0.05$) total goblet cell area and perimeter compared to fish fed the control diet (Fig. 4; Fig. 5C-5D). No differences in goblet cell density or distribution pattern were observed in either gut region.

4. Discussion

Optimizing growth and feed conversion ratios by promoting GI function and health is of foremost importance in ensuring cost-effective production in aquaculture (Cho and Bureau, 1997). It is well known that feeding habits determine the length of the GI tract, with carnivorous and omnivorous fish having shorter GI than herbivorous fish, and, consequently a lower efficiency in utilizing alternative diets with low FM contents and high plant raw materials, such as wheat or soybean (Montero and Izquierdo, 2010; Oliva-Teles, 2012; Aragão et al., 2022). Therefore, it is hypothesised that OAs, through their potential as gut pH optimizers, may play a role in improving the utilisation of plant-based diets in farmed fish. OAs include SCFAs, such as lactic acid, acetic acid, succinic acid, propionic acid, and butyric acid, as well as formic acid. Formic acid has a stronger acidifiying potential and higher density when compared to the previously mentioned OAs, which may favour the

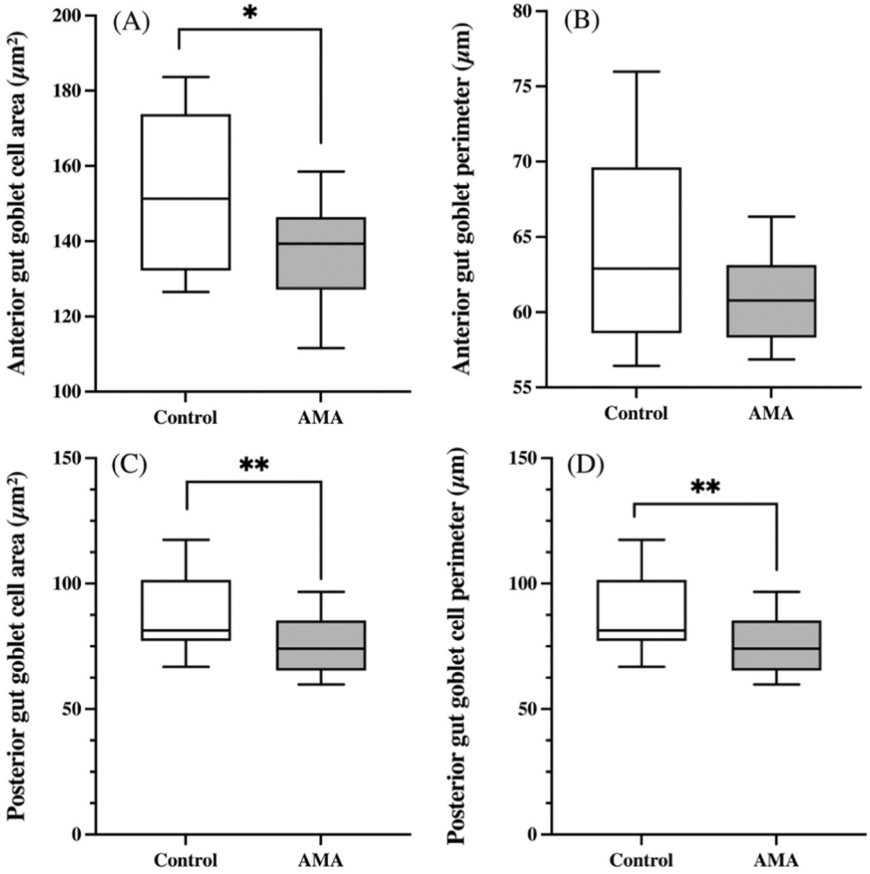

Fig. 4. Intestinal goblet cells morphometric characteristics of fish fed the experimental diets at the end of the feeding trial (8 weeks). Anterior gut (A) goblet cell area (μm^2) and (B) goblet cell minimum perimeter (μm). Posterior gut (C) goblet cell area (μm^2) and (D) goblet cell minimum perimeter (μm). Different symbols indicate significant differences (*$p < 0.1$; **$p < 0.05$) between supplemented fish and control fish. Control= control diet; AMA= Amasil NA® at 3 g/kg.

digestive processes by supporting protein and mineral digestion (Sardar et al., 2020). In this sense, Amasil NA® (BASF, Germany) is a commercial product composed of a mixture of formic acid and sodium formate, and thus has a potential activity as a gastric and intestinal pH balancer/optimizer and, consequently, theoretically increasing dietary nutrient availability, which may ultimately lead to better growth performance and feed utilization. In the present study, no effect on fish growth was observed after 8 weeks of dietary supplementation with Amasil NA®. However and since, typically, studies involving additives are conducted over a three-month period and/or extend to three times the initial biomass, it is important to note that this limitation was intentional, as the primary aim of the study was to assess the effect on gut morphology and disease resistance. Despite this lack of effect on growth performance, fish supplementatedwith Amasil NA® at 0.3% for 8 weeks presented a trend toward optimized feed utilization by 8%, suggesting that this OA may have a positive effect in high plant protein-based diet digestion. Consistent with our results, Amasil NA® and other sodium salts of formic acid have previously been shown to improve feed efficiency and nutrient retention in several tilapia species (Liebert et al., 2010; Sardar et al., 2020; Kore et al., 2019). In Atlantic salmon (*Salmo salar*), supplementation with potassium diformate (KDF) also promoted fish growth and improved feed conversion ratio (Christiansen et al., 2009). The lack of significant effects on growth in our study compared with other studies, could be related to differences in diet formulation, fish species, experimental conditions, and, in particular, the different supplementation times or doses tested, as described for many other functional ingredients. Indeed, the effects of several feed additives on fish growth performance are highly time- and dose-dependent. Although we did not measure fish intestinal pH or feed digestibility in the present study, the improved feed utilization observed in fish fed the AMA diet, could be a consequence of better digestion of the dietary protein content due to a direct effect of Amasil NA® on the gastric and intestinal pH of sea bream. Activation of acid protease zymogens is dependent on stomach pH, with pepsin being the enzyme responsible for the final cleavage of protein complexes to release oligopeptides. Other proteases such as trypsin depend on similar activation pathways, as trypsin is also involved in protein hydrolysis and further activation of chymotrypsinogen to chymotrypsin by proteolytic cleavage (Fabay et al., 2022). Therefore, the mechanism behind the mode of action of the AMA diet in the feed utilisation of sea bream could be related to a more suitable environment via pH optimisation for the action of acid and alkaline proteases, potentially favouring overall dietary protein digestion and thus compensating for the poorer digestion when sea bream are fed a low FM and high vegetable meal diet, resulting in better feed efficiency. Indeed, in other fish species such as rainbow trout, dietary supplementation with sodium-based salts of FA and/or FA itself has improved the digestibility of macronutrients, amino acids and minerals (Vielma et al., 1997; Sugiura et al., 1998; Morken et al., 2011). Furthermore, the supplementation with other OAs, such as citric acid at 1.5%, has been shown to increase the activity of digestive enzymes, including pepsin, trypsin, lipase, and amylase, in other marine warm-water species such as red drum (*Scianops ocellatus*) (Castillo et al., 2014). In the present study, the longer folds observed in the anterior gut of fish fed Amasil NA® -supplemented diet, could also be an indicator of an increased nutrient absorption area and, consequently, improved absorptive capacity (Vizcaíno et al., 2014). This is in agreement with the observed improved feed utilization in these fish and supports the hypothesis of a potential effect of the AMA functional diet as a promoter of intestinal absorption and gut health, as previously reported in other animal production species such as broiler chickens (Adeniji et al., 2015).

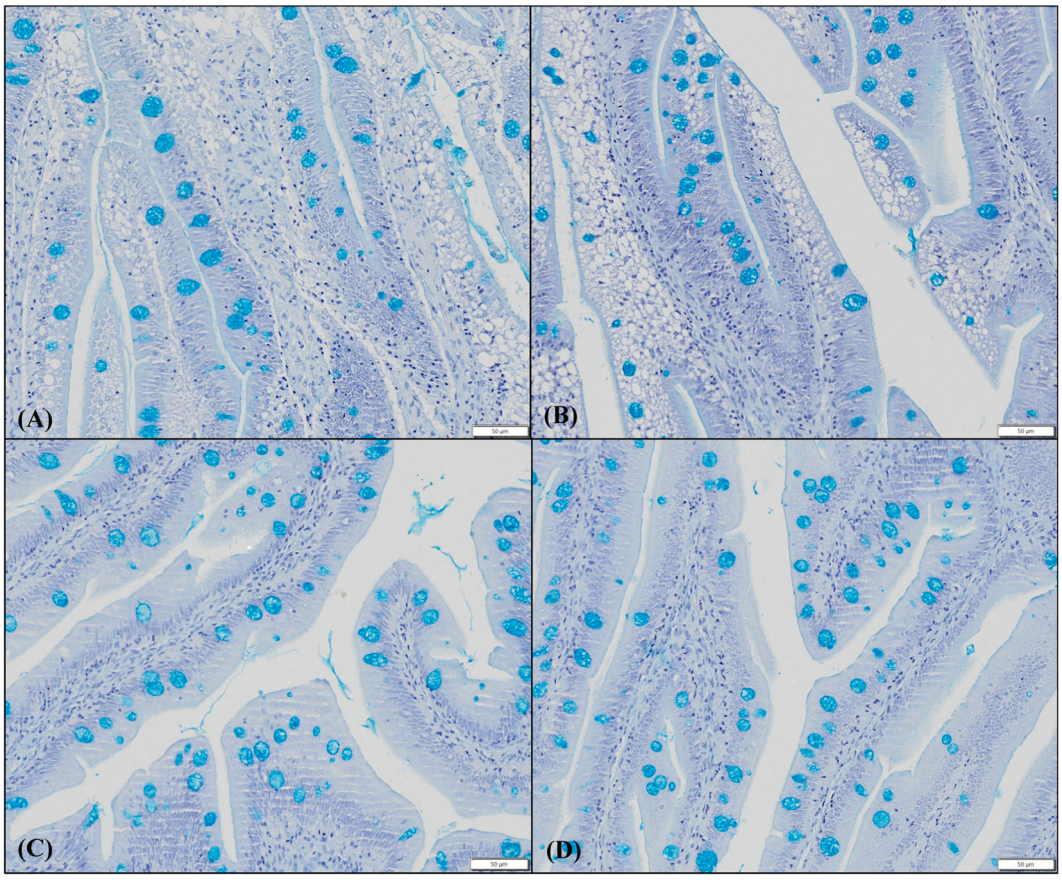

Fig. 5. Detailed micrographs of gilthead sea bream (*Sparus aurata*) anterior and posterior gut goblet morphology after 8 weeks of feeding stained with Alcian-Blue (pH=2.5). For both intestinal sections, the larger goblet cells pattern is represented in Figs. 5A and 5C and correspond to fish fed the control diet compared to fish fed AMA diet (E, D) as confirmed by morphometric analyses. Scale bar 50 μm. Control= control diet; AMA= Amasil NA® at 3 g/kg.

In addition to a reduced feed utilization, the high content levels of plant raw materials in the current practical aquafeeds is likely to induce a moderate gut-like inflammatory process in several carnivorous fish species (Oliva-Teles, 2012; Torrecillas et al., 2017; Aragão et al., 2022). Some typical indicators of this gut-like inflammatory state, induced by high dietary plant protein and lipid sources, include engrossed lamina propria and submucosa, sparse and shorter microvilli, exposure of tight junctions and impaired lipid transport, among others. As a result, these structural changes in the fish intestine affect not only intestinal health, but also nutrient digestibility (Torrecillas et al., 2017). In this sense, Amasil NA® dietary supplementation at 0.3% helped to modulate the gut health status of gilthead sea bream compared to fish fed the unsupplemented diet, by reducing posterior gut submucosa width and goblet cell size, which in other similar Mediterranean species have been associated with a high dietary vegetable meal content in relation to associated changes in the gut microbiome (Torrecillas et al., 2017). Therefore, these results indicate a protective function of this feed additive in gilthead sea bream fed practical diets, which is particularly important in the current scenario of new emergent available ingredients as potential FM replacers. In this line, some functional ingredients have been described as potential tools to facilitate the incorporation of emergent or alternative ingredients to traditional raw materials of marine origin, with the aim of providing the aquaculture sector with reliable management strategies. For example, Pérez-Sánchez et al. (2015)) reported the feasibility of using an encapsulated combination of carvacrol and thymol (Next Enhance ®, Novus, USA) as a promoter of gut health in combination or not with a prebiotic, by inducing an anti-inflammatory and anti-proliferative transcriptomic profile with a probable improvement of the intestinal absdorption capacity of fish. Similarly, other authors have pointed out the beneficial effects of including prebiotics in the diet for marine warm-water species in restoring gut homeostasis after a nutritional challenge with a total replacement of FO by soybean oil (Torrecillas et al., 2015), as well as the potential of supplementing bile salts in favour of a high dietary saturated fat intake for Mediterranean species. All of these highlight the potential use of functional ingredients not only as health promoters, but also as effective tools to facilitate the inclusion of new/emergent/alternative raw materials in aquafeeds.

In addition to the above-mentioned potential properties of FA on fish nutrient digestion and absorption, or as gut health promoter (Ng et al., 2009), it also has potent antifungal, antimicrobial, and anti-inflammatory activities (Ng and Koh, 2016; Fabay et al., 2022) with implications for the regulation of the gut microbiome (Ng et al., 2009). Previous studies have reported a reduction in intestinal pathogenic bacteria (*Aeromonas* and *Streptococcus*) in red and hybrid tilapia fed Amasil NA® (0.3%) (Kore et al., 2019), as well as in shrimp post larvae (Chuchird et al., 2015). Moreover, NaF, as well as other short and medium-chain fatty acids, have also been suggested to selectively modulate the intestinal microbiota and to promote the presence of intestinal lactic acid bacteria. Consequently, the parallel beneficial effects associated with this genus of bacteria are related to a modulation or contribution to the intestinal homeostasis of fish after challenged with low FM/FO based diets, via regulation of GALT humoral and cellular response (Pérez-Sánchez et al., 2014; Rimoldi et al., 2018; Mohammadian et al., 2020). Although we did not examine the intestinal microbiota in the present study, the AMA diet increased the survival of gilthead sea bream to *V. anguillarum*, as well as showing a tendency to increase the systemic humoral immune response, suggesting a potential

immunomodulatory effect of the AMA diet, not only locally but also systemically (Chuchird et al., 2015; Fabay et al., 2022). In previous studies, Amasil NA® (0.6%), likeother FA-based supplements, has also increased fish disease resistance when challenged against *Enterocytozoon hepatopenaei* and *Vibrio parahaemolyticus* or reduced the abundance of intestinal *Vibrio* spp. in farmed shrimp (Ng and Kho, 2017). A similar improvement in survival rate following the *V. anguillarum* challenge test was also reported in tilapia fed a KDF-supplemented diet (Ramli et al., 2005). Therefore, the present results further support the use of dietary OAs as a proactive strategy to reduce disease incidence, but also as a potential tool to facilitate the incorporation of new emergening alternative protein sources in practical diets of Mediterranean fish species.

5. Conclusions

The results of the present study showed that dietary supplementation with a commercial mixture of formic acid and sodium formate at a dietary level of 0.3% for 8 weeks, did not affect gilthead sea bream growth performance, but helped to improve the utilization of a high plant protein-based diet by 8%. This optimization is probably related to better efficiency in protein digestibility due to the potential gastric and intestinal pH-balancing effect of Amasil NA®. In addition, the AMA-diet also increased the folds length of gilthead sea bream anterior gut, increasing intestinal absorption area, and decreasing the submucosa width and goblet cell size in the posterior gut when included in a high dietary plant protein content diet. Furthermore, fish fed the AMA-diet showed increased survival when challenged with *V. anguillarum* compared to fish fed with a control diet. These results highlight the potential of Amasil NA® as a feed efficiency enhancer, as well as a promoter of gut and general health status in diets for gilthead sea bream.

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CRediT authorship contribution statement

Aboelsaadat Ehab: Conceptualization, Funding acquisition, Resources, Writing – original draft. **Torrecillas Silvia:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing. **Acosta Felix:** Formal analysis, Investigation, Supervision. **Carvalho Marta:** Formal analysis, Investigation, Writing – original draft. **Gordillo Alvaro:** Funding acquisition, Resources. **Monzón-Atienza Luis:** Formal analysis. **Montero Daniel:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Visualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests Ehab Aboelsaadat reports financial support was provided by BASF SE. Ehab Aboelsaadat reports a relationship with BASF SE that includes: employment. This work was partially supported by BASF, FZE (Dubai, United Arab Emirates), which provided the functional product and diets, and support in the form of salaries for authors EA and AG, but did not have any additional role in the study design, data collection and analyses.

Data availability

Data will be made available on request.

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