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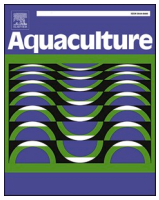

Novel chitinolytic *Bacillus* spp. increase feed efficiency, feed digestibility, and survivability to *Vibrio anguillarum* in European seabass fed with diets containing *Hermetia illucens* larvae meal

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A B S T R A C T

Insect meals (IM) are being pointed out as a promising sustainable protein source. However, chitin in IM may negatively interfere with fish performance and nutrient availability. Given that carnivorous fish have a low or inexistent ability to digest chitin, novel strategies are needed to overcome chitin-imposed bottlenecks. Carbohydrolytic sporeforming probiotics offer a competitive industrial approach, being able to resist the harsh feed manufacturing process, transport, storage, and animal's gastrointestinal tract, while being able to increase the digestibility of otherwise indigestible components. Aiming to increase IM chitin digestibility, two chitinolytic sporeforming fish isolates (Fish isolates 645 (FI645) and 658 (FI658)), closely related to *Bacillus licheniformis*, were isolated from European sea bass (ESB) gastrointestinal tract, and incorporated (individually or as a mixture) in ESB diets with high defatted *Hermetia illucens* larvae meal (HM) levels (30% inclusion). FI were evaluated regarding their effects on ESB growth performance and digestibility. The spores were able to maintain their viability throughout the 180 days of diet storage at room temperature. Dietary inclusion of FI645 led to higher chitin digestibility. Concomitantly, the digestibility of dry matter, protein, and energy increased, overall leading to higher feed efficiency and protein efficiency ratio. Dietary inclusion of FI645 led to increased plasma *N*-acetylglucosamine (GlcNAc) levels. This is the first evidence for diet-mediated modulation of plasma GlcNAc concentrations. Dietary inclusion of FI645 also increased the expressions of the genes coding for *N*-acetylglucosamine kinase (*nagk*) and GlcNAc phosphomutase (*pgm3)*, key enzymes from the GlcNAc salvage pathway and the hexosamine biosynthetic pathway (HBP). On the other hand, no differences were found for the expression of *N*-acetyl-D-glucosamine-6-phosphate deacetylase (*amdhd2)*, a precursor enzyme for GlcNAc redirection to glycolysis. These results hint that free GlcNAc resulting from increased chitin digestibility might be preferentially redirected to protein O-GlcNAcylation processes through HBP in fish cells. Additionally, when challenged with *Vibrio anguillarum*, and after being feed for one month with the same diets, D645 also increased ESB survival from 52.5% to 77.5%, when compared to the control. Together, these results establish the potential of using FI645 as a probiotic for enhancing chitin digestion and acting as a prophylactic agent for ESB protection against *V. anguillarum* infection. This strategy opens novel possibilities for using commodities, including high chitin levels in aquafeeds, and reducing the use of antibiotics in aquaculture.

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1. Introduction

Insect meals (IM) have a high potential to be used as ingredient in aquafeeds due to their suitable protein content and amino acid profile, and moldable fatty acid profile and content (Hua et al., 2019; Riekkinen et al., 2022; Siddiqui et al., 2022; Tran et al., 2022). Among the different insect species authorized for use in animal feed, *Hermetia illucens* (HI) has been the most studied and reared worldwide (Tran et al., 2022; van Huis, 2020; Weththasinghe et al., 2022), demonstrating high potential as a sustainable protein source in aquafeeds (Basto et al., 2020; Caimi et al., 2021; Hossain et al., 2021; Melenchón et al., 2021). While in some cases, high HI meal inclusion (>25%) in fish diets has no apparent drawback (Biasato et al., 2022; Melenchón et al., 2022; Moutinho et al., 2022; Rema et al., 2019), inclusion levels over 25% usually lead to poorer protein and/or lipid apparent digestibility coefficients (ADC) and growth performance (Dumas et al., 2018; Fisher et al., 2020; Stejskal et al., 2020; Stejskal et al., 2023). This is mainly attributed to the increased dietary chitin content, which has been negatively correlated with protein and lipid digestibility both *in vitro* (Marono et al., 2015) and *in vivo* (Eggink et al., 2022; Karlsen et al., 2017; Mastoraki et al., 2020; Piccolo et al., 2017).

Chitin, a polymer mainly composed of β-1-4 linked *N*-acetylglucosamine monomers (GlcNAc), is a structural component of the insect's exoskeleton (Muthukrishnan et al., 2016). Through a process called sclerotization, crystalline chitin forms a tightly bound matrix with cuticular proteins, severely limiting both chitin and associated protein enzymatic hydrolysis (Muthukrishnan et al., 2016). The breakdown of this chitin-protein matrix is mostly attributed to the combined action of two enzymes: chitinase, which, cleaves chitin into smaller molecules called chitooligosaccharides (COS); and chitobiase, which further hydrolyses these molecules into GlcNAc monomers (Fines and Holt, 2010; Henry et al., 2015). To date, while chitinase expression was reported in some fish species (Gao et al., 2017a; Holen et al., 2023; Ikeda et al., 2017; Koch et al., 2014), the extent of the potential endogenous or microbiota enzymatic contribution to chitin breakdown remains to be elucidated (Eggink et al., 2022; Hua, 2021; Panteli et al., 2021; Rangel et al., 2022a). Additionally, GlcNAc uptake and metabolism are also overlooked in fish. GlcNAc biological relevance is, however, well documented for prokaryotic and eukaryotic organisms where, through a highly conserved pathway called hexosamine biosynthetic pathway (HBP), GlcNAc can have a major impact on metabolic and immune gene expression, cellular signaling, or protein activation processes (Abdel Rahman et al., 2013; Chang et al., 2020; Groves et al., 2013; Hardivillé & Gerald, 2014; Holt et al., 1987).

Chitin digestion can be particularly challenging for carnivorous species (NRC, N, 2011). Upon dietary HI meal inclusion, low or inexistent chitin digestibility was observed in economically important carnivorous fish species, like Atlantic salmon (*Salmo salar*) (13–40%) (Olsen et al., 2006), rainbow trout (*Oncorhynchus mykiss*) (2–5%) (Lindsay et al., 1984), or meagre (*Argyrosomus regius*) (not detected) (Guerreiro et al., 2021). Moreover, as HI meal seems unable to promote the emergence of chitinolytic bacteria in the fish gut (Rangel et al., 2022a; Rangel et al., 2022b), alternative strategies are needed to increase chitin digestibility of IM-based diets.

Probiotics with digestive-enhancing abilities have been proven to be an efficient strategy to improve fish performance, potentially carrying additional benefits for the immunological status and gut microbiota composition (Butt and Volkoff, 2019; Van Doan et al., 2020; Wuertz et al., 2021). Several bacteria belonging to the *Lactobacillus*, *Carnobacterium*, *Bacillus*, *Streptomyces*, and *Enterococcus* genus have been successfully used as probiotics in aquaculture (Van Doan et al., 2020). Among these, *Bacillus* spp. are particularly attractive, as they can form spores, highly robust structures that withstand extreme conditions (Nicholson and Setlow, 1990; Nicholson et al., 2000; Setlow, 2006). This ability constitutes a potential advantage for the animal feed industry, as *Bacillus* spp. spores can resist the harsh extrusion process and gastrointestinal conditions (Cutting, 2011; Niu et al., 2018).

Aiming to improve dietary chitin digestibility, a selective dietary pressure to modulate European sea bass (ESB) gut microbiota towards enrichment with chitinolytic bacteria was performed (Rangel et al., 2022a). For that purpose, in a previous study, three IM were used and, through an *in vitro* sequential screening, two fish isolates (FI) closely related to *Bacillus licheniformis,* namely FI645 and FI658, were selected based on their sporeforming ability, total chitinolytic activity, hemolytic activity, antibiotic resistance, sporulation yield, and survival in gastrointestinal-like conditions (Rangel et al., 2022b). In the present study, the selected FI were supplemented individually or as a mixture in diets for ESB containing 30% inclusion of HI, to evaluate their probiotic potential to enhance growth performance, digestibility, and ability to protect fish from a *Vibrio anguillarum* infection. Moreover, this study also aimed to provide the first insights into potential routes for GlcNAc metabolism, in fish.

2. Materials and methods

2.1. Probiotic spores' production and purification

Each fish isolate (FI645 and FI658), whose selection was previously described in Rangel et al. (2022b), was induced to sporulate at high yields in liquid Difco Sporulation Medium according to Tavares et al. (2013). Sporulation occurred for 48 h at 37 °C in an orbital shaker at 200 rpm, and sporulation efficiency in each production cycle was determined by plating serial dilutions in Bott & Wilson (B&W) salts solution (1.24% K_2HPO_4, 0.76% H_2PO_4, 0.1% trisodium citrate, 0.6% $[NH_4]2SO_4$, pH 6.7) isotonic buffer on Luria Bertani (LB) agar, before and after 20 min heat treatment at 80 °C, to eliminate remaining vegetative cells. Following 24 h incubation at 37 °C, visible colonies were counted, and sporulation efficiency was calculated as the titer of Colony-Forming Units (CFU mL^{-1}) before and after the heat treatment.

The preparation of highly purified spores was done according to Tavares et al. (2013). Briefly, spore preparations of each isolate were centrifuged for 10 min at 10,000 ×g and 4 °C. Cell pellets were suspended in 1 volume of 50 mM Tris-HCl (pH 7.2) containing 50 μg mL^{-1} of lysozyme, and incubated for 1 h at 37 °C. After a single wash with 1 volume of distilled water (10 min at 10,000 ×g, 4 °C), cell pellets were suspended in 0.05% SDS (sodium dodecyl sulfate) solution by vortexing. Samples were then washed 3× with distilled water, suspended in 1 volume of distilled water, and lyophilized. Before dietary incorporation, serial dilutions of the lyophilized spores were carried out in B&W salts, and the number of CFU mg^{-1} was determined as described above.

2.2. Experimental diets and proximate analysis

Four experimental diets were formulated to be isoproteic (44%) and isolipidic (17%): a control diet, containing 30% HI meal (CTR diet); two CTR-like diets supplemented with 2×10^9 CFU kg^{-1} of either FI645 (D645 diet) or FI658 (D658 diet); and a CTR-like diet supplemented with 1×10^9 CFU kg^{-1} of both FI645 and FI658 (MIX diet). All dietary ingredients were finely ground, well mixed, and dry pelleted in a laboratory pellet mill (California Pellet Mill, CPM Crawfordsville, IN, USA) through a 2 mm die. The pellets were dried in an oven at 40 °C for 24 h and stored at room temperature in airtight bags until used. For the digestibility trial, 5 g kg^{-1} chromic oxide (Cr_2O_3) was added to the diets as an inert marker, substituting the same amount of wheat meal. Ingredients and proximate composition of the experimental diets are presented in Table 1. Chemical characterization (dry matter, protein, lipid, and ash contents) of the ingredients and experimental diets was carried out following the guidelines of the Association of Official Analytical Chemists methods (AOAC, 2000). Diets were analyzed for chitin composition as described by Guerreiro et al. (2020). Spore quantification and viability were measured by collecting 100 mg of each diet stored in airtight bags at room temperature (20–25 °C) at different

Table 1
Ingredient composition and proximate analysis of the experimental diets for the growth trial.

	Diets			
	CTR	D645	D658	MIX
Ingredients (% dry weight basis)				
Fish meal[a]	20.0	20.0	20.0	20.0
Soluble fish protein concentrate[b]	2.0	2.0	2.0	2.0
Hermetia illucens larvae meal[c]	30.0	30.0	30.0	30.0
Corn gluten[d]	7.5	7.5	7.5	7.5
Soybean meal[e]	5.6	5.6	5.6	5.6
Wheat meal[f]	16.2	16.2	16.2	16.2
Fish oil	13.1	13.1	13.1	13.1
Vitamin premix[g]	1.0	1.0	1.0	1.0
Mineral premix[h]	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5
Binder[i]	1.0	1.0	1.0	1.0
Taurine[j]	0.5	0.5	0.5	0.5
Dibasic calcium phosphate	1.7	1.7	1.7	1.7
Probiotic inclusion (10^9 CFU Kg^{-1})				
FI645	–	2	–	1
FI658	–	–	2	1
Proximate analyses (% dry weight basis)				
Dry matter	96.0	94.1	93.5	95.2
Crude protein	44.4	44.2	44.6	44.2
Crude fat	17.0	17.1	17.1	17.0
Ash	9.7	9.9	9.8	9.8
Chitin	1.7	1.7	1.7	1.7

[a] Sorgal, S.A. Ovar, Portugal (CP: 70.3% DM; GL: 12.0 %DM).
[b] Sorgal, S.A. Ovar, Portugal (CP: 79.7% DM; GL: 7.14% DM).
[c] Black soldier fly larvae meal (CP: 62.4% DM; GL: 6.3% DM).
[d] Sorgal, S.A. Ovar, Portugal (CP: 62.8% DM; GL: 1.2% DM).
[e] Sorgal, S.A. Ovar, Portugal (CP: 55.4% DM; GL: 1.9% DM).
[f] Sorgal, S.A. Ovar, Portugal (CP: 15.5% DM; GL: 1.9% DM).
[g] Vitamins (mg kg^{-1} diet): retinol, 18,000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); α- tocopherol, 35; menadione sodium bisulphate, 10; thiamine, 15; riboflavin, 25; Ca pantothenate, 50; nicotinic acid, 200; pyridoxine, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbyl monophosphate, 50; inositol, 400.
[h] Minerals (mg kg^{-1} diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.44 (g kg^{-1} diet).
[i] Aquacube. Agil, UK.
[j] Feed-grade taurine, Sorgal, S.A. Ovar, Portugal.

time points (days 0, 30, 60, 120, and 180). Thereafter, each sample was diluted in 1 mL of B&W solution, heat-treated (20 min at 80 °C), and CFU determined as described above. Triplicates were used for each time point.

2.3. Animals and growth trial conditions

European sea bass (*Dicentrarchus labrax*) (ESB) juveniles were obtained from Portuguese Institute for Sea and Atmosphere (IPMA), Olhão, Portugal. After arrival at CIIMAR experimental facilities (Matosinhos, Portugal) the fish were submitted to a 2-week quarantine period and fed with a commercial diet (44% protein and 18% lipids, Aquasoja Sustainable Feed: Sorgal, Ovar, Portugal). After 4 weeks of acclimation to the experimental system, 12 groups of 20 fish with an initial mean body weight of 24.6 ± 0.2 g were established and the experimental diets were randomly allocated to triplicate tanks. The experimental system consisted of 12 fiberglass tanks of 100 L water capacity in a recirculating life support system with artificial illumination (photoperiod set to 12:12 h light: dark), and water parameters maintained at 35.6 ± 0.6 g L^{-1} salinity, 7.6 ± 0.4 mg L^{-1} O_2, 7.3 ± 0.1 pH, thermoregulated to 23 ± 0.5 °C. The fish were hand-fed to apparent visual satiation twice daily, 6 days per week. The growth trial lasted 91 days.

2.4. Digestibility trial

At the end of the growth trial, 8 fish from each tank with a mean body weight of 75 ± 3 g were transferred to a digestibility system consisting of a battery of 12 fiberglass tanks of 60 L capacity designed according to Cho et al. (1982) and with a settling column connected to the outlet of each tank for feces collection. Tanks were supplied with a continuous flow of seawater (36.0 ± 0.5 g L^{-1} salinity, 7 mg L^{-1} oxygen) thermoregulated to 23.0 ± 1.0 °C. The first 8 days were used for fish to adapt to the experimental conditions, followed by 30 days of feces collection. During the trial, fish were hand-fed to apparent visual satiation twice a day, 7 days a week, and feces were collected from the settling column once a day, before the morning meal. Immediately after collection, feces from each tank were centrifuged at 3000 ×*g* for 10 min and then stored at 4 °C. Before proximal analysis determinations, feces were dried at 40 °C for one day, grounded, and stored at room temperature until analysis.

2.5. Sampling

Fish in each tank were bulk weighed at the beginning and end of the growth trial, after one day of feed deprivation. For that purpose, fish were anesthetized with 0.3 mL L^{-1} ethylene glycol monophenyl ether. After the final weighing, the fish were fed for 2 more days to minimize the stress caused by manipulation. Then, six fish from each tank were sampled 5 h after the morning meal, sacrificed with a sharp blow to the head, and dissected on chilled trays. The whole intestine with pyloric caeca and contents was freed from the adjacent adipose and connective tissues, snap-frozen in liquid nitrogen, and stored at −80 °C until digestive enzyme analyses. Three fish were used for measuring total alkaline protease, trypsin, lipase, and α-amylase activities, and the other three fish were for accessing total chitinase activity. Additionally, approximately 1 cm of the distal intestine (DI, distinct from the mid intestine by its darker mucosa, greater diameter, and presence of an annular ring) was collected from 3 of these fish for histological evaluation. The samples were washed in phosphate-buffered saline solution (PBS) and fixed in phosphate-buffered formalin (4%, pH 7.4). After 24 h, the samples were transferred to a 70% ethanol solution until further processing. The DI from the other three fish was collected, washed in PBS, and stored in RNA Latter for 24 h at 4 °C, being subsequently transferred at −80 °C until further processing for gene expression analyses.

2.6. Digestive enzymes

For total alkaline protease (TAP), trypsin (EC3.4.21.4), lipase (EC3.1.1.3), and α-amylase (EC3.2.1.1) activities, samples were homogenized in ice-cold 50 mM Tris-HCl buffer pH 7.5 (1:6 w:v). Homogenates were centrifuged at 33,000 ×*g* for 15 min at 4 °C, and the resultant supernatants were stored at −80 °C until digestive enzyme activity evaluation as described by Couto et al. (2016). Results are presented as specific activity. Protein concentration was ascertained as depicted by Bradford (1976) using bovine serum albumin (Sigma-Aldrich) as standard.

For total chitinase activity, samples were homogenized (1:6 w:v) in ice-cold McIlvaine buffer (0.15 M citric acid, 0.3 M sodium phosphate dibasic, pH 7). The homogenates were centrifuged for 5 min at 5000 ×*g* and the supernatants were aliquoted and stored at −80° until further processing. Total chitinase activity was measured as described by Guerreiro et al. (2021). All enzyme assays were performed at 37 °C with Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

2.7. Plasma metabolites and innate immune parameters

Plasma glucose and cholesterol were determined using Spinreact

(Girona, Spain) commercial kits (glucose kit, ref. 1,001,191; cholesterol kit, ref. 1,001,091). Plasma *N*-acetylglucosamine (GlcNAc) was assessed using a colorimetric method for the determination of *N*-acetylamino sugars, as proposed by Reissig et al. (1955), with some modifications. Briefly, 0.05 mL of plasma was transferred to a tube containing 0.01 mL of 0.8 M borate buffer ($K_2B_4O_7$), pH 9.3. The solution was boiled for exactly 3 min and thereafter it was cooled in a water bath at room temperature. Color development was attained by adding 0.5 mL of *p*-dimethyl-amino-benzaldehyde (DMAB) solution (15 g L^{-1} DMAB in glacial acetic acid with 1.25% (v:v) 12 N hydrochloric acid), and incubated at 37 °C for 20 min. A GlcNAc standard curve ranging from 0.4 to 250 μM was prepared. Plasma total proteins and total immunoglobulins were quantified using Pierce™ BCA Protein Assay Kit (ref. 23,225; Thermo Scientific) and performed as described by Siwicki (1993). Peroxidase and lysozyme activities were carried out as described by Machado et al. (2015). All metabolites concentration and enzyme activity readings were conducted in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

2.8. Histological processing and morphological assessment

The DI samples were processed in a tissue processor (Model Citadel 2000, Thermo Scientific, Nanjing, China), and sectioned with a microtome (Model Jung RM 2035, Leica Instruments GmbH, Wetzlar, Germany). The samples were stained with hematoxylin and eosin using an automatic slide stainer (Model Shandon Varistain 24–4, Thermo Scientific, Nanjing, China). A blind assessment of the DI slides was carried out taking particular attention to any inflammatory changes (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003), namely: changes in mucosal folds height; width and cellularity of the lamina propria and submucosa; number of intraepithelial lymphocytes and eosinophilic granulocytes; enterocyte overall organization, including nucleus position and supranuclear vacuolization (Fig. 1). A semi-quantitative scale scoring was applied as described in Couto et al. (2016), where scores ranged from 0 (normal) to 5 (highly modified), regarding the degree of structural alterations. The overall histomorphological alterations were estimated by averaging scores of the parameters described above. Images were acquired with Zen software (Blue edition; Zeiss, Jena, Germany).

2.9. Primer design and gene expression analysis

Pairs of oligonucleotide PCR primers were designed to target genes coding for key enzymes of the hexosamine biosynthetic pathway (HBP), namely: glutamine:fructose-6-phosphate amidotransferase (*gfat*; E.C. 2.6.1.16), *N*-acetylglucosamine kinase (*nagk*; E.C. 2.7.1.59), GlcNAc phosphomutase (*pgm3*; E.C. 5.4.2.3), *N*-acetyl-D-glucosamine-6-phosphate deacetylase (*amdhd2*; E.C. 3.5.1.25), and O-GlcNac transferase (*ogt*; E.C. 2.4.1.255). The primers were designed based on predicted mRNA sequences of ESB and other fish species (e.g., *Salmo salar*, *Morone saxatilis*, *Sparus aurata*, *Oncorhynchus mykiss*) available at the National Center for Biotechnology Information nucleotide database (http://www.ncbi.nlm.nih.gov, last access on June 15th, 2022). Primers were designed using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). To avoid unspecific genomic DNA binding, primers were designed to span an exon-exon junction.

For each diet, total RNA was extracted from the samples of DI using

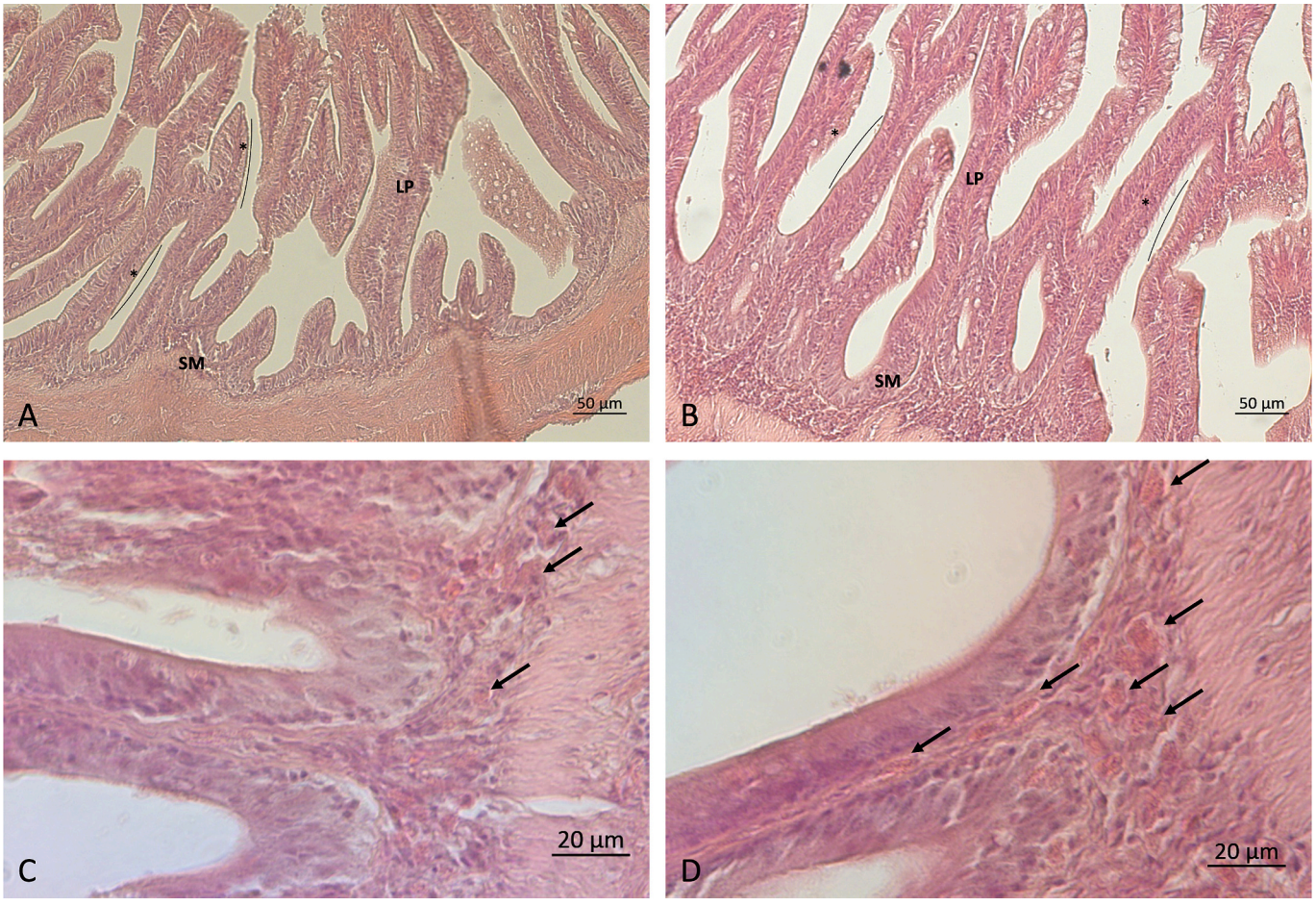


Fig. 1. Histological features of European seabass distal intestine fed Control (A, C) and Mix (B, D) diets. Lamina propria (lp), submucosa (SM), supranuclear vacuolization (*), eosinophilic granulocytes (arrows). Hematoxylin-eosin (HE) staining.

Direct-zol™ RNA MiniPrep Kit (Zymo Research, Irvine, CA, USA), following manufacturer instructions. Briefly, samples were homogenized in 500 μL TRI Reagent using Precellys 24 homogenizer (Bertin Technologies, Montigny-Le-Bretonneux, France), in a 2 mL vial, and then centrifuged at 13,000 ×*g* for 1 min at 4 °C. The supernatant was collected and mixed with an equal volume of absolute ethanol (PanReac, Barcelona, Spain). The mixture was transferred into a spin column (supplied with the kit), and centrifuged for 1 min at 13,000 ×g, at 4 °C. The samples were then washed with 400 μL of wash buffer and centrifuged for 1 min at 13,000 ×g, followed by a DNase treatment. The resulting RNA was washed twice and eluted in 50 μL of DEPC-treated water. RNA quantity was measured spectrophotometrically using μDrop™ Plate (Thermo Scientific, Courtaboeuf, France) in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). 1 μg of total RNA was used to generate cDNA following the NZY First-Strand cDNA Synthesis Kit (NZYTech, Lisbon, Portugal) protocol. The final product was stored at −20 °C until further analyses.

Gene expression was assessed using real-time quantitative PCR (CFX Connect™ Real-Time System, Bio-Rad, California, USA). cDNA amplification was performed using specific PCR primers (Table 2). Primer efficiency was determined as described by Monteiro et al. (2021). Real-time qPCR reactions were performed using 3.5 μL of ultrapure water (Sigma-Aldrich, Taufkirchen, Germany), 5 μL of SsoAdvanced Universal SYBR® Green supermix (Bio-Rad, California, USA), 0.5 μL of each primer and 0.5 μL cDNA from each sample, in a final volume of 10 μL. The different transcripts were amplified under the following conditions: 95 °C for 30 s for denaturation, followed by 40 cycles of 95 °C for 15 s and 55 °C for 30 s. Melting curve analysis was performed to verify the presence of a specific amplicon, and that no primer dimers were formed. Relative expression of each transcript was normalized using the comparative threshold cycle (Ct) methodology with only one housekeeping gene, namely elongation-factor 1-alpha gene (*ef1α*), due to its expression and stability (Monteiro et al., 2021), and calculated using the Pfaffl method (Pfaffl, 2001).

2.10. Bacterial challenge trial

The challenge trial was performed using 10 groups of 20 fish with a mean body weight of 54 ± 10.6 g, in a recirculating aquaculture system equipped with 100 L fiberglass tanks. Four groups were fed the CTR diet and two groups were fed each of the tested diets (D645, D658, and MIX). Fish were maintained with an artificial illumination photoperiod set to 12:12 h light:dark and controlled water parameters (35.6 ± 0.6 g L^{-1} salinity, 7.6 ± 0.4 mg L^{-1} O_2, 7.3 ± 0.1 pH, 23 ± 0.5 °C). Fish were hand-fed to apparent visual satiation twice daily, 6 days per week for 31 days. Next, the fish were fasted for 24 h and then were intraperitoneally injected with 100 μL of a solution containing 1.5 × 10^7 CFU mL^{-1} of pathogenic *Vibrio anguillarum* DSMZ 21597 (a concentration previously established as LD50). Two groups previously fed with the CTR diet were intraperitoneally injected with PBS and served as a negative control. Fish mortality was monitored for 8 days.

Table 2
Oligonucleotide primer-pairs used in this study.

Primer name	Primer sequence (5′- 3′)	Amplicon size (bp)	Reference
gfat_esb_FW	CGAGGTTACGACGTGGACTG	93 bp	This study
gfat_esb_REV	TGCCAGGCTTTTGTTGTTAGC		
nagk_esb_FW	ATCAGGGACCGGCTCTAACTG	103 bp	This study
nagk_esb_REV	CCAGAATGCTGAGCCCTCAT		
naga_esb_FW	TCTGGAGGGTCCGTTCATC	219 bp	This study
naga_esb_REV	GGCCACAGAGTGACCTAAGGAC		
pgm3_esb_FW	AGCTCAAAGTCAAGGTGTCGG	176 bp	This study
pgm3_esb_REV	CCTCTGCGTAAACTCTCACGA		
ogt_esb_FW	ATGCCCTGAAGGAGAAAG	190 bp	This study
ogt_esb_REV	AACTCTGGGAACACCTCTAGT		
ef1α_esb_FW	GCTTCGAGGAAATCACCAAG	153 bp	Geay et al. (2010)
ef1α_esb_REV	CAACCTTCCATCCCTTGAAC		

2.11. Statistical analysis

Data are represented as means ± standard deviation. All data were checked for normal distribution and homogeneity of variances using Shapiro-Wilk and Levene tests, respectively, and when necessary transformed. Spores' viability and variation was analyzed using a repeated measure analysis of variance (ANOVA). Data corresponding to the fish growth performance, digestibility, enzymatic activities, plasma metabolites and immune parameters, and gene expression was analyzed by two-way ANOVA with fish isolates (FI645 and FI658) as factors. When interaction was observed, a Dunnett's multiple comparisons test was performed to identify differences between the supplemented diets and the control. Histological data were not normal nor homogeneous and could not be normalized, thus the non-parametric Kruskal-Wallis test was performed followed by all pairwise comparisons with *p* values adjusted by the Bonferroni correction for multiple tests. The cumulative survival in the challenge trial was evaluated by the Kaplan-Meier method (Log rank), comparing each supplemented diet with the CTR. Differences were considered statistically significant when $p < 0.05$. All statistical analysis was done using IBM SPSS Statistics v28 (IBM Corp., New York, USA).

3. Results

Fish isolates (FI) spore quantification immediately after diet manufacture (day 0), showed that the isolates were within the expected supplemented concentrations. Additionally, spores' viability was maintained up to 180 days (Fig. 2).

Fish promptly accepted the experimental diets, and no mortality was recorded throughout the growth and digestibility trials. Dietary probiotic supplementation did not affect final body weight, daily growth index, or feed intake (Table 3). In contrast, dietary supplementation with FI645 significantly decreased feed conversion ratio. Protein efficiency ratio was higher in fish fed the FI645 supplemented diets (D645 and MIX diets).

Total alkaline protease (TAP) activity was increased in fish fed the D645 diet and decreased in fish fed the D658 diet (Table 4). No effect of dietary probiotic supplementation was observed in trypsin, lipase, and α-amylase activities.

The apparent digestibility coefficients (ADC) of dry matter, protein, and energy were higher in the D645 diet than in the control (Table 5). The ADC of chitin was significantly higher in the diets including the FI645 probiotic, being twice higher in the D645 diet than in the control (47.8% vs. 23.8%, respectively). The ADC of lipids was not affected by diet composition.

No effects of diet composition were observed in any distal intestine histological parameter analyzed (Table 6). The mean score of all evaluated parameters also did not differ among groups.

Dietary supplementation with FI645 led to higher plasma *N*-acetylglucosamine levels than with the other diets (Table 7). No further differences were observed in the levels of the other plasma metabolites measured. Dietary supplementation with FI645 also led to higher plasma total immunoglobulins levels, while no differences were observed in lysozyme and peroxidase activities.

Distal intestine expression of *nagk* and *pgm3* was significantly induced in fish fed with diets supplemented with FI645 (Fig. 3). No further differences were observed in the expression of the other genes measured.

No mortality was observed in the negative control group (PBS-treated fish) during the bacterial challenge (Fig. 4). Diet D645 significantly increased fish cumulative survival from 52.5% to 77.5%, when compared to the CTR diet. Additionally, while not statically different, diets D658 and MIX showed higher cumulative survival values (62.5% and 65%, respectively) than the CTR diet (52.5%).

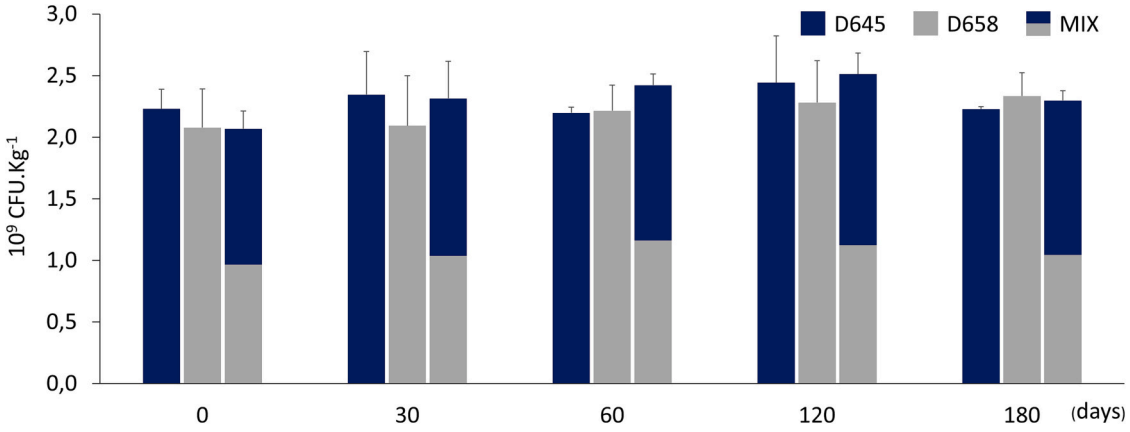


Fig. 2. Spores' viability in the experimental diets during storage time at room temperature for 180 days.

Table 3
Growth performance and feed utilization efficiency of European sea bass fed the experimental diets.

	Diets				*Two way-Anova*		
	CTR	D645	D658	MIX	*FI645*	*FI658*	*INT*
FBW[a]	74.3 ± 1.2	75.9 ± 1.3	74.9 ± 1.7	77.9 ± 4.5	0.245	0.506	0.708
DGI[b]	1.42 ± 0.03	1.46 ± 0.03	1.44 ± 0.03	1.49 ± 0.09	0.245	0.517	0.730
FI[c]	13.4 ± 0.2	13.2 ± 0.1	13.5 ± 0.2	13.2 ± 0.4	0.262	0.848	0.791
FCR[d]	1.20 ± 0.01	1.18 ± 0.01	1.21 ± 0.01	1.16 ± 0.01	<0.001	0.389	0.070
PER[e]	1.86 ± 0.00	1.92 ± 0.02**	1.85 ± 0.01	1.96 ± 0.01**	<0.001	0.145	0.040

Mean and standard deviation (± SD) are presented for each parameter ($n = 3$). When interaction was significant, Dunnett's test was performed to compare the probiotic supplemented diets to the control. Statistical differences are represented by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

[a] FBW (Final body weight) (g).

[b] DGI (Daily Growth Index) (%): ((final body weight$^{1/3}$ − initial body weight$^{1/3}$)/time in days) × 100.

[c] FI (Feed intake) (g kg ABW^{-1} day^{-1}) with ABW (Average body weight) = (initial body weight + final body weight)/2.

[d] FCR (Feed conversion ratio): feed intake/weight gain.

[e] PER (Protein efficiency ratio): weight gain/protein intake.

Table 4
Specific activities of total alkaline protease (TAP), trypsin, lipase, α-amylase (mU/mg protein) and total chitinolytic activity (TCA) (μg NAG / h / g wet tissue) in the intestine of European sea bass fed the experimental diets.

	Diets				*Two way-Anova*		
	CTR	D645	D658	MIX	*FI645*	*FI658*	*INT*
TAP	481.4 ± 217.2	551.6 ± 250.0	379.2 ± 132.2	408.3 ± 150.7	0.028	0.001	0.346
Trypsin	138.7 ± 56.4	134.4 ± 50.5	144.8 ± 42.5	122.4 ± 24.2	0.241	0.791	0.423
Lipase	13.3 ± 2.1	14.7 ± 4.0	17.2 ± 9.0	13.8 ± 3.2	0.694	0.681	0.289
α-Amylase	74.9 ± 25.8	64.2 ± 29.69	51.7 ± 25.8	66.4 ± 25.6	0.317	0.271	0.031
TCA	3.04 ± 0.86	4.34 ± 0.47	3.17 ± 1.44	3.61 ± 1.50	0.052	0.484	0.323

Mean and standard deviation (± SD) are presented for each parameter ($n = 9$).

4. Discussion

Dietary inclusion of *Bacillus* spp. as probiotics has been demonstrated to be an efficient strategy to increase feed digestibility, as the exoenzymes produced by these bacteria are proficient at hydrolyzing a large array of carbohydrates, lipids, and proteins (Van Doan et al., 2020). Chitin is among the carbohydrates postulated to be hydrolyzed by probiotic bacteria (Tran et al., 2022). Present in insects' exoskeleton, chitin is considered indigestible by most fish, being the main responsible for the decrease in protein and energy digestibility in IM-based diets (Henry et al., 2015). This negative correlation between dietary chitin content and protein digestibility was confirmed both in vitro (Marono et al., 2015) and in vivo (Eggink et al., 2022; Karlsen et al., 2017; Mastoraki et al., 2020; Piccolo et al., 2017). In fish, upon IM inclusion in the diets two main chitin antinutritional modes of action are proposed: chitin can increase viscosity within the fish intestine, decreasing feed exposure to digestive enzymes (Razdan and Pettersson, 1994); and/or the chitin matrix can entrap the sclerotized protein, thus reducing enzymatic access (De Marco et al., 2015). Removing chitin from IM, however, was shown to increase protein digestibility (Belghit et al., 2019). Present results corroborate these observations, as dietary supplementation with FI645 increased chitin digestibility and, concomitantly, increased protein digestibility. In turn, increased chitin digestibility is most likely driven by an increase in the total chitinolytic activity, which, although no statistical differences were found ($p = 0.052$), shows a tendency towards a concomitant increase with FI645 inclusion. Coupled with FI658's inability to increase the fish chitinolytic activity when compared with fish fed the CTR diet, it is plausible to speculate that the apparent increase observed in the MIX and D645 diets occurred in a FI645 concentration-dependent manner, opening promising prospects for further inclusion level optimization.

On the other hand, both intestinal chitinolytic activity and chitin digestibility were observed in fish fed the CTR diet. While several carnivorous fish species possess only vestigial or even no chitin digestibility (Lindsay et al., 1984; Guerreiro et al., 2021), in some cases, species such as Atlantic salmon and rainbow trout have been verified to digest chitin to a reasonable extent (Eggink et al., 2022; Olsen et al., 2006). However, whereas endogenous chitinase expression and chitinase activity have been detected in these species (Eggink et al., 2022; Holen et al., 2023) the full extent of the endogenous and microbiota contributions remains to be elucidated. In ESB, is plausible that chitin digestibility results from HI-driven ESB intestinal microbiota modulation, as previously shown by Rangel et al. (2022a). However, chitinolytic activity stemming from endogenously expressed chitinases cannot be ruled out, as ESB is known to express acidic mammalian chitinase in its intestine (Calduch-Giner et al., 2016). Independently of the endogenous and microbiota contributions, as dietary FI645 supplementation

Table 5
Apparent digestibility coefficients (%) of the European sea bass fed the experimental diets.

	Diets				*Two way-Anova*		
	CTR	D645	D658	MIX	*FI645*	*FI658*	*INT*
Dry matter	69.8 ± 1.3	76.4 ± 1.7**	71.9 ± 1.7	72.5 ± 1.0	0.003	0.319	0.009
Protein	90.0 ± 0.4	92.4 ± 0.6**	90.8 ± 0.5	91.1 ± 0.4	0.002	0.452	0.008
Lipids	98.7 ± 0.0	99.0 ± 0.3	98.9 ± 0.1	98.8 ± 0.1	0.225	0.649	0.060
Energy	83.0 ± 0.8	87.1 ± 0.9**	84.4 ± 1.1	85.0 ± 0.9	0.002	0.568	0.012
Chitin	23.8 ± 8.9	47.8 ± 7.5	18.7 ± 9.3	34.4 ± 10.5	0.005	0.116	0.451

Mean and standard deviation (± SD) are presented for each parameter (n = 3). When interaction was significant, a Dunnett's test was performed to compare the probiotic supplemented diets to the control. Statistical differences are represented by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001).

Table 6
Histomorphological scores of the distal intestine (DI) of the European sea bass fed the experimental diets.

	Diets				Kruskal-Wallis
	CTR	FI645	FI658	MIX	*P*-value*
FH	1.3 ± 0.5	1.2 ± 0.4	1.6 ± 0.7	1.6 ± 0.5	0.506
LP	1.2 ± 0.4	1.3 ± 0.5	1.3 ± 1.0	1.2 ± 0.4	0.819
SM	1.8 ± 0.8	2.0 ± 0.7	2.1 ± 0.8	2.7 ± 0.5	0.086
IEL	1.9 ± 0.8	2.4 ± 0.7	1.7 ± 0.7	2.2 ± 0.7	0.144
EGC	1.8 ± 0.4	2.2 ± 0.4	1.8 ± 0.4	2.1 ± 0.3	0.069
ENT	1.0 ± 0.0	1.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.0	0.392
SNV	1.0 ± 0.0	1.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.0	0.392
Mean Score	1.4 ± 0.3	1.6 ± 0.2	1.5 ± 0.3	1.7 ± 0.2	0.103

Mean values and standard deviation (± SD) are presented for each parameter (*n* = 9). Mucosal folds height (FH), width and cellularity of the lamina propria (LP) and submucosa (SM), number of intraepithelial lymphocytes (IELs) and eosinophilic granulocytes (EGC), enterocyte overall organization including nucleus position (ENT) and supranuclear vacuolization (SNV), and average of all the parameter evaluated (Mean Score).

was able to double chitin digestibility (47.8% vs. 23.8% in the CTR diet), these results also support the crucial role of the microbiota in the degradation of chitin in fish, as previously suggested by several other authors (Itoi et al., 2006; Ray et al., 2012; Sugita et al., 1999). Moreover, whereas in this study, only total chitinolytic activity was measured, to provide a better understanding of the enzymatic landscape present in the fish's digestive tract, subsequent analysis should also seek clarify *endo*- and *exo*-chitinases as well as chitobiase activity.

On the other hand, while increased chitin digestibility can partially explain the increase in dry matter digestibility in fish fed diet D645, it is important to mention that the latter can also be a result of increased digestibility of the dietary starches and sugars, present in diets containing plant-based ingredients. In fact, probiotics *Bacillus* spp. have been demonstrated to increase the digestibility of plant-based diets (Adorian et al., 2019; Hamza et al., 2016; Serra et al., 2019). Moreover, as *B. licheniformis* spores can survive extrusion processes (Niu et al., 2018), the digestibility of these water-soluble carbohydrates could potentially be further increased in extruded diets, as the high temperatures to which these components are exposed can increase their bioavailability (Enes et al., 2011).

Finally, probiotics can also modify intestinal architecture characteristics by modification of enterocyte tight junctions, thus limiting or enhancing macromolecules and pathogen permeability through the paracellular diffusion route (Maas et al., 2021; Parassol et al., 2005; Smith et al., 2004; Ward et al., 2000); by modifying enterocytes villi length (Nikiforov-Nikishin et al., 2022); by altering enterocyte division rate (Maas et al., 2021; Rawls et al., 2004); and by maintaining or disrupting mucosal integrity (Butt and Volkoff, 2019; Davis et al., 2016). Moreover, as depending on their size, chitin can be recognized by cells and trigger several immunophysiologic and morphologic changes, the formation of smaller chitin molecules or chitooligosaccharides resulting from increased digestion, could imact on the ESB intestinal architecture (Cuesta et al., 2003; Nurmalasari et al., 2022). However, the present data shows that intestinal architecture was unchanged by neither dietary probiotic supplementation nor concomitantly with chitin digestibility. Moreover, the lack of morphologic alterations indicates that the increases in feed efficiency and protein efficiency ratio in fish fed the diets supplemented with FI645 seem to be unrelated to modifications in intestinal architecture characteristics.

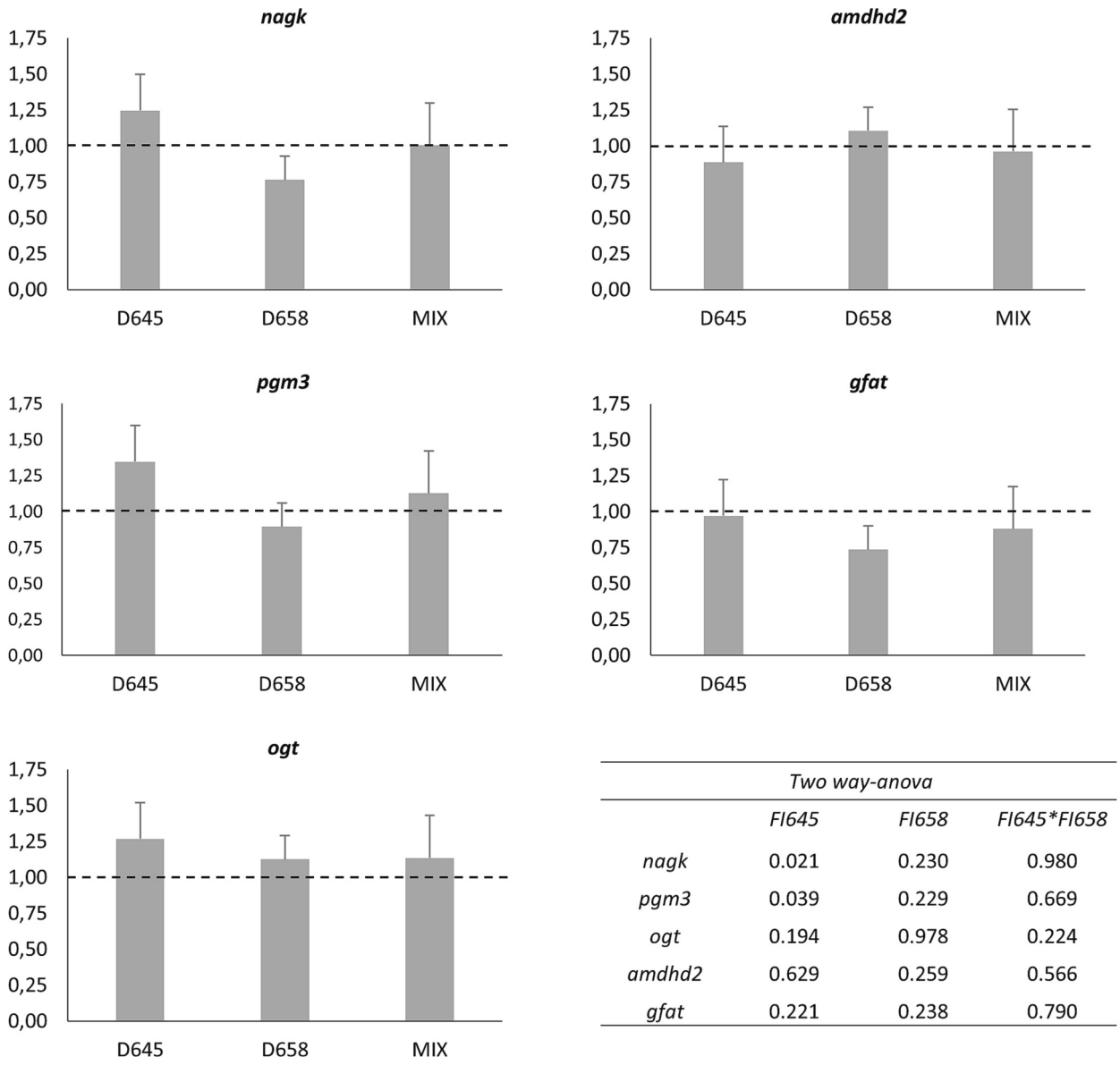
Smaller molecules available after chitin digestion, such as COS and GlcNAc, might be used by fish although their metabolism is still poorly understood. While GlcNAc uptake is hypothesized to occur by passive diffusion through the cell membrane (Krogdahl et al., 2005) (Fig. 5), no pathway has been proposed for its cellular metabolic integration. In the present study, several genes involved in GlcNAc salvage pathway and HBP were for the first time detected in fish, namely *nagk, pgm3, ogt, amdhd2, and gfat*, and an overview of GlcNAc pathway is proposed.

In humans, after entering the cells, GlcNAc can modulate the HBP (Abdel Rahman et al., 2013) (Fig. 5). The HBP is considered a nutrient-sensing pathway as it integrates glucose, amino acids, fatty acids, nucleotides, and energy metabolism (Hardivillé & Gerald, 2014). Alterations in the HBP can modulate physiological cell response to nutrients and infection (Chang et al., 2020; Hardivillé & Gerald, 2014). Integration of GlcNAc in the HBP can be achieved by *N*-acetylglucosamine

Table 7
Plasma metabolites, namely glucose, cholesterol (mmol L^{-1}), *N*-acetylglucosamine (GlcNAc) (μmol L^{-1}), total plasma proteins (mg mL^{-1}) and innate immune parameters, namely lysozyme (mg mL^{-1}), peroxidase (U mL^{-1}) and total plasma immunoglobulins (IGs) (mg mL^{-1}) of European sea bass fed the experimental diets.

	Diets				*Two way-Anova*		
	CTR	D645	D658	MIX	*FI645*	*FI658*	*INT*
Metabolites							
Glucose	6.29 ± 0.74	6.06 ± 1.31	6.12 ± 0.63	6.69 ± 0.51	0.585	0.457	0.207
Cholesterol	233.6 ± 40.1	235.2 ± 38.8	239.7 ± 35.7	248.7 ± 64.2	0.747	0.553	0.823
GlcNAc	22.9 ± 4.8	32.9 ± 12.3	18.6 ± 7.3	22.4 ± 5.1	0.017	0.100	0.170
Protein	46.9 ± 3.4	50.4 ± 7.8	48.1 ± 3.0	49.0 ± 4.7	0.209	0.964	0.449
Innate immunity							
Lysozyme	7.57 ± 0.79	7.67 ± 1.13	7.75 ± 1.15	7.08 ± 1.23	0.583	0.656	0.093
Peroxidase	0.34 ± 0.12	0.57 ± 0.21	0.55 ± 0.27	0.51 ± 0.28	0.459	0.256	0.115
IGs	21.6 ± 2.6	26.8 ± 5.8	21.0 ± 2.1	22.1 ± 3.0	0.032	0.074	0.193

Mean values and standard deviation (± SD) are presented for each parameter (n = 9). Data was analyzed using a two-way analysis of variance (ANOVA). Statistical significance was considered at p < 0.05.



	Two way-anova		
	FI645	*FI658*	*FI645*FI658*
nagk	0.021	0.230	0.980
pgm3	0.039	0.229	0.669
ogt	0.194	0.978	0.224
amdhd2	0.629	0.259	0.566
gfat	0.221	0.238	0.790

Fig. 3. Gene expression of *N*-acetylglucosamine kinase (*nagk*), GlcNac phosphomutase (*pgm3*), O-GlcNac transferase (*ogt*), *N*-acetyl-D-glucosamine-6-phosphate deacetylase (*amdhd2*), and glutamine:fructose-6-phosphate amidotransferase (*gfat*) in fish fed the experimental diets. Values are expressed relative to the control group. Data are presented as mean ($n = 8$). The error bars represent the standard deviation.

kinase (NAGK), which converts GlcNAc into *N*-acetylglucosamine-6-phosphate (GlcNAc-6P) (Fig. 5) (Chang et al., 2020; Marshall et al., 2004). Accordingly, in this study, dietary supplementation with FI645 significantly increased *nagk* expression concomitantly with increased chitin digestibility. Given that increased chitin hydrolysis potentially leads to the increased free GlcNAc levels, these results hint for a potential modulation of this gene in a GlcNAc-dependent manner, as previously reported in human cell lines Campbell et al. (2021). In fact, a similar hypothesis was also raised by Chien et al. (2020) which, when supplementing *Bacillus subtilis* E20 into *Litopenaeus vannamei* diets, verified a HBP enzyme expression modulation via glutamine metabolism.

In the cells, GlcNAc-6P can follow two pathways (Fig. 5). It may be converted into fructose-6-phosphate (F-6P) and enter the glycolysis pathway, through a route involving the deacetylation of GlcNAc-6P into GlcN-6P by the enzyme *N*-acetylglucosamine 6-phosphate deacetylase (AMDHD2) (Kroef et al., 2022; Świątek et al., 2012). Alternatively, GlcNAc-6P can be isomerized into *N*-acetylglucosamine-1-phosphate (GlcNAc-1P) by the action of GlcNAc phosphomutase (PGM3), ultimately leading to the formation of uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc), a substrate of O-GlcNAc transferase (OGT). Ultimately, this leads to protein O-GlcNAcylation, a dynamic post-translational modification that impacts several important physiologic processes (Chang et al., 2020; Groves et al., 2013; Hardivillé & Gerald, 2014). In the present study, while *amdhd2* expression was unaffected by dietary probiotic supplementation, *pgm3* expression was significantly induced by FI645 presence. Additionally, the increased expression is concomitant with the increased chitin digestibility and *nagk* expression, thus suggesting that GlcNAc might be channeled to UDP-GlcNAc production, although it was not possible to detect an increase of *ogt* expression.

Plasmatic GlcNAc was also significantly affected by FI645 in D645 diet. Independently of the absorption route, these results suggest that free GlcNAc resulting from chitin digestion can potentially travel through the blood stream to the liver and/or brain, impacting key processes such as nutrient sensing, animal behavior, energy homeostasis,

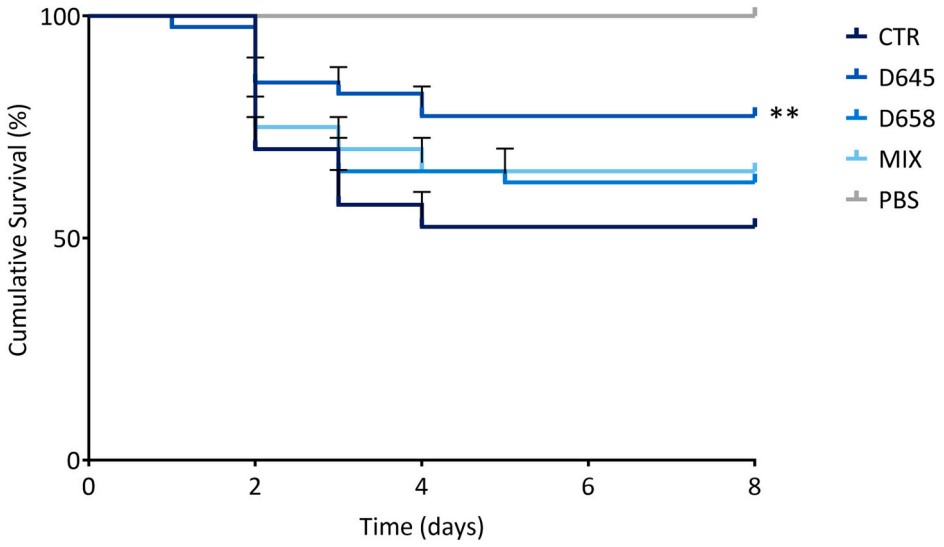


Fig. 4. Cumulative survival of European sea bass ($n = 40$) fed the experimental diets when challenged with *Vibrio anguillarum*. Fish treated with PBS was used as a negative control (PBS). *p < 0.05; **p < 0.01; ***$p < 0.001$.

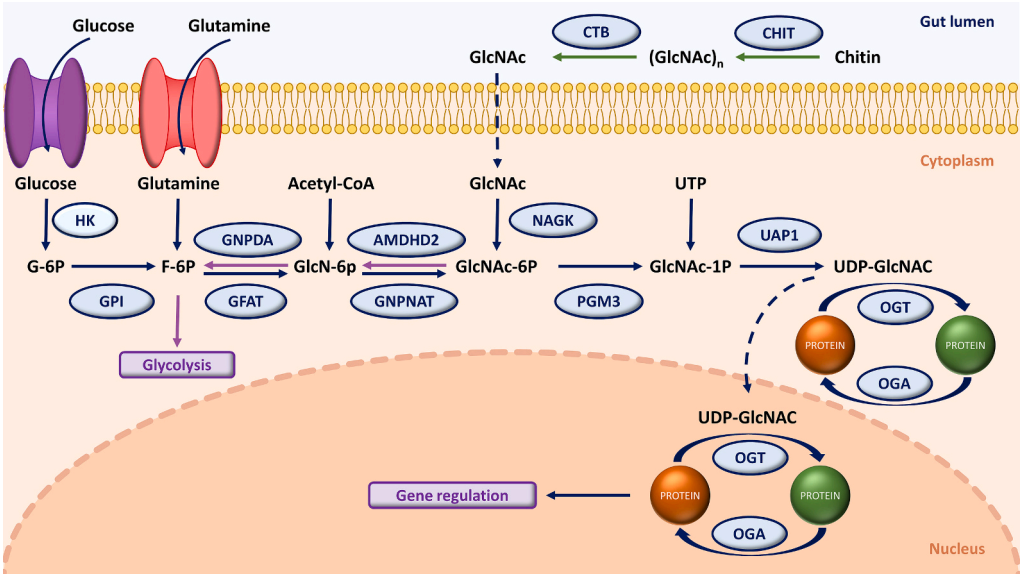


Fig. 5. Overview of chitin digestion and potential pathways followed by N-Acetylglucosamine (GlcNAc) upon entering the cells. Green arrows represent the steps that comprise chitin digestion into GlcNAc. Purple arrows represent the potential pathway followed by GlcNAc assimilation into the glycolysis pathway. Dark blue arrows represent the Hexosamine Biosynthetic Pathway (HBP) and O-GlcNAcylation modifications occurring on the cell cytoplasm and/or nucleus. Dashed arrows represent unexplored uptake mechanisms. Blue ovals represent enzymes involved in the HBP and O-GlcNAcylation processes. Green spheres represent O-glycosylated proteins whereas orange spheres represent non-O-glycosylated proteins. GlcNAc - *N*-acetylglucosamine; G-6P – glucose-6-phosphate; F-6P – fructose-6-phosphate; GlcN-6P – glucosamine-6-phosphate; GlcNAc-6P – *N*-acetylglucosamine-6-phosphate; GlcNAc-1P – *N*-acetylglucosamine-1-phosphate; UTP – Uridine-5′-triphosphate; UDP-GlcNAc - uridine diphosphate *N*-acetylglucosamine; CHIT - chitinase (EC: 3.2.1.14); CTB - chitobiase (EC: 3.2.1.52); HK - hexokinase (EC: 2.7.1.1); GPI - glucose-6-phosphate isomerase (EC: 5.3.1.9); GFAT - glutamine:fructose-6-phosphate amidotransferase (EC: 2.6.1.16); GNPNAT - glucosamine-phosphate *N*-acetyltransferase (EC: 2.3.1.4); PGM3 - GlcNAc phosphomutase (EC:5.4.2.3); UAP1 - UDP-*N*-acetylglucosamine (EC:2.7.7.23); OGT - O-GlcNAc transferase (EC:2.4.1.255); OGA -; NAGK - *N*-acetylglucosamine kinase (EC:2.7.1.59); AMDHD2 - *N*-acetylglucosamine 6-phosphate deacetylase (EC:3.5.1.25); GNPDA - glucosamine-6-phosphate deaminase (EC:3.5.99.6). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cell growth and proliferation, and appetite regulation (Dai et al., 2018; Hardivillé & Gerald, 2014; Hwang and Rhim, 2018; Issad et al., 2022; Lau et al., 2007). Moreover, metabolites of chitin degradation, namely COS or GlcNAc, also have a broad array of biological activities, such as antioxidant, antimicrobial, anti-inflammatory, and immunostimulatory (Kumar et al., 2020; Liaqat and Eltem, 2018), thus potentially contributing to improve disease resistance (Mohan et al., 2019).

In fish, dietary supplementation with COS or GlcNAc has been demonstrated to be an efficient strategy to enhance fish survival against several bacterial pathogens (Cheng et al., 2014; Lin et al., 2017; Lin et al., 2012). Accordingly, in this study, diet D645 increased the survival of ESB when challenged with *V. anguillarum*, one of the most prevalent pathogens in ESB aquaculture (Muniesa et al., 2020). This may be related to the increased GlcNAc available in these fish, which could be entering the HBP and increasing protein O-GlcNAcylation, as indicated by the gene expression data. Indeed, in mammalian cells, it was shown

that O-GlcNAcylation modulates oxidative stress (Groves et al., 2013; Issad et al., 2022) and both the innate and adaptative immune system (Božič et al., 2018; de Jesus et al., 2018; Kneass and Marchase, 2004; Lee et al., 2020; Newsholme and Parry-Billings, 1990; Wu et al., 2017). All these processes have been the target of immunostimulant strategies for aquaculture (Huynh et al., 2017; Wang et al., 2017), as several homologous mechanisms are present in fish (Bjørgen and Koppang, 2022), namely in ESB (Chistiakov et al., 2007; Picchietti et al., 2021). Further studies are however necessary to unravel the relevance of these processes on the fish's immune system.

Probiotic-mediated effects, however, cannot be discarded. Increased ESB survival with diet D645 could also be the result of other probiotic mechanisms of action. In fact, *Bacillus* spp. are able to increase fish survivability to *Vibrio* spp. (Gao et al., 2017b; Kuebutornye et al., 2020; Monzón-Atienza et al., 2022; Santos et al., 2021) due to its well documented antibiofilm, anti-adhesive and antimicrobial activities (Giri et al., 2019; Nithya and Pandian, 2010; Santos et al., 2021). However, while these claims are also documented for *B. licheniformis* (Hamza et al., 2016; Vinoj et al., 2014), the closest known species of FI645, in a preliminary colony overlay diffusion assay, FI645 and FI658 showed no anti-growth effect against *V. anguillarum* (data not shown). This indicates that, in this case, any *Bacillus* spp. induced protection mechanisms against pathogens is potentially reliant on anti-quorum sensing mechanisms (Santos et al., 2021; Vinoj et al., 2014), modulation of immune gene expression (Meloni et al., 2015; Monzón-Atienza et al., 2022), or through the enhancement of innate immune parameters (Akhter et al., 2015; Liu et al., 2012; Liu et al., 2017; Midhun et al., 2019; Monzón-Atienza et al., 2022). In this study, dietary supplementation of FI645 increased plasma total immunoglobulins levels. Moreover, despite no statistical difference verified, when compared to the control group, probiotic inclusion increased plasma peroxidase activity by 67.6%, 61.8%, and 50% for D645, D658 and MIX diets, respectively. Regardless of the mechanisms, present results showed that dietary supplementation with 2×10^9 CFU kg^{-1} of FI645 in IM containing diets appears to be an effective response against *V. anguillarum* infections in ESB. Thus, FI645 seemed to have potential to mitigate the economic losses caused by *V. anguillarum* in ESB (Muniesa et al., 2020), serving as an alternative to reduce the excessive antibiotics usage in aquaculture (Cabello, 2006; Dawood et al., 2018; Preena et al., 2020).

5. Conclusion

In this study, spores' viability was maintained for at least 180 days at room temperature, confirming their resilience during long storage stages. Overall, this study provides evidence of the potential of a chitinolytic probiotic bacteria (FI645) to promote chitin, protein, and energy digestibility in ESB, thus contributing to increasing feed efficiency and protein efficiency ratio in IM-based diets. Further, dietary supplementation with FI645 probiotic also significantly increased ESB survival when challenged with *V. anguillarum*, ascertaining its potential as an effective prophylactic agent for ESB protection against this pathogen. This study also provides, for the first time in fish, hints at the potential routes for GlcNAc metabolism, and how it contributes to increase disease resistance.

Ethics approval and consent to participate

Animal trials were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) and performed in a registered installation (N16091.UDER). The growth, digestibility and bacterial challenge trials were overseen by accredited scientists (following Federation of European Laboratory Animal Science Associations (FELASA) category C recommendations) and performed in accordance with the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes, as established by the European Parliament and the European Union Council.

Consent for publication

Not applicable.

Authors contributions

F.R., C.R.S., P.E., L.G, F.G., and A.O.T. designed and conceived the study. F.R., R.C., R.A.S., M.M., D.F.M., and P.E. performed the experiments. L.G. and F.G. bought the *Hermetia illucens* meal from MUTATEC. P.P.F. provided the European sea bass. F.R., C.R.S., and P.E. wrote the manuscript with contributions from all authors. All authors have read and agreed to the published version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the results of this study is available from the corresponding author upon reasonable request.

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