

Novel probiotics isolated from European sea bass (*Dicentrarchus labrax*) gut microbiota for improving insect meal utilization and gut health

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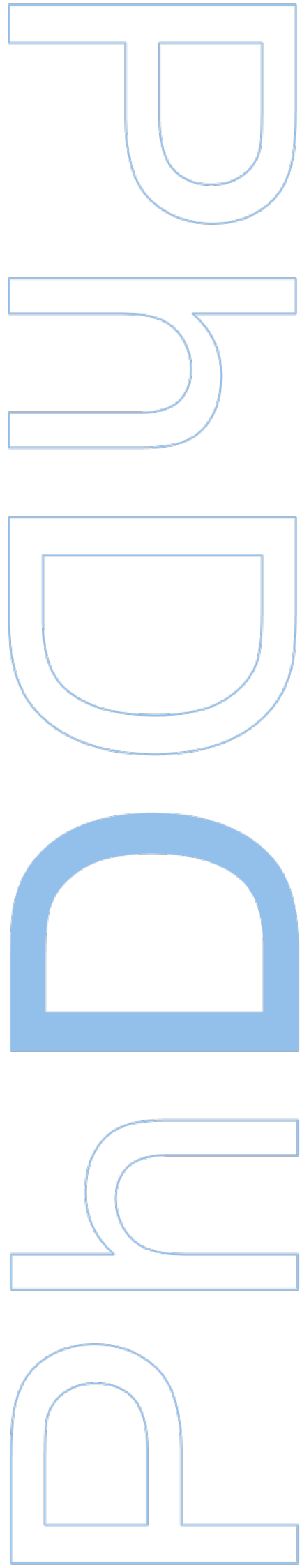
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À minha família,

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Porto, 18 de Janeiro de 2023

Previous Note

Thesis presented to the Faculty of Sciences of the University of Porto to obtain the Doctor Degree within the scope of the doctoral program in Biology

Supervisor – Dr. Paula Enes

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This thesis was done according to paragraph 2 of Article 4 of the General Regulation of Third Cycle of Studies, University of Porto and Article 31 of Decree 74/2006, of March 24, with new wording introduced by Decree 230/2009 of September 14, the total utilization of a coherent set of research papers already published or submitted for publication in international journals indexed and peer review, which comprises some of the chapters of this thesis. Given that such work was done in collaboration with other authors, the candidate clarifies that all of them actively participated in its design, obtention of results, data analysis, discussion, as well as in preparing its published form.

The present study was carried out in CIIMAR (Interdisciplinary Centre for Marine and Environmental Research) in Porto (Portugal) and Faculty of Sciences (Porto University), specifically in Fish Nutrition and Immunobiology Group (NUTRIMU).

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Sumário

Impulsionada pela crescente exigência de produtos de aquacultura e pelo aumento da dependência da mesma por rações animais, nos últimos anos, várias fontes alternativas de proteína têm sido estudadas exaustivamente. Entre as matérias-primas disponíveis está a farinha de inseto (IM) que, devido ao alto valor nutricional e baixa pegada ecológica, posiciona-se como uma forte alternativa para incorporação em rações para peixes, algo que tem acontecido gradualmente nos últimos anos. No entanto, a inclusão de IM nas rações para peixes (>15%) geralmente resulta em diminuição da digestibilidade da ração. Este resultado advém maioritariamente da incapacidade apresentada pelos peixes carnívoros em digerir a quitina que, por sua vez, aprisiona proteínas e lípidos com os quais estava associada no exosqueleto do inseto, levando assim a uma menor digestibilidade de proteínas, lípidos e energia.

Com o objetivo de aumentar a digestibilidade da IM em peixes, o presente trabalho procurou obter um probiótico ou uma mistura de probióticos com alta capacidade de degradação de quitina que, futuramente, possa ser incorporada em rações que contêm IM, no sentido de aumentar a digestibilidade de quitina. Para tal, com base na adaptabilidade das comunidades microbianas do intestino do peixe à sua dieta, rações contendo quitina foram usadas para alimentar o robalo europeu (ESB), visando assim aumentar o número de bactérias capazes de metabolizar quitina. Cinco dietas isoprotéicas (45%) e isolipídicas (18%) foram formuladas: uma dieta controlo (CTR); uma dieta baseada na CTR com 5% de suplementação de quitina (CHIT5); e 3 outras dietas com 25% de inclusão de farinha de larvas de *Hermetia illucens* (HI) (HM25), farinha de larvas de *Tenebrio molitor* (TM25) ou farinha de exúvia de HI (HEM25). ESB com um peso corporal inicial de 53,7 g foram distribuídos em tanques de 100L num sistema de recirculação aquático (RAS), termorregulado a 22,7 °C e o ensaio decorreu durante 8 semanas. No final do ensaio, para perceber as modulações induzidas pela dieta da microbiota intestinal do ESB, as comunidades autóctones (associadas à mucosa) e alóctones (associadas à digesta), foram analisadas por sequenciação na plataforma Illumina Miseq platform (região V4 do 16S rRNA).

As comunidades intestinais do ESB apresentaram-se representadas maioritariamente por organismos do filo Proteobacteria, seguido dos filos Firmicutes, Actinobacteria e Bacteroidota. Uma análise da diversidade beta demonstrou que local de amostragem teve um efeito mais forte na composição microbiana que o efeito da composição da dieta. Enquanto os microrganismos associados à mucosa foram mais resistentes a mudanças induzidas pela

dieta, as comunidades associadas à digesta foram mais diversas e significativamente alteradas em peixes alimentados com dietas HM25 e HEM25. No geral, ESB alimentados com dietas HM25 ou HEM25 demonstraram um aumento em várias famílias pertencentes aos filos Firmicutes e Actinobacteria, capazes de degradar (*Bacillaceae*) e/ou fermentar (*Lachnospiraceae* e *Bacillaceae*) a quitina, com a última dieta a apresentar uma influência mais forte. Finalmente, enquanto o potencial genómico para a atividade quitinolítica é reconhecido para bactérias dos filos Firmicutes e Actinobacteria, uma abordagem de PCR quantitativa visando o gene conservado *chia* (marcador comumente usado para a determinação do potencial quitinolítico em bactérias), mostrou que apenas a dieta HEM25 promoveu uma mudança para taxa com a capacidade de produzir quitinase, estando principalmente relacionado com um aumento da família Paenibacillaceae.

Sendo ambas *Bacillaceae* e *Paenibacillaceae* famílias compostas por organismos capazes de produzir esporos, estruturas altamente resistentes a injúrias externas com grande potencial de aplicação em aquacultura, foram utilizadas técnicas clássicas de isolamento para este tipo de bactérias nas digestas dos ESB utilizadas para sequenciação. Com uma seleção feita com base na dieta de origem e morfologia colonial, foram obtidos 366 isolados (FI). De seguida os FI foram exaustivamente avaliados *in vitro* tendo em conta potenciais características probióticas, como: a sua capacidade de esporular; a sua atividade quitinolítica; características relativas a questões de biossegurança (como por exemplo a sua capacidade hemolítica e a sua resistência a antibióticos); e a sua aptidão para sobreviver ao trato gastrointestinal. Desta avaliação foram selecionados os 4 FI com maior potencial. Por fim, após uma avaliação da sua atividade quitinolítica em condições semelhantes às encontradas no trato gastrointestinal do ESB, 2 FI, nomeadamente o FI645 e o FI658, espécies próximas filogeneticamente de *Bacillus licheniformis*, apresentaram a melhor performance.

Os melhores candidatos a probióticos foram posteriormente avaliados *in vivo* em relação à sua capacidade de manter a viabilidade durante o armazenamento e a sua capacidade de melhorar o desempenho zootécnico e a digestibilidade da quitina por parte do ESB. Para isso, os FI645 e FI658 foram incorporados (individualmente ou como uma mistura) em dietas ESB com altos níveis de farinha de larva de HI desengordurada (30%). Quatro dietas isoprotéicas (44%) e isolipídicas (17%) foram formuladas: foram formuladas: uma dieta controlo contendo 30% (CTR) de farinha desengordurada de larva de HI (CTR); duas dietas semelhantes à CTR, suplementadas com 2×10^9 CFU kg^{-1} de FI645 (D645) ou FI558 (D658); uma dieta semelhante à CTR suplementada com uma mistura (1:1) de 1×10^9 CFU kg^{-1} (cada) de FI645 e FI658 (DMIX). Peixes com peso inicial de 24,6 g foram distribuídos em tanques de 100L num sistema

RAS, termorregulado a 23 °C e o ensaio teve a duração de 13 semanas. Adicionalmente, para testar a capacidade dos FI de aumentar a resistência a doenças do ESB, os peixes foram alimentados com as mesmas formulações por um período de 4 semanas e submetido a um desafio bacteriano com *V. anguillarum* DSMZ 21597 por injeção intraperitoneal a uma concentração de $1,5 \times 10^7$ CFU mL⁻¹ (concentração previamente estabelecida como LD50).

Os FI645 e FI658 mantiveram a viabilidade por pelo menos 180 dias durante o armazenamento das dietas à temperatura ambiente. ESB alimentados com as dietas contendo FI645 aumentaram a sua atividade quitinolítica total no intestino, tendo este resultado numa maior digestibilidade da quitina. Consequentemente, a digestibilidade da matéria seca, proteína e energia também aumentou, levando a uma maior eficiência alimentar e maior índice de eficiência proteica. O aumento da digestibilidade da quitina também resultou no aumento da N-acetilglucosamina plasmática (GlcNAc), sendo este trabalho a primeira indicação da modulação mediada pela dieta das concentrações plasmáticas de GlcNAc. ESB alimentados com as dietas suplementadas com FI645 também mostraram um aumento nas expressões no intestino distal dos genes que codificam para N-acetilglucosamina quinase (*nagk*) e GlcNAc fosfomutase (*pgm3*), enzimas chave da via biossintética da hexosamina (HBP). Finalmente, quando desafiados com *V. anguillarum*, ESB alimentados com dietas suplementadas com FI645 tiveram um aumento de sobrevivência de 52,5% para 77,5%, quando comparado com a dieta CTR.

Em conjunto, estes resultados apresentados nesta tese de doutoramento estabelecem o potencial FI645 como uma solução multifuncional capaz de aumentar a digestão da quitina e reduzir a mortalidade por ESB quando desafiado com uma infeção por *V. anguillarum*. Esta estratégia abre novas possibilidades para a formulação de dietas contendo IM, permitindo assim a inclusão de níveis mais altos de quitina em rações aquáticas, podendo também ser utilizado como uma ferramenta para reduzir o uso de antibióticos na aquacultura.

Palavras-chave: Farinha de inseto, *Hermetia illucens*, *Hermetia illucens* exuvia, *Tenebrio molitor*, *Bacillus*, Probióticos, Quitina, Quitinases, Robalo, Microbiota, Microrganismos esporulantes

Abstract

Driven by the incremental demand for aquaculture products and the increased reliance on aquafeeds for fish production, alternative protein sources have been exhaustively explored over the last years. Among these, insect meal (IM), due to its high nutritional value and low environmental footprint, positions itself as a strong candidate for incorporation into fish feeds, being, in the last years, gradually introduced in fish dietary regimes. However, IM inclusion on fish feeds (>15%) often results in decreased feed efficiency and lower growth performance. This is mainly the result of fish's inability to digest chitin which, in turn, by entrapping proteins and lipids within the insect exoskeleton, leads to lower protein, lipid, and energy digestibility.

With the final goal of increasing IM digestibility in fish, the present work aimed to obtain a probiotic strain or a probiotic mixture with a high chitin-degrading ability which, in the future, could be incorporated in IM-containing aquafeeds to increase chitin digestibility. To do so, based on the adaptability of fish gut microbial communities to their dietary regime, chitin-containing diets were used to feed European seabass (ESB) to promote gut microbial shifts towards chitin-metabolizing bacteria. Five isoproteic (45%) and isolipidic (18%) diets were formulated: a control diet (CTR); a CTR-like diet with 5% chitin supplementation (CHIT5); and 3 other diets with 25% inclusion of either *Hermetia illucens* (HI) larvae meal (HM25), *Tenebrio molitor* larvae meal (TM25) or HI exuviae meal (HEM25). Fish with an initial body weight of 53.7 g were distributed in 100L tanks in a recirculating aquatic system (RAS), thermoregulated to 22.7 °C and the trial lasted for 8 weeks. At the end of the trial, to understand the diet-induced modulation of the gut microbiota triggered by dietary IM and chitin, the allochthonous (digesta) and autochthonous (mucosa) microbial communities were assessed by sequencing with the Illumina Miseq platform (V4 region of the 16S rRNA).

The ESB gut microbiota was dominated by the Proteobacteria phylum, followed by Firmicutes, Actinobacteria, and Bacteroidota. A beta-diversity analysis of the digesta-associated and mucosa-associated communities showed that the sampling site had a stronger effect on microbial composition when compared to diet composition. While the mucosa-associated microorganisms were more resilient to dietary changes, digesta-associated communities were more diverse and significantly altered in fish fed HM25 and HEM25 diets. Overall, ESB fed either HM25 or HEM25 diets showed an increase in several families belonging to the Firmicutes and Actinobacteria phyla, either able to degrade (e.g., *Bacillaceae*) and/or ferment chitin (e.g., *Lachnospiraceae* and *Bacillaceae*), with the latter diet presenting a stronger influence. Finally, while the genomic potential for chitinolytic activity is recognized for bacteria

in both Firmicutes and Actinobacteria, a quantitative PCR approach targeting the conserved *chiA* gene (commonly used marker for the determination of chitinolytic potential in bacteria), showed that only HEM25 diet promoted a shift towards chitinase-encoding taxa, primarily related to the increase of the *Paenibacillaceae* family.

Being both *Bacillaceae* and *Paenibacillaceae* families composed of microorganisms able to form spores, highly resilient structures that have great potential for probiotic applications in aquaculture, a classic bacterial isolation approach targeting aerobic bacterial sporeformers was done from the same fish gut samples. A total of 366 sporulating FI were obtained based on their origin (diet) and colony morphology. The FI obtained were then comprehensively screened *in vitro*, concerning their putative probiotic traits, namely: sporeforming ability; chitinolytic activity; biosafety characteristics (e.g., antibiotic resistance; hemolytic activity); and gut-survival aptitude, resulting in the selection of 4 FI with higher *in vitro* chitinolytic activity. The 4 FI were further assessed regarding their total chitinolytic activity in gut-like conditions, with 2 FI, namely FI645 and FI658, closely related to *Bacillus licheniformis*, presenting the best performance.

The best probiotic candidates were thereafter gauged regarding their ability to maintain viability through storage as well as their ability to enhance ESB zootechnic performance and chitin digestibility. For that, FI645 and FI658 were integrated (individually or as a mixture) in ESB diets with high defatted *Hermetia illucens* larvae meal (HM) levels. Four isoproteic (45%) and isolipidic (18%) diets were formulated: a control diet, containing 30% of HM (CTR); two diets like the CTR containing 2×10^9 colony forming units (CFU) kg^{-1} of either FI645 (D645) or FI558 (D658); a diet like the CTR containing 1×10^9 CFU kg^{-1} of each FI645 and FI658 (DMIX). Fish with an initial body weight of 24.6 g were distributed in 100L tanks in a RAS, thermoregulated to 23 °C and the trial lasted 13 weeks. Additionally, to test the FI ability to enhance ESB disease resistance, fish were fed these dietary formulations for 4 weeks and submitted to a bacterial challenge with the pathogenic *V. anguillarum* DSMZ 21597, intraperitoneally injected at 1.5×10^7 CFU mL^{-1} (a concentration previously established as LD50).

FI645 and FI658 maintained viability for at least 180 days when diets were stored at room temperature. ESB fed with the diets containing FI645 increased intestinal total chitinolytic activity, resulting in significantly higher chitin digestibility. Accordingly, dry matter, protein, and energy digestibility also increased, leading to higher feed efficiency and protein efficiency ratio. Increased chitin digestibility also resulted in increased plasma N-acetylglucosamine (GlcNAc), with this work being the first indication of diet-mediated modulation of GlcNAc plasma

concentrations. ESB fed with the diets supplemented with FI645 also showed an increase in the distal intestine expressions of the genes coding for N-acetylglucosamine kinase (*nagk*) and GlcNAc phosphomutase (*pgm3*), which are key enzymes of the GlcNAc salvage pathway and the hexosamine biosynthetic pathway (HBP). Whether this is a result of a probiotic-induced signal or of the rapid cell influx of GlcNAc, remains to be elucidated. Nonetheless, given that increased expression in these genes aligns with increased chitin digestibility, these results hint at a potential GlcNAc integration in the HBP. Finally, when challenged with *V. anguillarum*, ESB fed the diets supplemented FI645 increased survival from 52.5% to 77.5%, when compared to the CTR diet.

Together, the results presented in this Ph.D. Thesis highlighted the sporeforming fish isolate FI645, a *B. licheniformis*, as a potential multifunctional solution able to increase chitin digestion and reduce ESB mortality when challenged with a *V. anguillarum* infection. This strategy opens novel possibilities for using IM-containing diets, allowing for an efficient use of diets with higher dietary chitin levels, and reducing the use of antibiotics in aquaculture.

Keywords: Insect meal, *Hermetia illucens*, *Hermetia illucens* exuviae, *Tenebrio molitor*, *Bacillus*, Sporeformers, Probiotic, Chitin, Chitinase, European sea bass, Microbiota

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Abbreviation List

AA – Amino acids
ADC – Apparent digestibility coefficient
AMP - Antimicrobial peptides
APr - Anti-protease
APh - Alkaline phosphatase
BA - Bactericidal activity
CO - Ceruloplasmin
COS – Chitooligosaccharides
CRP - C-reactive protein
DHA - Docosahexaenoic acid
DM – Dry matter
DNA - Deoxyribonucleic acid
EAA - Essential amino acids
EPA - Eicosapentaenoic acid
EFA – Essential fatty acids
EFSA - European Food Safety Authority
ESB - European sea bass
EU – European Union
EUMOFA - European Market Observatory for Fisheries and Aquaculture Products
FA - Fatty acids
FAO - Food and Agriculture Organization of the United Nations
FBW - Fish body weight
FCR - Feed conversion ratios
FE - Feed efficiency
FM - Fish meal
FO - Fish oil
GALT - Gut-associated lymphoid tissue
GlcN - D-glucosamine
GlcNAc - N-acetylglucosamine
Hb - Hemoglobin concentration
HCT - Hematocrit count
HI – *Hermetia illucens*

HSP70 - Heat-shock protein 70

IFN- γ - Gamma interferon

Ig - Immunoglobulin

IL-1 β -Interleukin-1 β

IL-8 - Interleukin-8

IL-10 - Interleukin-10

IM - Insect meals

INE - Instituto Nacional de Estatística

IPIFF - International Platform of Insects for Food and Feed

IRF-1-Interferon regulatory factor 1

LAB – Lactic acid bacteria

LC-PUFA - Long-chain polyunsaturated fatty acids

LER- Lipid efficiency ratio

LPMO - Lytic polysaccharide monooxygenase

LYZ - Lysozyme

MAMP - Microbe-associated molecular patterns

MCH - Mean corpuscular hemoglobin~

MCHC - Mean corpuscular hemoglobin concentration

MCV - Mean corpuscular volume

MPO - Myeloperoxidase

MYD88 - Myeloid differentiation primary response 88

NF- κ B-Nuclear factor- κ B

NF- κ Bem1a-Nuclear factor kappa-B kinase essential modifier

NO-Nitric oxide

NOS - National Ocean Strategy

NSP - Non-starch polysaccharides

OTU - Operational taxonomic unit

PA - Phagocytic activity

PER- Protein efficiency ratio

PF - Plant feedstuffs

PI - Phagocytic index

ProstD - Prostaglandin D

PRR - Pattern recognition receptors

QPS - Qualified presumption of safety

RB - Respiratory burst

RBCC - Red blood cell count

RNA - Ribonucleic Acid

SCFA - Short-chain fatty acids

SGR- Specific growth rate

TGFβ1-Transforming growth factor beta 1

TNFα - Tumor necrosis factor alpha

TM - *Tenebrio molitor*

TLR - Toll-like receptor

WBCC-White blood cell count

WG - Weight gain

WHO - World Health Organization

Chapter 1.

General introduction

1.1 Aquaculture in numbers

Nutritional balance is a foundational requirement for human population development (FAO et al., 2022). Healthier diets are often associated with lower rates of pathological conditions emergence (e.g., heart disease, diabetes, strokes, cancer) as well as higher cognitive development (WHO, 2020; Roberts et al., 2022). Among the elements of the human diet, fish is one of the most valuable, providing high-quality protein and lipids (Tacon and Metian, 2013). Numerous fish species are exceptionally rich in n-3 long-chain polyunsaturated fatty acids (LC-PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), a subset of lipids with demonstrated benefits on brain development (Innis, 2007), heart condition (Russo, 2009), and reproduction (Wathes et al., 2007). It is, therefore, no surprise that the increment populational growth is accompanied by an increase in fish consumption, as well as a rapid expansion of the aquaculture industry (FAO, 2022c).

From 1961 to nowadays, while the world's population grew at a 1.6% rate, consumption of aquatic animal foods per capita increased at a 3% rate, jumping from 9.9 Kg in 1961 to 20.2 Kg in 2020 (FAO, 2022c). Mirroring this growth, from 1990 to 2020, aquaculture grew at an annual rate of 6.7%, providing, in 2020, 49.6% of all consumed aquatic fisheries products, a significant increase from the 25.7% produced in 2000 (FAO, 2022c). While the relative aquaculture growth rate has been slowly decreasing (9.5% from 1990-2000 vs 3.3% from 2015-2020), the absolute growth has been maintained at relatively steady levels, with the decline of relative growth rate reflecting the higher total production (FAO, 2022c).

In the last two decades, fish production presented the highest annual growth rate among farmed aquaculture animals (9.9% vs 5.7% and 3.5% for mollusks and crustaceans, respectively), reaching a volume of 56 million tons in 2019 (46.6% of all fish produced) (Figure 1.1A) (Naylor et al., 2021; FAO, 2022b). Overall, farmed fish is the most valuable sector among the aquatic farmed animals, having generated over 130 thousand million euros in 2019, corresponding to approximately 56% of all animal aquaculture value (FAO, 2022b). Asia remains the major producer of aquaculture fish, followed by Europe, America, and Africa (FAO, 2022c). Asian production far surpasses the European one, with 49.08 million tons produced in 2019, generating 96.03 thousand million euros (87.1% and 78.5% of the total finfish production volume and value, respectively), while Europe only produced 2.59 million tons with a market value of 11.91 thousand million

euros (4.6% and 9.7% of the total finfish production volume and value, respectively) (Figure 1.1B) (FAO, 2022b).

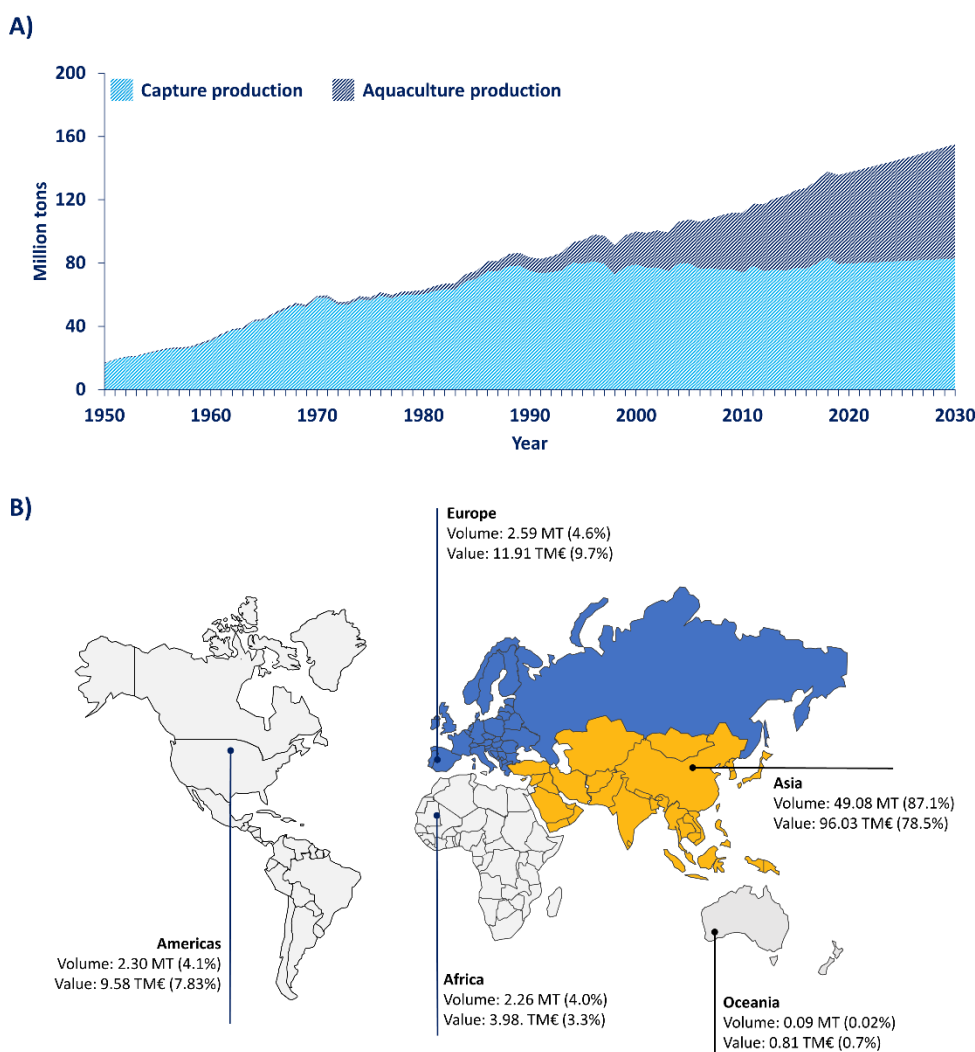


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In 2020, European Union aquaculture finfish produced 511 377 thousand tons, valued at 2 150 million euros (EUMOFA, 2022). Finfish production was mainly dominated by two marine species, gilthead seabream (*Sparus aurata*) (representing 18.2% and 21.1% of Europe's total finfish production volume and value, respectively), and European sea bass (ESB) (*Dicentrarchus labrax*) (15.8% volume, 21.9% value), and one freshwater species, rainbow trout (*Oncorhynchus mykiss*) (36.1% volume, 30.0% value) (Figure 1.2) (EUMOFA, 2022).

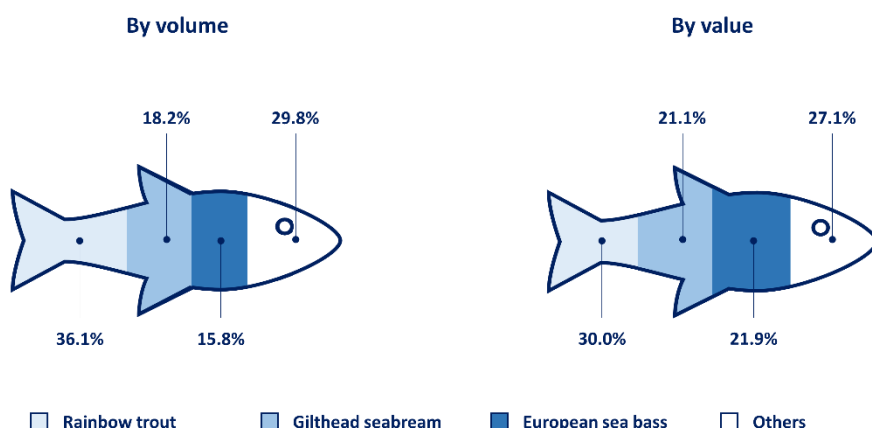


Figure 1.2 Relative production volume and value of four important fish species in the European Union aquaculture finfish production landscape. Data obtained in EUMOFA (2022)

1.1.1 The Portuguese aquaculture industry

Located in the western part of Europe, Portugal has an extensive coastline of 1 214 km distributed by its mainland and Azores and Madeira archipelagos, totaling 1.8% of the EU's coastline (EUMOFA, 2021). Portugal is the major fish consumer in the EU, having registered, in 2019, a total consumption of 59.91 kg per capita, with fish consumption having a large social and cultural relevance in certain traditional fishing towns (EUMOFA, 2021). Fish consumption habits, however, are not attended by Portuguese aquaculture, which, in 2020, only produced a total of 16 999 tons. This value represented less than 1% of the total EU-28 production (United Kingdom included), and less than 0.1% of the World's production (FAO, 2022c).

Nonetheless, from 2010-2020 the sector has registered a considerable evolution, doubling its production volume from 8 225 tons to 16 999 tons and increasing its sales value from 42.8 million euros to 100 million euros (INE, 2020). In 2020, the Portuguese aquaculture finfish production represented 42.2% (7 137 tons) of the total Portuguese aquaculture production, being mainly carried out in marine and brackish waters (87.3%, 6232 tons) under an extensive regime (60.8% extensive vs. 32.6% intensive, and 6.6% semi-intensive), and was mainly dominated by 3 species: turbot (*Psetta maxima*), with 3 407 tons produced (corresponding to 54.7% of the total Portuguese finfish production); gilthead seabream, with 1 768 tons produced (28.4%); and ESB, with 904 tons produced (14.5%) (INE, 2020).

The Portuguese aquaculture growth was mainly pushed by the implementation of a series of Portuguese official policies such as the National Ocean Strategy (NOS)

(DGRM, 2014), or the Strategic Plan for Portuguese Aquaculture 2014-2020 (DGPM, 2013). These policies were aligned with both the European Commission Strategic Guidelines (European Commission, 2013), and the reform of the Common Fisheries Policy (EU Regulation No. 1380/2013), establishing a framework to potentiate the blue economy and, with it, aquaculture (Salas-Leiton et al., 2021). The Portuguese aquaculture industry development will continue to be considered a priority within the blue economy framework, as stated by the new NOS for 2021-2023 (DGPM, 2021).

1.1.2 Aquaculture challenges: aquafeed issue

Nowadays, it is well established that aquaculture is an indispensable animal production sector for a social, economic, and environmentally sustainable future, being frequently addressed as a solution to mitigate the environmental impacts of capture fisheries and the global food needs (Duarte et al., 2009; FAO, 2022c). However, despite aquaculture's continuous growth and the restrictions imposed on fisheries, the biological sustainability of fish stocks continues to drop, with 35.5% of species still being overfished in 2019 (Naylor et al., 2000; FAO, 2022c). Moreover, the rapid and sometimes unchecked aquaculture growth led to the emergence of several fish production, environmental, and public-health challenges (Naylor et al., 2000; Franks et al., 2021; FAO, 2022c).

Forced to tackle these obstacles, the aquaculture industry experienced several technological leaps over the last years: fed aquaculture outpaced non-fed aquaculture, accounting for 69.5% (57 million tons) of all aquaculture in 2018, increasing both nutritional control and animal growth rates (FAO, 2020); application of intelligent engineering-based monitorization and control allowed the development of novel concepts such as Precision Fish Farming (Føre et al., 2018; Antonucci and Costa, 2020; Wang et al., 2021a); increased accessibility to multi-omics tools, namely metabolomics (Alfaro and Young, 2018), transcriptomics (Chandhini and Rejish Kumar, 2019), proteomics (Rodrigues et al., 2012), and metagenomics (Yukgehnaish et al., 2020) allowed for the development of customized nutritional and immunological strategies spurring fields like nutrigenomics (Leaver et al., 2008; Martin and Król, 2017), trained immunity (Zhang et al., 2019), and precision nutritional regulation (Zhang et al., 2020). However, despite the application of novel and innovative approaches, several challenges remain unsolved in the aquaculture industry.

Fish feeds, for example, remain a major bottleneck for sustainable aquaculture growth (Kok et al., 2020). Consistently increasing at an annual growth rate of 8% between 2000

to 2017, fish feeds correspond to 40-70% of the costs of marine fish aquaculture production, greatly due to the dietary inclusion of fish meal (FM) and fish oil (FO) (Boyd et al., 2020). While FM is mainly looked at as a highly valuable protein source with a well-balanced essential amino acids (EAA) content, FO is regarded as the major source of LC-PUFAs (e.g., EPA and DHA), indispensable dietary components in carnivorous fish feeds (Rosenlund et al., 2016). Being highly palatable, and highly digestible raw materials with a well-balanced amino acid profile, and no antinutritional factors, FM and FO saw their usage in fish feeds increasing concomitantly with aquaculture growth (Mitra, 2021).

FM and FO production peak was reached in 1994-1995 (Mitra, 2021), and excessive reliance on these commodities greatly contributed to the depletion of fish wild stocks (FAO, 2020; Kok et al., 2020). In an attempt to protect ecosystems, several restrictions on fisheries were put in place, limiting the availability of these commodities (Shepherd and Jackson, 2013). With increasing fish feed demand and insufficient FM and FO availability, in the last 25 years these ingredients saw an increasing trend in their prices, forcing aquaculture feeds to shift towards alternative ingredients (Kok et al., 2020; Mitra, 2021). Today, FM and FO are mainly looked upon as strategic ingredients, with FM being commonly used at low dietary inclusion levels (being on average for carnivorous species between 11%-23% in 2014 and expected to decrease to 6% by 2025), or at specific development stages (e.g., early life) (Shepherd and Jackson, 2013; Jannathulla et al., 2019). FO is, however, still considered a key ingredient to compensate for a dietary deficiency of LC-PUFAs resulting from the dietary inclusion of alternative ingredients (Rosenlund et al., 2016)

From a nutritional standpoint, feed formulation is the process of selecting a combination of ingredients that, collectively, can respond to the specific nutritional needs of the species to be cultured (Turchini et al., 2009). By understanding the fish dietary nutrient requirements (e.g., EAA, essential fatty acids (EFA), minerals, vitamins, etc.), researchers and industry were able to consistently reduce FM and FO dietary inclusion levels (Boyd et al., 2020). Over the years, marine raw materials have been gradually substituted by alternative ingredients such as plant feedstuffs (PF), vegetable oils, processed animal proteins, and, more recently, insect meal (IM) (Hua et al., 2019).

Incorporation of PF in fish feeds allowed for increasing aquafeed sustainability, as these ingredients usually have lower production costs and are more easily obtained (Boyd et al., 2020). Currently, soybean meal and plant-based protein concentrates, such as wheat

and corn gluten meals, are some of the main protein sources used in aquafeeds, while vegetable oils (e.g., canola, linseed, or soybean oils) are often used as FO substitutes (Yu et al., 2022). Although omnivorous and herbivorous species swiftly transitioned to PF and vegetable oils-based meals, incorporation of these commodities in carnivorous fish feeds often comes with several challenges associated (Turchini et al., 2009). This mostly derives from the PFs composition that, in several cases are deficient in certain EAA (Gatlin et al., 2007), have an unfavorable fatty acids profile (e.g., deficient composition in EFA) (Sales and Glencross, 2011), possess high levels of indigestible fibers, usually linked to lower nutrient digestibility and bioavailability (Ghosh and Ray, 2017), reduced palatability (Kokou and Fountoulaki, 2018), and are rich in antinutritional factors (Ghosh and Ray, 2017). On the other hand, vegetable oils are devoid of LC-PUFAs (e.g., EPA and DHA), which are required by marine fish, leading to decreases in these fatty acids in the farmed fish's final product (Sprague et al., 2017). Adding to the animal's performance and health issues posed by PF and vegetable oils, aquafeeds increased dependency on these products also increases aquaculture carbon footprint, as well as deforestation and arable land use, demanding, once again, alternative solutions (Woodgate et al., 2022).

Animal by-products, such as processed animal proteins (e.g., meat, blood, or feather meals), present themselves as protein-rich alternatives with high digestibility and palatability (Mitra, 2021; Woodgate et al., 2022). While processed animal protein supplementation can lead to amino acid imbalances in dietary nutritional profiles (Fasakin et al., 2005), their combination with other protein sources (e.g., PF) can help to offset nutritional imbalances (Mitra, 2021). On the other hand, additional animal by-products, such as rendered animal fats are also a valid alternative for partial FO replacement (Monteiro et al., 2018; Campos et al., 2019). Depending on their availability and price/quality ratio, the inclusion of these ingredients in fish feeds can be a cost-effective alternative with the additional benefit of reducing animal production wastes (Mitra, 2021).

Alternatively, the substitution of marine raw materials and PF in fish feeds can also be achieved using, for example, microbiologically produced single-cell proteins (Jones et al., 2020), aerobic/anaerobic fermentation ingredients (Dawood and Koshio, 2020), and IM, with the latter being increasingly explored in the recent years (Hua et al., 2019; Gasco et al., 2023).

1.2 Insect meal as a novel aquafeed ingredient

Over the last 5 years, research on IM's potential as a fish feed ingredient has been steadily increasing, coinciding with the release of the EU Directive (Regulation No 2017/893) that authorized the use of processed animal protein from insects in aquafeeds after July 1st, 2017. This directive encompassed seven insect species: Black Soldier Fly (*Hermetia illucens*; HI), Common Housefly (*Musca domestica*; MD), Yellow Mealworm (*Tenebrio molitor*; TM), Lesser Mealworm (*Alphitobius diaperinus*), House Cricket (*Acheta domesticus*), Banded Cricket (*Gryllodes sigillatus*), and Field Cricket (*Gryllus assimilis*). As of November 2021, the EU also framed silkworm (*Bombyx mori*) among the available species to be used in aquafeeds (Regulation No 2021/1925). These authorizations fast-tracked IM market availability and competitiveness, fomenting a new and fast-developing industry with high potential (Makkar et al., 2014; Van Huis et al., 2015). According to the International Platform of Insects for Food and Feed (IPIFF), in the first quarter of 2021, the insect production sector had already captured over 1 billion euros in investment and is expected to reach over 3 billion euros by 2025, with production reaching over 1 million tons by 2030 (IPIFF, 2021). Moreover, while IM remains expensive when compared with other ingredients, feed producers are receptive to its inclusion in fish feeds, dependable on a more cost-effective price proportional to its protein content and functional aspects (Gasco et al., 2023).

From a production standpoint, insects are highly efficient water and organic matter converters. They can grow on a wide array of substrates with significantly better feed conversion ratios (FCR) than those of traditional livestock systems (Berggren et al., 2019), and in lower amounts of time (5-10 days MD, 6-30 weeks HI, and 40-90 weeks TM; Makkar et al., 2014). These characteristics greatly improve insects' potential, which becomes further appealing by their ability to be fed with otherwise unused resources, such as agriculture by-products or food wastes (Miech et al., 2016). Insect production also requires less space than plant crops, lessening unsustainable environmental pressure applied by the mass production of plant proteins (Fry et al., 2016). Additionally, as insects are not directly applied as human food in Europe (Henry et al., 2015; Van Huis et al., 2015), insect production can be directed to the animal feed sector, being estimated that in 2030 it will allocate 40% of its production to aquafeeds (IPIFF, 2021).

Nutritionally, IM characteristics can vary, depending on several factors such as the insect species, development stage, and processing method (Henry et al., 2015). Overall, IM crude protein levels vary between 30-83% on dry matter (DM), while lipid levels usually

fluctuate between 10-30% DM (Henry et al., 2015; Nogales-Mérida et al., 2018). IM presents well-balanced amino acid profiles, similar to FM (Tran et al., 2022a), being, for example, an important source of methionine (Li et al., 2009; Nogales-Mérida et al., 2018). Its lipid profile, however, is less favorable for marine fish production as it is mostly lacking several key fatty acids such as EPA and DHA (Barroso et al., 2014). Although insect EPA and DHA levels can be modulated/enriched by using different rearing substrates, such as fish offal, this is economically less viable, as these by-products can be directly fed to fish (Henry et al., 2015). While in specific situations (e.g., FM substitution), IM EPA and DHA deficiencies can be considered as a formulation challenge, these can be mitigated by strategically incorporating alternative sources of ingredients rich in these LC-PUFAs, such as microalgae oil and genetically modified organisms (Tocher et al., 2019; Santigosa et al., 2021). Additionally, IM can also be a rich source of several minerals and vitamins (Koutsos et al., 2019). Among the different authorized IM, TM, and HI have been given the most attention (and will, therefore, be the main focus of analysis in this work), with the latter being the most studied and reared worldwide (van Huis, 2020; Weththasinghe et al., 2021a; Tran et al., 2022a).

Although both HI and TM meals have relatively high protein contents (31-59% and 47-60% on a DM basis, respectively), HI meal's amino acid profile is more balanced and closer to FM's profile, while TM meal is deficient in several amino acids such as cysteine, histidine, lysine, methionine, and threonine (Nogales-Mérida et al., 2018; Shafique et al., 2021). Lipid content is highly variable in both IM, varying between 15-49% and 31-43%, for HI and TM meals, respectively (Makkar et al., 2014; Shafique et al., 2021). Regarding lipid composition, while both IM contain high levels of palmitic (C16:0) and oleic (C18:1n7) acids, TM meal is usually richer in linoleic acid (C18:2 n6) (Tzompa-Sosa et al., 2014; Belforti et al., 2015; Adámková et al., 2017), whereas HI meal is usually richer in lauric acid (12:0) (Borgogno et al., 2017; Renna et al., 2017; Belghit et al., 2019; Guerreiro et al., 2020). However, as previously mentioned, IM fatty acids profile can be modulated by rearing conditions, as the insects' fatty acids usually reflect feed composition, thus being amenable to adjustment according to fish needs (Barroso et al., 2014; Makkar et al., 2014; Henry et al., 2015; Nogales-Mérida et al., 2018). Moreover, depending on the desired application, IM lipid content can also be reduced by defatting processes (from full fat to partially defatted or fully defatted), thus increasing IM protein relative content (Bußler et al., 2016). However, IM production for aquafeeds does not have yet a standardized process and thus disparate results obtained with the same

insect species should account for possible variable nutritional profiles reflecting the insect origins (Gasco et al., 2019), or processing methods (Zulkifli et al., 2022).

IM use in fish diets also introduces chitin, a structural carbohydrate of the insect's exoskeleton (Younes and Rinaudo, 2015; Muthukrishnan et al., 2016). Carbohydrates are particularly interesting dietary components in fish nutrition with sugars and starches offering the cheapest source of energy for fish (Krogdahl et al., 2005; NRC, 2011; Kamalam et al., 2017; Zhang et al., 2020). However, non-starch polysaccharides (NSPs), are mentioned as one of the main constraints for a successful replacement of FM by PF or IM (Sinha et al., 2011; Henry et al., 2015). Indeed, in IM, chitin is frequently pointed out as an indigestible carbohydrate with potential negative effects on protein and lipid digestibility, especially at higher IM inclusion levels (>15%) (Shiau and Yu, 1999; Karlsen et al., 2017; Basto et al., 2020; Guerreiro et al., 2021; Weththasinghe et al., 2021a; Tran et al., 2022a).

NSPs, however, are not inherently harmful dietary components as they can exhibit immunostimulant, prebiotic, and growth-promoting characteristics, and can also contribute to mitigate fish stress and diseases (Sinha et al., 2011; Mohan et al., 2019; Ciji and Akhtar, 2021). Chitin and its derivatives (chitosan and chitooligosaccharides (COS), for example, are biocompatible, biodegradable, and bioabsorbable (Khoushab and Yamabhai, 2010), with antioxidant, antimicrobial, chelating, growth-stimulating, and immunostimulant abilities (Kumar, 2000; Khoushab and Yamabhai, 2010; Hamed et al., 2016; Kumar et al., 2020) thus, being able to add value to the diet.

To properly utilize IM in fish feeds, it is paramount to understand chitin's impacts on IM digestibility, and its ability to operate as a functional ingredient. As such, the following sub-chapters will provide a detailed description of chitin rearrangement in the insect's exoskeleton (sub-chapter 1.2.1), as well as a general overview of the effects of HI and TM meals (the most used and produced IM) on fish growth performance and diet digestibility (sub-chapter 1.2.2), gut morphology (sub-chapter 1.2.3), and immune status and disease resistance (sub-chapter 1.2.4). Additionally, after introducing the microbiota functions within the fish as well as its establishment and variations (sub-chapter 1.4), HI and TM ability to modulate fish gut microbiota will also be discussed (sub-chapter 1.4.2). Finally, as carnivorous fish species have been the main target for IM inclusion and are often reported to present limitations on IM digestibility (Nogales-Mérida et al., 2019; Hua, 2021), this work will discuss all the above-mentioned parameters on carnivorous species.

1.2.1 Insect's exoskeleton: chitin structure and function

Chitin is a homo- or heteropolymer composed of β (1 $\rightarrow$ 4)-linked N-acetylglucosamine (GlcNAc) and D-glucosamine (GlcN) monomers (Figure 1.3) (Rinaudo, 2006; Younes and Rinaudo, 2015). It is the second most abundant natural polymer after cellulose, differing from it only at the C2 position of each monomer, where the hydrogen group is replaced by an acetamide group ($-\text{NHCOCH}_3$) (Figure 1.3) (Rinaudo, 2006).

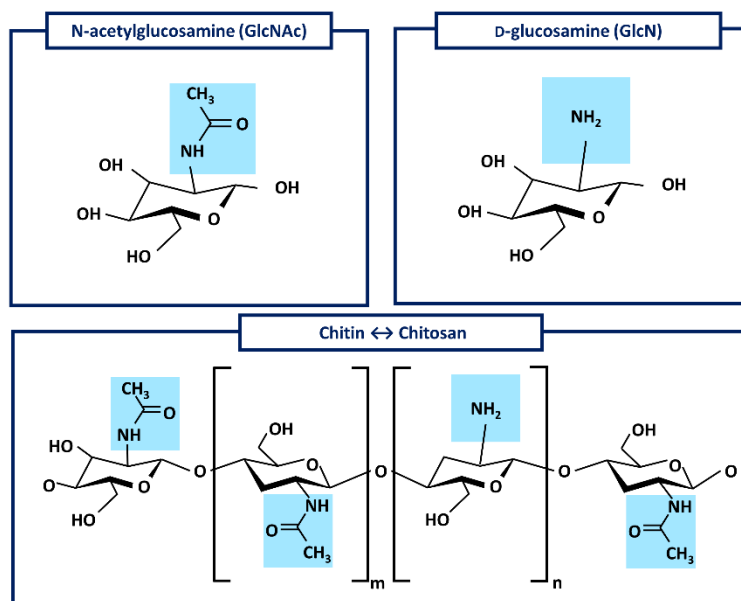


Figure 1.3 Schematic representation of N-acetylglucosamine (GlcNAc), D-glucosamine (GlcN), and a GlcNAc, GlcN heteropolymer. Variations on the degree of acetylation (R-group, represented by light blue squares) confer this heteropolymer different classifications: when m (representing GlcNAc monomers) $> 50\%$, is designated as chitin and when n (representing GlcN monomers) $> 50\%$ is designated as chitosan. Adapted from Zargar et al. (2015)

This molecule is an essential structural polysaccharide of the exoskeleton of several animals (e.g., phyla Arthropoda) and fungal cell walls (Younes and Rinaudo, 2015; Muthukrishnan et al., 2016), where linear chitin polysaccharides are usually stacked, forming a chitin microfibrils matrix with increased strength (Gooday, 1983; Zargar et al., 2015). Depending on the molecular rearrangement of these microfibrils, chitin can be classified in three polymorphic forms: antiparallel (α), the most abundant form in nature; parallel (β); or mixed (γ) (Gooday, 1983; Resh and Cardé, 2009).

In almost any case, chitin is covalently or non-covalently linked with other structural molecules, such as proteins, minerals, other polysaccharides, lipids, vitamins, and phenols (Figure 1.4) (Kurita, 2006; Resh and Cardé, 2009). Protein sclerotization forms a chitin-protein matrix which, depending on its function, has different chitin: protein ratios

(Muthukrishnan et al., 2020). Cuticular zones with increased flexibility tend to have 1:1 chitin: protein ratios, whereas 1:3 to 1:4 chitin: protein ratios are present in harder cuticle zones (Resh and Cardé, 2009; Muthukrishnan et al., 2020).

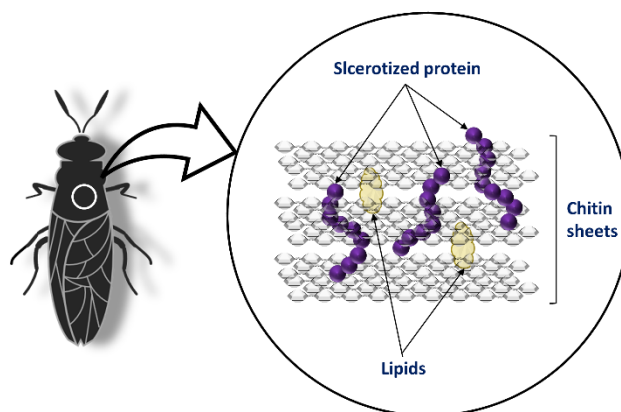


Figure 1.4 Schematic representation of protein and lipid covalent bonding within the chitin matrix present in the insect's exoskeleton.

Contrary to crustaceans' exocuticles, insects do not possess a calcium carbonate coating on top of their chitin-protein matrix (Nagasawa, 2012), a feature lost in favor of lighter cuticles for terrestrial and aerial environments adaptation (Bentov et al., 2016). Depending on the insect's species and development stage, the cuticular structure can have fluctuation of chitin contents (usually between 20-40% on DM), variable degrees of acetylation, and different compositions of chitin-associated proteins (Muthukrishnan et al., 2020). Ultimately, these variations alter chitin's porosity, solubility, and structure, thus modifying its bioaccessibility (Zargar et al., 2015; Waśko et al., 2016; Muthukrishnan et al., 2019; da Silva Lucas et al., 2021).

1.2.2 HI and TM meals effect on growth performance and digestibility

From a nutritional standpoint, HI meal demonstrated a high potential as a sustainable protein source in aquafeeds for several carnivorous fish species (Table 1.1). For example, Magalhães et al. (2017) showed that in ESB dietary inclusion of up to 19.5% of HI meal, corresponding to 45% FM substitution, could be achieved without any negative effects on fish final body weight (FBW). This was further supported by studies of Abdel-Tawwab et al. (2020) and Mastoraki et al. (2020) in the same species, where HI was included in the diets at 14.8% (50% FM substitution) and 19.5% (30% FM

substitution) without affecting growth performance. Other studies confirmed similar trends for several other carnivorous fish species, for example, gilthead seabream (up to 45% inclusion, 100% FM substitution) (Randazzo et al., 2021a; Moutinho et al., 2022); rainbow trout (up to 32%, 100% FM substitution) (Terova et al., 2019; Hoc et al., 2021b; Biasato et al., 2022); Atlantic salmon (*Salmo salar*) (14.8%, corresponding to 100% FM substitution or 30%, corresponding to 30% FM substitution) (Belghit et al., 2019; Fisher et al., 2020); Siberian sturgeon (*Acipenser baerii*) (18.5%, 25% FM substitution) (Caimi et al., 2020b); meagre (*Argyrosomus regius*) (20%, 35% FM substitution) (Guerreiro et al., 2020); and European perch (*Perca fluviatilis*) (40%, 42% FM substitution).

TM meal appears to be equally promising as an FM substitute (Table 1.1). For example, no negative effects on FBW were reported when feeding diets with TM meal to ESB (25% inclusion and 36% FM substitution, or 80% inclusion and 36% FM substitution) (Gasco et al., 2016; Basto et al., 2021b), gilthead seabream (25% inclusion and 36% FM substitution) (Piccolo et al., 2017), rainbow trout (25% inclusion and 100% FM substitution) (Rema et al., 2019; Chemello et al., 2020), pearl gentian grouper (*Epinephelus fuscoguttatus* ♀ × *Epinephelus lanceolatus* ♂) (12.5% inclusion and 31% FM substitution), blackspot seabream (*Pagellus bogaraveo*) (40% inclusion and 50% FM substitution) (Iaconisi et al., 2017), and black head seabream (*Acanthopagrus schlegelii*) (33.6% inclusion and 60% FM substitution) (Jeong et al., 2022).

Despite the promising growth results, improving feed efficiency is key for the development of economically and environmentally sustainable aquaculture (Verdal et al., 2018). It is here, however, that HI and TM meals see one of their main bottlenecks. Overall, the biggest impact of HI or TM meals on diet digestibility is noted in protein digestibility (Table 1.1). Dietary inclusion levels of HI and TM meals higher than 15% often led to significantly poorer protein digestibility, as verified by Basto et al. (2020, 2021a) in ESB (HI meal at 20% and TM meal at 60%, corresponding to 20% and 100% FM substitution, respectively), Belghit et al. (2018) in Atlantic salmon (HI meal at 60%, 85% FM substitution), Piccolo et al. (2017) in gilthead seabream (TM meal at 50%, 71% FM substitution), Belforti et al. (2015) in rainbow trout (TM meal at 50%, 66% FM substitution), Caimi et al. (2020b) in Siberian sturgeon (HI meal at 18.5%, 25% FM substitution), and Ge et al. (2022) in largemouth seabass (*Micropterus salmoides*) (TM meal at 48%, 100% FM substitution). However, in studies with several species (e.g., meagre, pikeperch (*Sander lucioperca*), European perch, Siberian sturgeon, and rainbow trout), protein digestibility can be decreased with dietary HI or TM meals levels as low as 6-10% (Caimi et al., 2020b; Chemello et al., 2020; Coutinho et al., 2021;

Guerreiro et al., 2021; Tran et al., 2022b; Stejskal et al., 2023). It should be noted, however, that in some studies high dietary HI or TM meals levels did not affect protein digestibility, such as in ESB fed with diets containing 36% TM meal (80% FM substitution) (Basto et al., 2021b), Atlantic salmon fed diets containing 30% HI meal (30% FM substitution) (Fisher et al., 2020), or rainbow trout fed diets containing 30% (33% FM substitution) and 40% (50% FM substitution) HI meal (Renna et al., 2017; Terova et al., 2019), or even increased protein digestibility (e.g., rainbow trout fed diets containing 10.3% TM meal, 30% FM substitution) (Melenchón et al., 2021).

While not as common as decreased protein digestibility, dietary HI or TM meal inclusion can also lead to decreased lipid digestibility (Table 1.1). This effect is mainly observed Belghit et al. (2018) in Atlantic salmon (HI meal at 60%, 85% FM substitution), Stejskal et al. (2023) in pikeperch (HI meal at 36%, 60% FM substitution), Piccolo et al. (2017) in gilthead seabream (TM meal at 50%, 71% FM substitution), Ge et al. (2022) in largemouth seabass (TM meal at 48%, 100% FM substitution), and Tran et al. (2022b) in European perch (TM meal at 20.3%, 75% FM substitution).

Low protein and lipid digestibility often led to higher FCR, which, in some cases, were also reported to be correlated with HI or TM meal inclusion levels (Song et al., 2018; Coutinho et al., 2021; Guerreiro et al., 2021; Weththasinghe et al., 2021b; Gu et al., 2022; Tran et al., 2022b). Given that chitin is ubiquitous in HI and TM meals, authors postulated that it was the main responsible for the negative correlation between HI or TM meal inclusion level and protein and lipid digestibility. This hypothesis was further supported by results obtained *in vitro* (Marono et al., 2015) and *in vivo*, with IM-containing diets (Karlsen et al., 2017; Piccolo et al., 2017; Renna et al., 2017; Mastoraki et al., 2020; Guerreiro et al., 2021; Eggink et al., 2022).

The negative correlation between dietary chitin and protein and lipid digestibility is not surprising. NSPs, such as chitin, are assumed to be indigestible by carnivorous fish, hindering protein and lipid hydrolysis and absorption (Kroghdahl et al., 2010; Sinha et al., 2011; Kokou and Fountoulaki, 2018). Several mechanisms can explain the negative effects of chitin on diets digestibility, such as an increase in digesta viscosity within the fish gut, which can lead to a decrease in feed exposure to digestive enzymes (Razdan and Pettersson, 1994), or pH-dependent establishment of ionic bonds with free fatty acids or bile acids, which limits hydrolysis by lipases (Tharanathan and Kittur, 2003). However, in the case of HI and TM meals, researchers consider that the main cause for the lower protein and lipid digestibility, is the entrapment of sclerotized proteins and or

lipids within the chitin matrix, obstructing the access of proteases and lipases to the substrates, thus limiting their digestibility (De Marco et al., 2015).

To sum up, dietary inclusion of defatted or partially defatted HI or TM meal was able to substitute up to 50% FM without a negative impact on fish FBW or FCR. In the case of HI meal, it has been reported as a viable alternative to completely substitute FM in diets for gilthead seabream (45% defatted HI inclusion), rainbow trout (32% partially defatted HI meal inclusion) (Biasato et al., 2022), and Atlantic salmon (14.8% partially defatted HI meal inclusion) (Belghit et al., 2019). On the other hand, dietary inclusion of full-fat HI or TM has been reported to lead to poor results growth and FCR, even at low substitution levels, mainly due to the unbalanced fatty acid profile or reduced palatability of these ingredients (Sealey et al., 2011; Weththasinghe et al., 2021b; Belforti et al., 2015; Piccolo et al., 2017; Mastoraki et al., 2020; Huang et al., 2022 Karapanagiotidis et al., 2023). Finally, dietary inclusion of HI or TM above 15% has often been linked to lower protein and/or lipid digestibility. Several authors have highlighted a negative correlation between chitin levels and nutrient digestibility (Dumas et al., 2018; Coutinho et al., 2021; Guerreiro et al., 2021; Weththasinghe et al., 2021b). As such, to increase HI and TM digestibility in aquafeeds, strategies that increase chitin digestibility must be developed.

1.2.3 HI and TM meals effect on the gastrointestinal tract (gut) morphology

Maintenance of gut homeostasis is crucial for fish growth performance and well-being, and disruptive changes in gut morphology can lead to poor nutrient digestion and absorption, and a decrease in immunity (Krogdahl et al., 2005; Grosell et al., 2010). As such, morphometric features such as villi number and length, muscular layer, enterocyte vacuolization, or immune cell infiltrations are often used as useful indicators of the fish's functional and health status (Krogdahl et al., 2005).

Upon HI or TM meal inclusion in fish feed, several modifications in the gut morphology of carnivorous fish were noted. For example, Chaklader et al. (2019) reported that in juvenile Asian seabass (*Lates calcarifer*) fed diets containing 12% of HI meal, villi and enterocyte width and microvilli height increased compared to the control diet. Józefiak et al. (2019) and Dumas et al. (2018) reported a shortening of the villi length in the intestine of rainbow trout fed with diets including either 15% TM meal or 26.4% HI meal. Cardinaletti et al. (2019), also in rainbow trout, observed a reduction in the intestinal fold

Table 1.1 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on growth performance, feed utilization, and apparent digestibility coefficients (ADC) of diets of carnivorous fish species.

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (Treatment)	Results	Reference
<i>Hermetia illucens</i>				
<i>Dicentrarchus labrax</i> (European sea bass)	20% (20%)	Larvae meal (Untreated and defatted)	↓ADC _{DM} , ADC _P , ADC _L , and ADC _E	Basto et al. (2020)
	7.25% (25%), 10.1% (35%), 14.8% (50%)	Larvae meal (Partially defatted)	→ FBW, WG, SGR, and FCR	Abdel-Tawwab et al. (2020)
	19.5% (30%)	Larvae meal (Partially defatted)	→ FBW, WG, SGR, and FCR → ADC _{DM} , ADC _P , and ADC _L	Mastoraki et al. (2020)
	6.5% (15%), 13% (30%), and 19.5% (45%)	Pre-pupa meal (Defatted)	→ FBW, WG and FE → ADC _{DM} , ADC _P , and ADC _L (up to 30%)	Magalhães et al. (2017)
<i>Sparus aurata</i> (Gilthead seabream)	9.5% (9%), 19.4% (17%), and 27.6% (24%)	Larvae meal (Full fat)	↓FBW and SGR → FCR and PER	Karapanagiotidis et al. (2023)
	5.8% (10%), 11.6% (20%), and 17.4% (30%)	Larvae meal (Defatted)	↓FBW, SGR, PER (17.4%) ↑FCR (17.4%)	Karapanagiotidis et al. (2023)
	15% (22%), 30% (60%), and 45% (100%)	Larvae meal (Defatted)	→ FBW, FE, and PER → ADC _P , ADC _L , and ↑ADC _{DM} , ADC _E ,	Moutinho et al. (2022)
	16.2% and 32.4%*	Larvae meal (Partially defatted)	↑FBW, SGR and ↓ FCR	Randazzo et al. (2021a)
<i>Oncorhynchus mykiss</i> (Rainbow trout)	8% (25%), 16% (50%), and 32% (100%)	Larvae meal (Partially defatted)	→ FBW, SGR, FCR, and PER → ADC _{DM} , ADC _P , ADC _L , and ADC _E	Biasato et al. (2022)

Table 1.1 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on growth performance, feed utilization, and apparent digestibility coefficients (ADC) of diets of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (Treatment)	Results	Reference
<i>Oncorhynchus mykiss</i> (Rainbow trout)	6.2% (25%) and 12.4% (50%)	Larvae meal (Partially defatted)	→ FBW, FCR and ↓SGR → ADC _{DM} , ADC _P , ADC _L , and ADC _E	Bordignon et al. (2022)
	30% (75%)	Pre-pupae (Untreated and ω3-enriched)	↓ FBW and SGR	Hoc et al. (2021a)
	5.6% (15%) and 10.9% (30%)	Larvae meal (Full fat)	→ FBW, SGR, FCR, and PER → ADC _P	Melenchón et al. (2021)
	7.8% (25%) and 10% (50%)	Larvae meal (Full fat)	→ FBW, SGR, and FCR	Cardinaletti et al. (2019)
	10% (9%), 20% (20%) and 30% (33.3%)	Pre-pupa meal (Partially defatted)	→ FBW, WG, SGR, and FCR → ADC _{DM} , ADC _P , and ADC _L	Terova et al. (2019)
	6.6% (25%), 13.2% (50%) and 26.4% (100%)	Larvae meal (Partially defatted)	↓FBW (26.4%) and WG (26.4%) ↑FCR (correlated ↑HI)	Dumas et al. (2018)
	20% (25%), 40% (50%)	Larvae meal (Partially defatted)	→ FBW, WG, SGR, and FCR → ADC _{DM} , ADC _P , ADC _L , and ADC _E	Renna et al. (2017)
	20,1% (46%)	Larvae meal (Partially defatted)	→ FBW, SRG, and FCR ↓PER and ↑LER	Stadtlander et al. (2017)
	16.4% (25%) and 32.8% (50%)	Pre-pupae meal (Full fat)	↓WG (16.4% and 32.8%) → FCR	Sealey et al. (2011)
	18.12% (25%) and 36.24% (50)	Pre-pupae meal (Fish offal-enriched)	→ WG and FCR	Sealey et al. (2011)
	8.07% (6.25%), 16.13% (12.5%), and 32.27% (25%)	Larvae meal (Full fat)	↓FBW, SGR and ↑FCR (correlated ↑HI) ↓ ADC _P and ADC _L linearly correlated with HI	Weththasinghe et al. (2021b)
<i>Salmo salar</i> (Atlantic salmon)				

Table 1.1 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on growth performance, feed utilization, and apparent digestibility coefficients (ADC) of diets of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (Treatment)	Results	Reference
<i>Salmo salar</i> (Atlantic salmon)	30% (30%)	Larvae meal (n/s)	→ FBW, SGR, FCR, and PER ↑ ADC _{DM} , → ADC _P , ADC _L , and ADC _E	Fisher et al. (2020)
	4.91% (33%), 9.84% (66%), and 14.75% (100%)	Larva meal (Partially defatted)	→ FBW, SGR, and FCR → ADC _{CP} , ADC _L , ADC _{EAA}	Belghit et al. (2019)
	60% (85%)	Larvae meal (Defatted)	→ FBW, SGR, and FCR ↓ ADC _P , ADC _L , ADC _{EAA}	Belghit et al. (2018)
<i>Perca fluviatilis</i> (Eurasian perch)	20% (21%), 40% (42%), and 60% (63%)	Larvae meal (Partially defatted)	→ SGR, FCR and PER ↓ FBW (60%)	Stejskal et al. (2020)
<i>Sander lucioperca</i> (Pikeperch)	9% (25%), 18% (50%), and 36% (60%)	Larvae meal (defatted)	↓ FBW, PER and ↑ FCR (36%) ↓ ADC _{DM} (18% and 36%), ADC _P (9%, 18%, and 36%), and ADC _L (36%)	Stejskal et al. (2023)
<i>Argyrosomus regius</i> (Meagre)	10% (17%), 20% (35%), and 30% (52%)	Larvae meal (defatted)	↓ FBW, FE, and PER linearly correlated with HI meal inclusion ↓ ADC _{DM} (10% and 30%), ADC _P (10%, 20%, and 30%), and ADC _E (36%)	Guerreiro et al., 2020, 2021
<i>Lates calcarifer</i> (Asian seabass)	22.1% (30%)	Larvae meal (Defatted)	→ FBW, SGR, FCR, PER	Hender et al. (2021)
	11.9%** for larvae meal 9.83%** for pre-pupae meal	Larvae and pre-pupae meal (Full fat)	↓ FBW and ↑ FCR	Fischer et al. (2021)

Table 1.1 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on growth performance, feed utilization, and apparent digestibility coefficients (ADC) of diets of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (Treatment)	Results	Reference
<i>Lates calcarifer</i> (Asian seabass)	35% (n/a) for full fat 27.83 (n/a) defatted	Larvae meal (Full fat and defatted)	↓FBW, SGR and ↑FCR (defatted) →FBW, SGR and FCR (full fat)	Chaklader et al. (2021)
<i>Epinephelus coioides</i> (Grouper)	2.5% (n/a), 5% (n/a), 10% (n/a)	Larvae meal (Full fat)	→FBW and ↑FCR	Jiang et al. (2022)
<i>Epinephelus fuscoguttatus</i> ♀ × <i>Epinephelus lanceolatus</i> ♂ (pearl gentian grouper)	7% (10%), 14% (20%), and 21% (30%)	Larvae meal (Full fat)	↓FBW, SGR and ↑FCR	Huang et al. (2022)
<i>Lateolabrax japonicus</i> (Japanese sea bass)	9.6% (32%), 14.4% (42%), and 19.2% (64%)	Larvae meal (Defatted)	→ FBW, SGR and FCR	Wang et al. (2019b)
<i>Acipenser baerii</i> (Siberian sturgeon)	18.5% (25%), 37.5% (50%), and 75% (100%; not tested for ADC)	Larvae meal (defatted)	↓ FBW (37.5%) and SGR (37.5%) ↓ ADC _P (18.5%, 37.5%)	Caimi et al. (2020b)
<i>Tenebrio molitor</i>				
<i>Dicentrarchus labrax</i> (European sea bass)	18% (40%), 36% (80%), 60% (100%)	Larvae meal (Defatted)	→ FBW or FCR (up to 36% inclusion) ↓ FBW or FCR (60% inclusion) ↓ADC _{DM} and ADC _E (18%) and ↓ADC _{CP} (60%)	Basto et al. (2021a)
	19.5% (30%)	Larvae meal (Full fat)	↓FBW ↑FCR	Mastoraki et al. (2020)
	20% (20%)	Larvae meal (Defatted)	↑ADC _{DM} , → ADC _P , and ↓ ADC _L and ADC _E	Basto et al. (2020)

Table 1.1 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on growth performance, feed utilization, and apparent digestibility coefficients (ADC) of diets of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (Treatment)	Results	Reference
<i>Dicentrarchus labrax</i> (European sea bass)	20% (20%)	Larvae meal (Defatted)	→ ADC _P , ADC _L and ADC _E and ↓ ADC _{DM}	Basto et al. (2020)
	25% (36%) and 50% (71)	Larvae meal (Full fat)	↓ FBW and SGR (50%) → FCR and PER	Gasco et al. (2016)
<i>Sparus aurata</i> (Gilthead seabream)	25% (36%) and 50% (71%)	Larvae meal (Full fat)	↑ FBW (25%), SGR (25%), and PER (25%) and ↓ FCR (25%) ↓ ADC _P (50%) and ADC _L (50%)	Piccolo et al. (2017)
<i>Oncorhynchus mykiss</i> (Rainbow trout)	5.1% (15%) and 10.3% (30%)	Larvae meal (Full fat)	→ FBW, SGR, FCR, and PER ↑ ADC _P	Melenchón et al. (2021)
	5% (25%), 10% (50%), and 20% (100%)	Larvae meal (Partially defatted)	→ FBW, SGR, FCR, PER → ADC _{DM} and ADC _L and ↓ ADC _P (10%, 20%)	Chemello et al. (2020)
	5% (20%), 7.5% (30%), 15% (60%), or 25% (100%)	Larvae meal (Defatted)	↑ FBW and SGR ↓ FCR and ↑ PER → ADC _{DM} , ADC _P , ADC _L , and ADC _E	Rema et al. (2019)
	25% (35%) and 50% (66%)	Larvae meal (Full fat)	→ FBW, ↑ SGR and PER and ↓ FCR	Belforti et al. (2015)
<i>Micropterus salmoides</i> (Largemouth bass)	4% (11.1%), 8.1% (22.2%), 12.2% (33.3%), 16.3% (44.4%), 20.4% (55.5%), and 24.5% (66.6%)	Larvae meal (Defatted)	↓ FBW and SGR (above 8.1%) and FCR (above 12.2%)	Gu et al. (2022)

Table 1.1 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on growth performance, feed utilization, and apparent digestibility coefficients (ADC) of diets of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (Treatment)	Results	Reference
<i>Micropterus salmoides</i> (Largemouth bass)	24% (50%) and 48% (100%)	Larvae meal (Defatted)	↓ FBW and SGR (48%) ↑ FCR (24% and 48%) ↑ ADC _{DM} (24%) ↓ ADC _P , ADC _L , and ADC _E (48%)	Ge et al. (2022)
<i>Salmo trutta</i> m. <i>trutta</i> (Sea Trout)	20% (10%)	Larvae meal (Untreated or hydrolyzed with peptidases at 0.5% and 1%)	↓ WG → SGR, FCR, and PER	Hoffmann et al. (2021)
	10% (42%)	Larvae meal (Hydrolyzed)	→ FBW, SGR, FCR, and ↓ PER	Mikołajczak et al. (2020)
<i>Epinephelus fuscoguttatus</i> ♀ × <i>Epinephelus lanceolatus</i> ♂ (Pearl gentian grouper)	2.5% (6%), 5% (12%), 7.5% (18%), 10% (25%), and 12.5% (31%)	Larvae meal (Defatted)	↑ FBW (5%, 7.5%, 10%, and 12.5%) ↓ FBW (2.5%) → SGR ↑ FCR (2.5%, 10%, and 12.5%)	Song et al. (2018)
<i>Argyrosomus regius</i> (Meagre)	10% (25%), 20% (50%), and 30% (75%)	Larvae meal (defatted)	↓ FBW, FE, and PER linearly correlated with inclusion level ↓ ADC _{DM} (10% and 30%), ADC _P (10%, 20%, and 30%), and ADC _E (36%)	Coutinho et al. (2021)
<i>Siniperca scherzeri</i> (Mandarin fish)	10% (10%), 20% (20%), and 30% (30%)	Larvae meal (Full fat)	↑ WG and SGR (10% and 20%) ↑ PER (20%) ↓ FCR (10% and 20%)	Sankian et al. (2018)

Table 1.1 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on growth performance, feed utilization, and apparent digestibility coefficients (ADC) of diets of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (Treatment)	Results	Reference
<i>Acanthopagrus schlegelii</i> (Black head seabream)	8.4% (15%), 16.8% (30%), 25.2% (45%), and 33.6% (60%)	n/s	→ WG, SGR, and FCR	Jeong et al. (2022)
<i>Pagellus bogaraveo</i> (Black spot seabream)	21% (25%) and 40% (50%)	Larvae meal (Full fat)	→FBW, SGR and FCR	Iaconisi et al. (2017)
<i>Pargus major</i> (Red seabream)	25% (38.5%), 40% (61.5%), and 65% (100%)	Larvae meal (Defatted)	↑ WG and SGR → FCR	Ido et al. (2019)
<i>Perca fluviatilis</i> (Eurasian perch)	6.8% (25%), 13.5% (50%) and 20.3% (75%)	Larvae meal (defatted)	↓WG, SGR, FCR and PER (13.5% and 20.3%) ↓ ADC _{DM} (6.8%, 13.5% and 20.3%), ADC _P (6.8%, 13.5% and 20.3%), and ADC _L (20.3%)	Tran et al. (2022b)

The symbols represent an increase (↑), decrease (↓), or no effect (→) on the tested parameters relative to the control.

*diets deprived of fish meal; ; **no fish meal substitution; n/s - not specified; IM - insect meal; ADC_{DM} - dry matter apparent digestibility coefficient; ADC_P - protein apparent digestibility coefficient; ADC_L - lipid apparent digestibility coefficient; ADC_E - energy apparent digestibility coefficient; FBW - final body weight; WG - weight gain; SGR- specific growth rate; FCR- feed conversion ratio; FE - feed efficiency; PER - protein efficiency ratio; LER- lipid efficiency ratio; ADC_{EAA} - essential amino acids apparent digestibility coefficient.

length when in fish fed with diets including 10.5% of HI meal. Li et al. (2020), in Atlantic salmon, observed a severe enterocyte hypervacuolization in fish fed a diet including 14.8% of HI meal.

The diet can also impact the gut inflammatory status (Grosell et al., 2010). In carnivorous fish, for example, dietary inclusion of HI meal at 37.5% and 50% had no effect on immune cell infiltration within the gut mucosa and submucosa of Siberian sturgeon and rainbow trout, respectively (Elia et al., 2018; Caimi et al., 2020a). On the other hand, Li et al. (2020), in Atlantic salmon, noted an increase in submucosa cellularity when including 14.8% HI meal in the diet. While this potentially indicates an inflammatory gut status, this was not confirmed by the expression of pro-inflammatory genes (Li et al., 2020). Acidic mucins, which are known for inhibiting the adherence of pathogenic bacteria in the gut, increased in juvenile Asian seabass fed with diets containing 12% HI meal, thus potentially enhancing fish gut immune response (Chaklader et al., 2019).

While few studies have addressed the impact of HI and TM meal inclusion in carnivorous fish diets, results seem to point towards a negative impact on the fish gut morphometric features at inclusion levels above 10% (Dumas et al., 2018; Cardinaletti et al., 2019; Józefiak et al., 2019; Li et al., 2020). This could be the result of poor chitin digestibility which, due to its chelating abilities, can limit the bioavailability of several important minerals or alter gut pH, thus leading to detrimental morphologic changes (Tharanathan and Kittur, 2003; Hamed et al., 2016). On the other hand, increasing chitin digestibility can potentially trigger positive alterations in the fish gut morphology. For example, when supplemented at 0.4% in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) diets, COS (a product resulting from chitin hydrolysis) was reported to improve fold height and width and lamina propria thickness, ultimately leading to better growth performance (Chen et al., 2021). Increasing chitin bacterial fermentation could also trigger positive gut morphology modifications as the higher formation of short-chain fatty acids (e.g., butyric, acetic, and propionic acids) can induce changes in enterocytes villi length (Nikiforov-Nikishin et al., 2022), division rate (Rawls et al., 2004) and enzymes secretion (Cani and Knauf, 2016), modulate gut motility (Luna et al., 2022), regulate glucose and fat homeostasis (Backhed et al., 2004; De Vadder et al., 2014), and maintain or disrupt mucosal integrity (Davis et al., 2016; Butt and Volkoff, 2019). Chitosan, another product resulting from chitin fermentation, when supplemented up to 4% in ESB diets was also observed to increase microvilli height (Zaki et al., 2015). Furthermore, chitosan can also modulate the epithelial cell tight junctions, increasing macromolecule

paracellular permeability, and immune cell migration ability, as verified *in vitro* with human cells (Ward et al., 2000; Smith et al., 2004). However, these effects require to be confirmed in fish.

1.2.4 HI and TM meals effects on the fish immune status and disease resistance

Diets can crucially affect the fish's immune status, either enhancing it by fulfilling the nutritional requirements or by containing immunostimulants molecules or hindering it by failing to provide enough nutrients or possessing antinutritional factors (Kiron, 2012; Mohan et al., 2019). This is one of the main arguments for IM usage in fish feeds as this commodity also carries numerous compounds that can improve fish health and increase disease resistance (Gasco et al., 2018).

In fish, assessment of the immune status is often carried out by measuring several key parameters of the nonspecific innate immune system (e.g., lysozyme, myeloperoxidase, complement system, and bactericidal activity) (Table 1.2). In carnivorous fish fed with diets containing HI meal, it was observed that serum lysozyme activity remained unchanged in several fish species, such as rainbow trout (5.6% and 10.9%, corresponding to 15% and 30% FM substitution) (Melenchón et al., 2021), Atlantic salmon (8%-32%, maximum FM substitution of 25%) (Weththasinghe et al., 2021c), and Asian seabass (22%, 30% FM substitution) (Hender et al., 2021). On the other hand, positive effects were verified in fish fed with diets containing HI meal, for instance in ESB where it was observed an increase of phagocytic activity, serum lysozyme activity, and respiratory burst (7%-15%, corresponding to FM substitution of 25%-50%) (Abdel-Latif et al., 2021), in rainbow trout, where serum lysozyme activity increased (16% inclusion, no dietary FM) (Kumar et al., 2021), and in Atlantic salmon, where C-reactive protein (an acute-phase inflammatory marker) linearly decreased with dietary HI meal inclusion levels (8%-32%, substituting between 6%-25% FM) (Weththasinghe et al., 2021c).

Similar results were obtained upon including TM meal in carnivorous fish feeds (Table 1.2). For example, in rainbow trout fed diets containing up to 50% TM meal (67% FM substitution), no differences were reported in serum lysozyme, nitric oxide, or ceruloplasmin (inflammation marker), while serum myeloperoxidase activity increased (Henry et al., 2018a; Melenchón et al., 2021). Mandarin fish (*Siniperca scherzeri*) fed diets containing 10%-30% TM meal maintained serum myeloperoxidase activity and total immunoglobulins, and increased serum lysozyme at 30% TM inclusion (Sankian et al.,

2018). In ESB fed with diets containing 25% TM meal (36% FM substitution), serum lysozyme and bactericidal activity remained unchanged, while a decrease in serum ceruloplasmin, myeloperoxidase activity, and nitric oxide was recorded (Henry et al., 2018b).

Expression of immune-related genes is also commonly used to assess the fish's immune status (Zou and Secombes, 2016). To date, in carnivorous fish, these parameters were only evaluated upon dietary HI meal inclusion, with pro-inflammatory cytokines IL-1 β , IL-8, and TNF α , and the anti-inflammatory cytokine IL-10 being the main objects of study (Table 1.2) (Zou and Secombes, 2016). In rainbow trout, Kumar et al. (2021) observed a decrease in IL-8 and TNF α expression in the head kidney of fish fed a diet with 8% HI meal when compared to a soybean-based diet. Randazzo et al. (2021b) described a similar effect on rainbow trout, where a decrease in IL-1 β and TNF α expression was observed in the distal intestine (22% and 45% HI meal inclusion, no dietary FM). In both cases, no FM was included in the diets, and the authors described the decrease in the expression of these pro-inflammatory cytokines as a positive outcome, arguing that HI meal inclusion could potentially mitigate the intestinal inflammatory state promoted by an excessive inclusion of PF in the diets (Kumar et al., 2021; Randazzo et al., 2021b). An increase in pro-inflammatory gene expression induced by HI inclusion in fish feeds was also reported. However, only Cardinaletti et al. (2019) reported an increase in proinflammatory genes (TNF α) associated with morphometrical indicators of inflammatory state, namely villi length reduction rainbow trout medium intestine (7.8% and 10%, 25% and 50% FM substitution, respectively) (Cardinaletti et al., 2019).

Hematological parameters (e.g., erythrocyte count and indices, leucocyte total and differential counts) can also be used to evaluate the fish's immune status. However, in the few studies that reported such parameters, no alterations were observed either in ESB (HI meal inclusion 7.3%-14.8%, substituting 25%-50% FM) (Abdel-Tawwab et al., 2020), or European perch (HI meal inclusion 20%-60%, substituting 21%-63% FM) (Stejskal et al., 2020) (Table 1.2).

Dietary enrichment with specific components (e.g., amino acids, fatty acids, vitamins, minerals, or immunostimulants) may also improve fish disease resistance (Oliva-Teles, 2012; Dawood et al., 2018). As HI and TM meals are known to have immunostimulant components (e.g., chitin and its derivatives or AMPs), these ingredients have also been evaluated regarding disease resistance. To date, only a few studies evaluated the relation between HI or TM meal inclusion in fish feeds and disease resistance in

carnivorous species, and most of the results pointed to a positive effect when compared to the control diets. Thus, ESB fed diets containing HI meal (7.3%, 10.1%, and 14.8%, substituting 25%, 35%, and 50% FM, respectively), increased survivability against *Vibrio alginolyticus* concomitantly with the dietary increase in HI meal (Abdel-Latif et al., 2021). Similarly, rainbow trout fed diets containing HI meal (5%, no FM substitution), increased their survivability against *Aeromonas salmonicida* at both low and high fish densities (Cho et al., 2022), and red seabream (*Pargus major*) fed diets containing TM meal (10%, 20% FM substitution) increased resistance against *Edwardsiella tarda* (Ido et al., 2019). On the other hand, in grouper and Asian seabass fed with diets containing HI meal, no increase in survivability against *V. harvey* was observed (Chaklader et al., 2021; Jiang et al., 2022), while in pearl gentian grouper survivability against *V. harvey* decreased in fish fed diets including 10% and 12.5% (corresponding to 25% and 31% FM) of TM meal (Song et al., 2018).

Overall, when included in fish diets, HI or TM meals are usually reported to either have no effect or to improve the fish's immune status. To date, however, the studies evaluating the impact of IM in carnivorous fish are mostly focused on HI meals, with TM meals having only a few studies. The results obtained to date showed that dietary inclusion of up to 30% HI meal (independently of the processing method) can increase several nonspecific innate immune parameters. Moreover, as demonstrated by Abdel-Latif et al. (2021) in ESB fed with diets including HI, enhancement of the innate immune parameters due to dietary HI inclusion resulted in higher survivability when challenged with bacterial pathogens. Gene expression was also modulable by the inclusion of up to 30% HI meal in the diet. When evaluated under stress or disease conditions, gene modulation was mostly associated with a positive impact on the fish's immune status, being either associated with reduced mortality against bacterial pathogens (Cho et al., 2022) or reduced inflammatory status prompted by PF inclusion in the diets (Kumar et al., 2021; Randazzo et al., 2021b). As such, results obtained so far present HI meal as a potential functional ingredient able to enhance fish health status.

While several components present in IM can play a role in the modulation of the fish immune system and disease resistance (e.g. AMP), authors mostly attribute these effects to chitin (Lopez-Santamarina et al., 2020; Mousavi et al., 2020; Gasco et al., 2023). In fact, chitin or its derivatives (chitosan and COS), when supplemented in fish feeds, can modulate the expression of several key genes related to stress/disease resistance and stimulate various hematological, antioxidant, and innate immune parameters as thoroughly reviewed by Mohan et al. (2019), Abdel-Ghany and Salem

(2020), and Kamilya and Khan (2020). To name a few examples: dietary chitin supplementation modulated the natural hemolytic complement, respiratory burst, and phagocytic activity in gilthead seabream head-kidney leukocytes (50 mg kg⁻¹ feed) (Esteban et al., 2001), and increased white blood cell count, serum lysozyme, and neutrophils respiratory burst activity in common carp (*Cyprinus carpio*) (10 g kg⁻¹ feed) (Gopalakannan and Arul, 2006). Dietary chitosan supplementation increased the alternative complement pathway, lysozyme, phagocytic, and neutrophils respiratory burst activity in the serum of Nile tilapia (*Oreochromis niloticus*) (10 g kg⁻¹ feed) (Abu-Elala et al., 2015), and increased red and white blood cell counts, and serum lysozyme, respiratory burst, phagocytic and bactericidal activity in Asian seabass (10 g kg⁻¹ feed) (Ranjan et al., 2014). Dietary COS supplementation increased the expression of pro-inflammatory interleukins TNF α , IL-1 β , and IL-8 genes in the head kidney and spleen, and the phagocytic capacity, serum lysozyme and serum bactericidal activities in Nile tilapia (4 g kg⁻¹ feed and 8 g kg⁻¹) (Meng et al., 2017; Nurmalasari et al., 2022), and increased the hemato-biochemical profile (e.g., white blood cell count) in silver carp (*Hypophthalmichthys molitrix*) (2 g kg⁻¹ feed and 3 g kg⁻¹) (Harikrishnan et al., 2022).

Moreover, dietary inclusion of chitin and its derivatives are often pointed as promising strategies to increase fish resistance to bacterial pathogens as verified in ESB, rainbow trout, and largemouth bass, challenged against *A. hydrophila* (Luo et al., 2009; El-Sayed and Barakat, 2016; Lin et al., 2017b); rainbow trout challenged against *A. salmonicida* (Siwicki et al., 1994); longtooth grouper (*Epinephelus bruneus*) challenged against *V. alginolyticus* (Harikrishnan et al., 2012); pearl gentian grouper and pompano (*Trachinotus ovatus*) challenged against *V. harveyi* (Lin et al., 2012; Zhang et al., 2021); and Asian seabass challenged against *V. anguillarum* (Ranjan et al., 2014). While chitin and its derivatives' ability to increase disease resistance is often postulated to result from their capability to positively modulate fish's immune system, these molecules also possess antimicrobial activity against Gram-positive and Gram-negative bacteria (Xia et al., 2011). The amino groups present in these molecules are pointed as the main contributors to antimicrobial activity (Fei Liu et al., 2001; Rabea et al., 2003). Through electrostatic interactions, these positively charged groups can bind to negatively charged molecules present in the bacterial cell surface (Chung et al., 2004; Kasaai, 2008). These interactions with the bacterial cell membrane can result in the formation of an impermeable layer that impedes efficient molecular transport and bacterial metabolism (Benhabiles et al., 2012), or alter bacterial cell membranes permeability, leading to leakages of intracellular components (Rabea et al., 2003; Kong et al., 2008).

Table 1.2 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the immune status and disease resistance of carnivorous fish species.

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (treatment)	Challenge type (dosage)	Results [tissue]	Reference
<i>Hermetia illucens</i>					
<i>Dicentrarchus labrax</i> (European sea bass)	7.25% (25%), 10.1% (35%), and 14.8% (50%)	Larvae meal (n/s)	IP with <i>Vibrio</i> <i>alginolyticus</i> (2.0×10^5 CFU*/fish)	↑PA, PI, serum LYZ, and RB ↑ HSP70, IL-1β and IL-10 [liver] ↑ survival	Abdel-Latif et al. (2021)
	7.25% (25%), 10.1% (35%), 14.8% (50%)	Larvae meal (Partially defatted)	n/a	→ RBCC, HCT, Hb, MCV, MCH, MCHC, WBCC, lymphocytes, monocytes, and neutrophils	Abdel-Tawwab et al. (2020)
<i>Sparus aurata</i> (Gilthead seabream)	16.2% and 32.4%*	Larvae meal (Partially defatted)	n/a	→ IL-10, IL-1β, TNFα, NF-κB, and MYD88 [mid and distal intestine]	Randazzo et al. (2021a)
<i>Oncorhynchus mykiss</i> (Rainbow trout)	5% (0%)	Pre-pupae (Partially defatted)	<i>Aeromonas</i> <i>salmonicida</i> (2.0×10^7 CFU/fish)	↑ IL-1β, IgM and LYZ gene expression at low fish densities [liver] → IL-1β, IgM and LYZ gene expression at low fish densities [liver] ↑ survival (both fish densities)	Cho et al. (2022)
	8% and 16%	Larvae meal (Full fat)	n/a	↓ IL-8 (8%), TNFα, → ProstD, and ↑ IRF-1 (8%) [head kidney] (HI meal vs Soybean meal diet → IL-8, TNFα, ProstD, and ↑ IRF-1 (8%) in kidney (HI vs FM) ↓ ProstD and IRF-1 [intestine and head kidney] (HI meal vs FM diet or Soybean meal diet) ↑ serum LYZ (16%)	Kumar et al. (2021)

Table 1.2 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the immune status and disease resistance of carnivorous fish species (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (treatment)	Challenge type (dosage)	Results [tissue]	Reference
<i>Oncorhynchus mykiss</i> (Rainbow trout)	7.8%*, 22%*, and 45%*	Larvae meal (defatted)	n/a	↑ IL-10, IL-1β, NF-κB, and MYD88 (7.8%) [distal intestine] ↓ IL-10, IL-1β, TNFα, and MYD88 (22% and 45%) and NF-κB (45%) [distal intestine]	Randazzo et al. (2021b)
	5.6% (15%) and 10.9% (30%)	Larvae meal (Full fat)		→ Serum LYZ, peroxidase, Igs ↑ Serum anti-protease (5.6%)	Melenchón et al. (2021)
	7.8% (25%) and 10% (50%)	Larvae meal (Full fat)	n/a	↑ HSP70 (10%, liver) ↑ IL-10, TNFα, and TLR-5 [medium intestine] ↑ IL-10 (7.8%) and → TNFα, and TLR-5 [hind intestine]	Cardinaletti et al. (2019)
<i>Salmo salar</i> (Atlantic salmon)	8.07% (6.25%), 16.13% (12.5%), and 32.27% (25%)	Larvae meal (Full fat)	n/a	→ Serum LYZ ↓ CRP linearly correlated with HI meal inclusion → PA [head-kidney macrophages]	Weththasinghe et al. (2021c)
	10% (66%) and 15% (100%)	Larvae meal (Partially defatted)	Head kidney leukocytes incubated with <i>Pseudomonas Aeruginosa</i> LPS (100 µg/mL) or Poly I:C (50 µg/mL)	→ IL-1β, IL-8, IL-10, TNF-α (both treatments) ↑ TLR 22 (LPS)	Stenberg et al. (2019)

Table 1.2 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the immune status and disease resistance of carnivorous fish species (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (treatment)	Challenge type (dosage)	Results [tissue]	Reference
<i>Lates calcarifer</i> (Asian seabass)	11.9%** for larvae meal 9.83%** for pre-pupae meal	Larvae and pre-pupae meal	n/a	→ TNFα vs FM [liver] ↑ TNFα vs SB [liver] ↓ TGFβ1 vs FM (9.83%) [liver] → TGFβ1 vs SB (9.83%) [liver] → TNFα and TGFβ1 [intestine]	Fischer et al. (2021)
	22.1% (30%)	Larvae meal (Partially defatted)	n/a	→ IL-8, TNFα and ↑ IL-10 and IL-1β [intestine] → HSP70, and HSP90 [liver] → serum LYZ and ↑ serum BA	Hender et al. (2021)
	35% (n/a) full fat 27.83 (n/a) defatted	Larvae meal (full fat and defatted)	IP with <i>V. harveyi</i> (1.1×10^7 CFU/fish)	→ serum lysozyme → disease resistance	Chaklader et al. (2021)
<i>Epinephelus coioides</i> (Grouper)	2.5% (n/a), 5% (n/a), 10% (n/a)	Larvae meal (Full fat)	IP with <i>V. harveyi</i> (6.0×10^6 CFU/fish)	↑ IL-1β (5%, and 10%) [head kidney and spleen] ↑ IFN-γ (5%, 10%) [spleen] → TNFα and HSP70 [spleen, liver, head kidney, and gills] → survival	Jiang et al. (2022)
<i>Epinephelus fuscoguttatus</i> ♀ × <i>Epinephelus lanceolatus</i> ♂ (Pearl gentian grouper)	7% (10%), 14% (20%), and 21% (30%)	Larvae meal (Full fat)	n/a	↑ MYD88 (21%) [distal intestine] ↑ NF-κB (7%, 14%, and 21%) [distal intestine] ↑ TLR22 (14% and 21%) [distal intestine] ↑ IL-10 (all) distal intestine → TNFα [distal intestine]	Huang et al. (2022)

Table 1.2 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the immune status and disease resistance of carnivorous fish species (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (treatment)	Challenge type (dosage)	Results [tissue]	Reference
<i>Perca fluviatilis</i> (Eurasian perch)	20% (21%), 40% (42%), 60% (63%)	Larvae meal (Partially defatted)	n/a	→ RBCC, HCT, Hb, MCV, MCH, and MCHC	Stejskal et al. (2020)
<i>Tenebrio molitor</i>					
<i>Oncorhynchus mykiss</i> (Rainbow trout)	5.6% (15%) and 10.9% (30%)	Larvae meal (Full fat)		→ serum LYZ, APr, MPO; ↓ APH; ↑ Total Igs (5.6%)	Melenchón et al. (2021)
	25% (35%) and 50% (67%)	Larvae meal (Full fat)	n/a	→ serum LYZ, CO, NO, trypsin inhibition; serum MPO (25%) ↑serum MPO (50%)	Henry et al. (2018a)
<i>Dicentrarchus labrax</i> (European sea bass)	25% (36%)	Larvae meal (Full fat)	n/a	→ serum lysozyme; serum BA ↑ serum trypsin-inhibition at the TM ↓serum CO, MPO and NO	Henry et al. (2018b)
<i>Epinephelus fuscoguttatus</i> ♀ × <i>Epinephelus lanceolatus</i> ♂ (Pearl gentian grouper)	2.5% (6%), 5% (12%), 7.5% (18%), 10% (25%), 12.5% (31%)	Larvae meal (Defatted)	IP with <i>V. harveyi</i> (5.58×10^8 CFU/fish)	→ survival (2.5%, 5%, 7.5%) ↓ survival (10% and 12.5%)	Song et al. (2018)
<i>Siniperca scherzeri</i> (Mandarin fish)	10% (10%), 20% (20%), and 30% (30%)	Larvae meal (Full fat)	n/a	→ serum MPO and total Igs →serum (10% and 20%) ↑serum LYZ (30%)	Sankian et al. (2018)

Table 1.2 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the immune status and disease resistance of carnivorous fish species (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (treatment)	Challenge type (dosage)	Results [tissue]	Reference
<i>Pargus major</i> (Red seabream)	5% (10%) and 10% (20%)	Larvae meal (Defatted)	IP with <i>E. tarda</i> (5.0×10^5 CFU/fish)	↑ survival (10%)	Ido et al. (2019)

The symbols represent an increase (↑), decrease (↓), or no effect (→) on the tested parameters on fish fed diets containing either HI or TM meal, relative to the control FM-containing diet.

IM - insect meal; IP - intraperitoneal injected; CFU – colony forming units; n/a – not applicable; diets containing no FM; ** no FM substitution; PA-Phagocytic activity; PI-Phagocytic index; LYZ-Lysozyme; RB-Respiratory burst; HSP-heat-shock protein; IL-1β-Interleukin-1β; IL-10-Interleukin-10; RBCC-Red blood cell count; HCT-Hematocrit count; Hb-Hemoglobin concentration; MCV-Mean corpuscular volume; MCH - Mean corpuscular hemoglobin; MCHC-Mean corpuscular hemoglobin concentration; WBCC-White blood cell count; TNFα-Tumor necrosis factor alpha; NF-κB-Nuclear factor-κB; MYD88-Myeloid differentiation primary response 88; ProstD-Prostaglandin D; IRF-1-Interferon regulatory factor 1; TLR-Toll-like receptor; IgM-Immunoglobulin M; CRP-C-reactive protein; IL-8 - Interleukin 8; BA-Bactericidal activity; TGFβ1-Transforming growth factor beta 1; IFN-γ-Gamma interferon; NF-κBem1a-Nuclear factor kappa-B kinase essential modifier; CO-Ceruloplasmin; NO-Nitric oxide; MPO-Myeloperoxidase; APr-Anti-protease; APh-Alkaline phosphatase.

Low molecular weight molecules, such as COS, can permeate through the bacteria cell membrane and bind to DNA, through adsorption mechanisms, thus inhibiting RNA transcription (Sudarshan et al., 1992; Fei Liu et al., 2001), or bind themselves to Gram-positive bacteria cell-wall teichoic acids and damage membrane lipids, leading to a cascade reaction that ends in cell death (Raafat et al., 2008). As such, increased chitin digestibility can also lead to increased performance of IM as a functional ingredient.

1.2.5 The chitin conundrum in IM

To circumvent the digestive limitations imposed by chitin in IM, this molecule can be biologically or physico-chemically removed before inclusion in feeds (Mohan et al., 2020; Sindermann et al., 2021). These strategies have long been used for the removal of other NSPs, such as lignin and cellulose from PF (Li et al., 2022). However, the treatments used have their bottlenecks: biologic processes have low hydrolysis rates, high consumption time, and low sugar yields (Rezania et al., 2020); and physico-chemical treatments are costly, harmful to the environment, can alter the ingredient composition, produce inhibitors, and can sometimes lead to the formation of novel antinutritional factors (Karlsen and Skov, 2022). Chitin removal pre-treatments for IM are still in the beginning, adding extra layers of complexity and industrial costs (Sindermann et al., 2021). On the other hand, chitin is also frequently pointed as positively modulating fish' immune status and disease resistance (Lopez-Santamarina et al., 2020; Mousavi et al., 2020; Gasco et al., 2023), creating a difficult conundrum to solve.

Ideally, to potentiate chitin's benefits upon IM inclusion in fish diets, researchers should aim to i) reduce chitin's indigestibility/antinutritional effect (especially in carnivorous fish); ii) increase chitin's functional effects. A potential strategy would therefore be to increase chitin digestibility within the fish gut, thus potentially increasing the bioaccessibility to entrapped proteins and lipids as well as increasing energy availability. Additionally, as chitin functional properties are correlated with its degree of polymerization (number of monomer units composing the molecule) and degree of deacetylation (molar ratio of GlcN in the chitin, chitosan, or COS molecule) (Halder and Mondal, 2018; Liaqat and Eltem, 2018), increasing chitin hydrolysis could also potentiate its positive effects. Small molecules with high degrees of deacetylation are often considered more bioactive and, thus, COS are often regarded as having increased bioactivity when compared to chitin/chitosan (Kumar et al., 2020). However, to effectively develop a solution that increases chitin digestibility within the fish, we must understand the two major players in the digestive process: fish gut physiology and microbiota.

1.3 Fish gut physiology

The gut is the largest endocrine organ in fish. Although primarily perceived as the place for nutrient digestion and absorption, it is now evident that the gut is a multifunctional organ that plays a crucial role in metabolic, homeostatic, and protective processes (Kuz'mina, 2008; Bakke et al., 2010). The gut anatomy of most fish species is subdivided into four zones: the head-gut, composed of the mouth and pharynx; the foregut, consisting of the esophagus, and stomach; the midgut, or intestine, usually including pyloric ceca; and the hindgut, which is the final part of the gut, encompassing the rectum (Wilson and Castro, 2010; Egerton et al., 2018). Headgut primary function is food capture and mechanical digestion, whereas chemical digestion starts in the foregut (Bakke et al., 2010). The midgut, which is the longest portion of the gut, is where most of the digestive processes and absorption occur. In this region, the epithelial-lumen interface is mediated by a mucus layer, containing several protective and antimicrobial compounds, aiding in the protection of potentially injurious actions from dietary or environmental factors, thus providing ideal conditions for efficient nutrient digestion and absorption (Bakke et al., 2010; Pérez et al., 2010). Although common for all fish species, these regions can present a degree of variation due to fish phylogeny and ontogeny, dietary habits, and the surrounding environment. Examples of these variations are the absence of the stomach in 20% of the fish species (Wilson and Castro, 2010), or the short gut length of carnivores (usually equal to or lower than their body size) when compared to omnivores (usually 1-3 times bigger than their body size) and herbivores (usually more than 3 times their body size) (Egerton et al., 2018).

Different fish species have different endogenous digestive abilities, with these being mainly affected by the species' trophic level, gut structure, developmental stage, temperature, and diet composition (Ray et al., 2012). However, despite these differences, the digestion process is similar in all fish, with each section of the gut secreting specific components that will aid food passage, increase digestibility and nutrient uptake, and protect the intestinal mucosa from harmful components (Bakke et al., 2010). The digestive process is progressive, starting in the foregut, where proteins, lipids, and carbohydrates are first exposed to an enzymatic breakdown (Bakke et al., 2010). It continues in the midgut, where pancreatic secretions and enzymes contained in the enterocyte brush border break large molecules resulting in smaller, absorbable molecules. Thus, proteins are digested into amino acids or small peptides; triglycerides into fatty acids and glycerol or monoglycerides; and polysaccharides into

monosaccharides (Ugolev, 1972; Bakke et al., 2010). The inability to decompose ingested material for absorption characterizes such dietary components as indigestible.

1.3.1 Endogenous chitin digestion

Chitin enzymatic degradation in fish is postulated to be mainly accomplished by two enzymes: chitinases and chitobiase (Jeuniaux, 1966; Fines and Holt, 2010). Chitinases (CHIT; E.C. 3.2.1.14) starts the process by hydrolyzing chitin into COS (Jeuniaux, 1966; Gutowska et al., 2004). COS have between 2 and 20 units (< 39kDa) (Islam et al., 2017), but the molecules resulting from chitinases action in fish are postulated to be mainly dimers and trimers (Fines and Holt, 2010). This process is followed by the action of β -N-acetylglucosamine-type enzymes (commonly designated chitobiase) (CTB; E.C. 3.2.1.52), which cleave the resulting COS into GlcNAc and GlcN monomers (Danulat, 1987; Krogdahl et al., 2005; Fines and Holt, 2010; Henry et al., 2015). Whereas both enzymes can be found in carnivorous and omnivorous fish inhabiting fresh and marine waters (Danulat and Kausch, 1984; Lindsay et al., 1984; Matsumiya and Mochizuki, 1996; Kurokawa et al., 2004; Fines and Holt, 2010), only chitinases were the target of an extensive molecular characterization (Molinari et al., 2007; Ikeda et al., 2017). Moreover, although chitinases and chitobiase are the main enzymes responsible for chitin digestion, other enzymes present in fish can use chitin as an unspecific substrate, such as lysozyme (Fines and Holt, 2010)

Carnivorous fish, however, are particularly NSP (NRC, 2011) as their endogenously secreted enzymes are usually incapable of effectively cleaving the anomeric β -glycosidic or α -galactosidic bonds present in NSPs, thus rendering polymers, such as chitin, as indigestible carbohydrates with potentially detrimental effects (Krogdahl et al., 2005; NRC, 2011; Sinha et al., 2011). This means that some economically important carnivorous species, such as Atlantic Salmon, gilthead seabream, and ESB have to potentially rely on their microbiota's ability to breakdown these otherwise indigestible dietary components (Kuz'mina, 2008; Roeselers et al., 2011; Ray et al., 2012; Tremaroli and Bäckhed, 2012).

1.4 The fish gut microbiota

Following the comprehensive description of the vastly complex and diverse microbial community inhabiting the human gut (Kroes et al., 1999), research of animal gut-associated microbiomes increased exponentially, evidencing their crucial roles in the

host's homeostasis (Jones, 2013; Huang et al., 2019). The microbiota, defined as the assemblage of living multispecies microorganisms in a contiguous, well-defined environment, contemplating bacteria, fungi, archaea, protists, and algae, is only a small portion of the microbiome (Marchesi and Ravel, 2015). The microbiome, as described by Berg et al. (2020), includes all the above-mentioned elements as well as their "*theatre of activity*", forming a "*dynamic and interactive micro-ecosystem prone to change in time and scale*" crucial for the functioning and health of its eukaryotic host. This includes all elements that are usually not considered living organisms such as viruses, phages, prions, and extracellular nucleic acids, such as relic DNA, viroids, and plasmids (Dupré and O'Malley, 2009), as well as all other structural elements or metabolites produced by the host and its microbiota (Berg et al., 2020).

Interpretation of these communities and their functions within the gut environment created several paradigm shifts. Nowadays, it is accepted that all animals establish symbiotic relationships with their microbiota and, therefore, should be considered a functional and inseparable unit, generally defined as a metaorganism or a holobiont (Bordenstein and Theis, 2015; Theis et al., 2016; Simon et al., 2019). In turn, host's fitness is the result of complex interactions between the combined expression of the host and its associated microorganism genetic information (hologenome), as a response to several biotic and abiotic environmental changes (Zilber-Rosenberg and Rosenberg, 2008).

Given the economic relevance of aquaculture (FAO, 2020), interest in the fish gut microbiome has accelerated exponentially. To date, approximately 150 teleost species have their gut bacterial communities accessed (Perry et al., 2020). The teleost's microbiome, however, is mainly analyzed in the context of sustainable aquaculture, having its main objectives to increase dietary utilization, or enhance fish fitness and immune status (Perry et al., 2020). Despite the diversity of organisms and molecules comprising the fish's gut microbiome, and the array of techniques available for its interpretation, studies are still heavily reliant on the evaluation of the collection of genomes (metagenome) of the bacteria present in the fish gut (Marchesi and Ravel, 2015; Berg et al., 2020; Yukgehnash et al., 2020). While insufficient to provide a full picture of the fish's microbiome, as bacteria are assumed to be the major component of teleost gut microbiota, and, thus, the evaluation of their community's metagenome is efficient for estimating both the individual and community functional potential within the host (Gómez and Balcázar, 2008; Perry et al., 2020; Yukgehnash et al., 2020; Infante-Villamil et al., 2021). Identification of gut bacterial communities allowed researchers to

establish the concept of fish gut core microbiota (Roeselers et al., 2011; Egerton et al., 2018; Kokou et al., 2019), and start understanding the crucial roles played by these microorganisms, namely: regulation of immune-related processes (Rakoff-Nahoum et al., 2004; Pérez et al., 2010; Llewellyn et al., 2014; Feng et al., 2018; López Nadal et al., 2020); modulation of energetic and metabolic homeostasis (Zhang and Davies, 2016; Butt and Volkoff, 2019); enhancement or impairment of effective digestion of dietary components (Roeselers et al., 2011; Ray et al., 2012; Tremaroli and Bäckhed, 2012; Wu et al., 2015); antipathogenic effects (Gómez and Balcázar, 2008; Yukgehnash et al., 2020); and modulation of behavioral and neurologic processes (Borrelli et al., 2016). Ultimately, understanding microbiota establishment and variation as well as its function is crucial to improve aquaculture practices.

1.4.1 Fish gut microbiota establishment and variation

Gut microbial communities are usually divided into two groups: allochthonous (transient) microbiota, generally associated with the digesta or intestinal lumen, and autochthonous (adherent) microbiota, closely attached to the host epithelial surfaces (Ringø et al., 2016). Colonization of fish gut starts after hatching, with the initial colonizing bacteria having their origin in the egg epibiota (microbial community present on the surface of the egg) (Llewellyn et al., 2014). As a result, the initial colonizing bacterial community is species-specific, being the result of variation in binding glycoproteins on the egg surface (Montalban-Arques et al., 2015; Egerton et al., 2018).

At this stage, the fish microbiota is far from complete, as the newly hatched larvae contain only a small portion of their final composition. From this point onwards, the gut will be subsequently occupied by a diverse community following the larvae's mouth opening, and thus the first water ingestion. However, the most relevant shaping of the gut microbial communities starts to occur after first feeding (Lauzon et al., 2010; Egerton et al., 2018; López Nadal et al., 2020). Within the first 50 days of life of several marine and freshwater fish species, the bacterial gut communities will settle, forming a relatively stable microbiota (Roeselers et al., 2011; Egerton et al., 2018; Kokou et al., 2019), mainly composed of Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes (Ghanbari et al., 2015; Yukgehnash et al., 2020).

Upon its establishment, the fish gut microbiota is instrumental in the maturation of fish immune response as it generates crucial immunophysiologic cues that train the gut-associated lymphoid tissue (GALT) for the distinction of commensal microorganisms

from pathogenic bacteria, allowing a rapid and effective assembly of a specific response against pathogens (Acheson and Luccioli, 2004; Salminen et al., 2005; Pérez et al., 2010). Moreover, once established, the autochthonous communities will immediately start acting as the first line of defense, either by competing with pathogens for nutrients, energy, and adhesion sites or by producing secondary metabolites that will inhibit or eliminate competitors' growth and potentially disrupt quorum sensing-mediated gene expression (e.g., expression of virulence-related genes) (Egerton et al., 2018; Legrand et al., 2020)

The fish gut microbiota constitution is, however, highly fluid, with spatial and temporal variations prompted by both biotic and abiotic factors (Sullam et al., 2012; Kokou et al., 2019; Legrand et al., 2020; Minich et al., 2020; Keating et al., 2021). Natural changes in the microbiota can occur both intra- and inter-specifically depending on host characteristics, such as genetic background (Li et al., 2018); sex- and age-dependent differential gene expression and metabolic requirements (Domazet-Lošo and Tautz, 2010; Piazzon et al., 2019); host gut motility (Wiles et al., 2016); immune status (Wang et al., 2018a); and physicochemical profile of the different gut regions (Kokou et al., 2019). Additionally, microbiota variation is also modulated by environmental conditions (e.g., seasonality, salinity, pH, temperature, water quality, and geographic location) (Sullam et al., 2012; Tarnecki et al., 2017; Legrand et al., 2020), diet composition (Ringø et al., 2016), and microorganisms characteristics (Prakash et al. 2011). A healthy gut microbiota is, therefore, highly dependent on a fine balance between the complex communication between hundreds of microorganisms, the host, and the environment. As such, disruption of this equilibrium can have three potential outcomes: reshaping the microbiota towards a more beneficial profile (e.g., increase in lactic acid bacteria (LAB) upon dietary fibers inclusion) (Ringø et al., 2020; Serra et al., 2021); result in stochastic events with no relevant repercussions on its function (Brüssow, 2020); negatively modify the microbiota leading to dysbiosis, a microbiota imbalance with harmful effects on the host's physiological processes (Infante-Villamil et al., 2021).

In the last decades, prompted by the progressive intensification of aquaculture and the subsequent increase of environmental and manipulation stressors, such as crowding, hypoxia, or excessive handling, dysbiosis is becoming a major challenge (Ciji and Akhtar, 2021). Outcomes vary accordingly to the time and intensity of exposition to stressors, being classified as chronic or acute (Ashley, 2007). Chronic stress has long been a key issue in aquaculture, undermining fish quality and production due to its immunosuppressive effect (Yada and Tort, 2016). Acute stress-mediated effects are still

poorly understood but are usually associated with exacerbated inflammatory responses (Guo and Dixon, 2021). Both types of stress, however, are known to promote marked shifts in the fish gut microbial community, destabilizing a key player of the host's physiology modulation (Butt and Volkoff, 2019; Kraimi et al., 2019).

Diet-induced dysbiosis is also an issue. The introduction of novel plant- or animal-based ingredients aiming to increase aquaculture's sustainability, can also generate a negative selective pressure that leads to fish gut microbiota dysbiosis (Ringø et al., 2016; Vargas-Albores et al., 2021). Independently of the microbiota disruption trigger, negatively altering the bacterial communities can lead to cascade reactions that disrupt fish homeostasis by altering gene expression patterns as well as metabolic, energetic, immune, and endocrine processes (Brugman et al., 2018; Butt and Volkoff, 2019; Kraimi et al., 2019). Moreover, microbial community imbalances are also linked to the development of multifactorial diseases, increasing susceptibility to pathogens and leading to intestinal inflammation (Brugman et al., 2018; Rinninella et al., 2019). Ultimately, aquaculture's inability to promote a healthy fish gut microbiota can lead to poor final product quality or even stress-driven or disease-related mass mortality events, causing major economic losses (Ciji and Akhtar, 2021).

Aiming to improve aquaculture sustainability, while maintaining the performance and well-being of the reared fish species, understanding the physiologic repercussions of aquaculture procedures on the fish gut microbiota is becoming a priority for the aquaculture industry (Brugman et al., 2018; Ciji and Akhtar, 2021; Vargas-Albores et al., 2021). As a result, one of the main topics of research within aquaculture microbiome has been the interpretation of the diet-driven bacterial community modulation and its effects on feed digestibility, nutritional uptake, and immune modulation (Ringø et al., 2016; Egerton et al., 2018; Butt and Volkoff, 2019; Vargas-Albores et al., 2021).

1.4.2 HI and TM meals effects on the fish gut bacterial microbiota

Most of the studies considering the impact of IM in the fish gut bacterial communities have been focused on the use of dietary HI meal, particularly in rainbow trout (Table 1.3) (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019; Rimoldi et al., 2021; Biasato et al., 2022). Overall, these studies observed that, independently of the insect life stage (larvae and pre-pupae) or processing method (full fat, partially defatted or defatted) dietary HI meal inclusion between 10% and 40% in rainbow trout diets, usually leads to an increase in Firmicutes and Actinobacteria in detriment of

Proteobacteria and Bacteroidetes in the digesta bacterial community (Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019). These changes reflect the enrichment of bacterial communities known to hydrolyze or ferment chitin, such as *Actinomyces* spp., *Corynebacterium* spp., and *Bacillus* spp. (Huyben et al., 2019; Terova et al., 2019), LAB (Bruni et al., 2018; Terova et al., 2019), or butyrate-producing bacteria, such as *Carnobacterium* spp. (Terova et al., 2019). Similar results were also observed in other fish species, such as Atlantic salmon (Weththasinghe et al., 2022) (Li et al., 2021), Siberian sturgeon (Józefiak et al., 2019), and Pikeperch (Tran et al., 2021). Data on TM meal's ability to modulate digesta communities is still scarce and up to now, results showed no major modulatory effects registered at the phylum level (Table 1.3) (Józefiak et al., 2019; Mikołajczak et al., 2020).

Contrary to digesta microbiota, no clear pattern was observed in the mucosa-associated communities in response to the inclusion of HI or TM meal in fish diets (Table 1.3). For example, when including HI meal (19.5%) in ESB or gilthead seabream diets, Panteli et al. (2021) observed an increase in Actinobacteria and a decrease in Firmicutes in the gut mucosa. However, upon feeding ESB with diets containing TM meal (19.5%), no decrease in Firmicutes was observed while the increase in Actinobacteria was also observed. In rainbow trout fed with diets containing HI meal (10%-30%), it was observed an overall increase in α -diversity, accompanied by a Proteobacteria decrease and a Mycoplasma increase (Rimoldi et al., 2019). In Atlantic salmon fed with diets containing HI meal (14.7%), no major differences were noted at the phylum level. The mucosa-associated communities of this species, however, were reported to be enriched in *Actinomyces*, *Brevibacterium*, *Corynebacterium*, and *Microbacterium*, which are bacteria from the Actinobacteria phylum, or *Enterococcus*, *Lactobacillales*, and *Oceanobacillus*, which are bacteria from the Firmicutes phylum (Li et al., 2021).

Given the complex and variable composition of IM meals, several factors were proposed to influence the fish gut microbiota communities upon IM feeding, such as different amino acids and fatty acids profiles, or species-specific antimicrobial peptides (AMPs) (Gasco et al., 2018; Terova et al., 2019; Mikołajczak et al., 2020) However, due to the reported emergence of carbohydrate-fermenting bacteria (e.g., LAB and/or *Carnobacterium* spp.) and, particularly, chitin-degrading bacteria (e.g., *Actinomyces* spp. and/or *Bacillus* spp.), it was postulated that chitin was a major player of the changes of microbiota communities, as this carbohydrate is known to act as a prebiotic (Bruni et al., 2018) and antibacterial/bacteriostatic agent (Rimoldi et al., 2019; Rimoldi et al., 2021), or to positively modify the host gut architecture or immune status (Zaki et al., 2015; Mohan et

Table 1.3 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the gut microbiota of carnivorous fish species.

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (IM treatment)	Microbial communities	Results	Reference
<i>Hermetia illucens</i>					
<i>Dicentrarchus labrax</i> (European sea bass)	19.5% (30%)	Larvae meal (n/s)	Mucosa- associated	↑Actinobacteria and ↓ Firmicutes ↑ <i>Staphylococcaceae</i>	Panteli et al. (2021)
<i>Sparus aurata</i> (Gilthead seabream)	19.5% (30%)	Larvae meal (n/s)	Mucosa- associated	↑Actinobacteria and ↓ Firmicutes ↑ <i>Staphylococcaceae</i>	Panteli et al. (2021)
<i>Oncorhynchus mykiss</i> (Rainbow trout)	8% (25%), 16% (50%), and 32% (100%)	Larvae meal (Partially defatted)	Digesta- associated	↑Actinobacteria (16% and 32%) ↓ Bacteroidetes (16 and 32%) ↑ <i>Actinomyces</i> , <i>Bacillus</i> , <i>Enterococcus</i> , <i>Oceanobacillus</i> , and <i>Staphylococcus</i> ↓ <i>Clostridium</i> , <i>Campylobacter</i> , <i>Listeria</i> , <i>Lactobacillus</i> , <i>Lactococcus</i> , <i>Leuconostoc</i> , and <i>Pediococcus</i>	Biasato et al. (2022)
	15% (50%)	Larvae meal (n/s)	Digesta- associated	↑ Firmicutes and ↓Proteobacteria ↑ <i>Oceanobacillus</i> , <i>Bacillus</i> , <i>Paenibacillus</i> , <i>Lactobacillus</i> and <i>Cetobacterium</i> ↓ <i>Aeromonas</i>	Rimoldi et al. (2021)
	10% (10%), 20% (20%) and 30% (30%)	Pre-pupae (Partially defatted)	Mucosa- associated	↑ α-diversity ↑ <i>Mycoplasma</i> ↓Proteobacteria	Rimoldi et al. (2019)
	10% (10%), 20% (20%) and 30% (30%)	Pre-pupae (Partially defatted)	Digesta- associated	↑ α-diversity ↑Actinobacteria and Firmicutes ↓Proteobacteria ↑ <i>Lactobacillaceae</i> , <i>Actinomyces</i> , <i>Corynebacterium</i> and <i>Clostridium</i>	Terova et al. (2019)

Table 1.3 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the gut microbiota of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (IM treatment)	Microbial communities	Results	Reference
<i>Oncorhynchus mykiss</i> (Rainbow trout)	30% (30%)	Larvae meal (Untreated and defatted)	Digesta- and mucosa-associated	↑ α -diversity ↑ Firmicutes and Actinobacteria ↓ Proteobacteria ↑ Bacillales, Actinomycetales, and Lactobacillales ↓ Pseudomonadales	Huyben et al. (2019)
	30% (30%)	Pre-pupae (Untreated and defatted)	Digesta- and mucosa-associated	↑ α -diversity ↑ Actinobacteria ↓ Proteobacteria ↑ Actinomycetales and Lactobacillales ↓ Pseudomonadales	
<i>Salmo salar</i> (Atlantic salmon)	20% (25%) and 40% (50%)	Larvae meal (Partially defatted)	Digesta-associated	↑ α -diversity ↑ Lactobacillaceae	Bruni et al. (2018)
	20.4% (17.5%)	Larvae meal (Full fat)	Digesta-associated	↑ Firmicutes and Actinobacteria ↓ Proteobacteria ↑ <i>Actinomyces</i> , <i>Bacillaceae</i>	Weththasinghe et al. (2022)
	14.9% (17.5%)	Larvae meal (Defatted)	Digesta-associated	↑ Firmicutes and Actinobacteria ↓ Proteobacteria ↑ <i>Actinomyces</i> , <i>Bacillaceae</i>	Weththasinghe et al. (2022)
	24.53% (17.5%)	Larvae meal (de-chitinized)	Digesta-associated	↑ Firmicutes and Actinobacteria ↓ Proteobacteria ↑ <i>Actinomyces</i> , <i>Corynebacterium</i> , <i>Bacillaceae</i>	Weththasinghe et al. (2022)
	7.2% (3.2%)	Larvae (exoskeleton)	Digesta-associated	↑ Actinobacteria ↑ <i>Actinomyces</i>	Weththasinghe et al. (2022)

Table 1.3 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the gut microbiota of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (IM treatment)	Microbial communities	Results	Reference
<i>Salmo salar</i> (Atlantic salmon)	14.75% (100%)	Larvae meal (Partially defatted)	Digesta- or mucosa- associated	↑ <i>Actinomyces</i> , <i>Bacillaceae</i> , <i>Beutenbergiaceae</i> , <i>Brevibacterium</i> , <i>Corynebacterium</i> , <i>Enterococcus</i> , <i>Lactobacillales</i> , <i>Microbacterium</i> , and <i>Oceanobacillus</i>	Li et al. (2021)
<i>Sander lucioperca</i> (Pikeperch)	9% (25%), 18% (50%), and 36% (100%)	Larvae (Defatted)	Digesta- associated	↑ α-diversity (18%) ↑ <i>Bacteroides</i> ↑ <i>Clostridium</i> (9% and 18%)	Tran et al. (2021)
<i>Acipenser baerii</i> (Siberian sturgeon)	15% (29%)	Larvae meal (Full fat)	Digesta- associated	↑ <i>Bacillus</i> , <i>Carnobacterium</i> , <i>Enterococcus</i> , <i>Lactobacillus</i> , <i>Enterobacteriaceae</i> and <i>Aeromonas</i> ↑ <i>Clostridium leptum</i> subgroup and <i>Clostridium coccoides</i>	Józefiak et al. (2019)
<i>Epinephelus fuscoguttatus</i> ♀ × <i>Epinephelus lanceolatus</i> ♂ (Pearl gentian grouper)	7% (10%), 14% (20%), and 21% (30%)	Larvae meal (Full fat)	n/s	↓ diversity and abundance with increased HI meal inclusion ↓ OTU (21%) ↑ Firmicutes (21%), ↑ Fusobacteria (14%), ↓ Spirochaetes (14%), and ↑ <i>Lactobacillus</i> (21%)	Huang et al. (2022)

Table 1.3 Effects of dietary inclusion of *Hermetia illucens* or *Tenebrio molitor* meals on the gut microbiota of carnivorous fish species. (continued)

Fish species (Common name)	Inclusion level (FM substitution)	Insect stage (IM treatment)	Microbial communities	Results	Reference
<i>Tenebrio molitor</i>					
<i>Dicentrarchus labrax</i> (European sea bass)	19.5% (30%)	Larvae meal (n/s)	Mucosa- associated	↑Actinobacteria	Panteli et al. (2021)
<i>Sparus aurata</i> (Gilthead seabream)	19.5% (30%)	Larvae meal (n/s)	Mucosa- associated	↑Actinobacteria	Panteli et al. (2021)
<i>Oncorhynchus mykiss</i> (Rainbow trout)	20% (100%)	Larvae meal (Partially defatted)	Mucosa- associated	↑ <i>Lactobacillaceae</i> (particularly <i>Lactobacillus</i>) ↓ <i>Vibrionaceae</i> , <i>Fusobacteriaceae</i> , <i>Staphylococcaceae</i> , and <i>Photobacterium</i>	Terova et al. (2021)
<i>Salmo trutta m. trutta</i> (Sea Trout)	10% (42%)	Larvae meal (Hydrolyzed)	Digesta- associated	↓ <i>Carnobacterium</i> and <i>Lactobacillus</i>	Mikołajczak et al. (2020)
<i>Acipenser baerii</i> (Siberian sturgeon)	15% (40%)	Larvae meal (Full fat)	Digesta- associated	↑ <i>Clostridium coccoides</i> , <i>Bacillus</i> , <i>Carnobacterium</i> and <i>Enterococcus</i>	Józefiak et al. (2019)

The symbols represent an increase (↑), decrease (↓), or no effect (→) on the tested parameters on fish fed diets containing either HI or TM meal, relative to the control.

FM- fish meal; IM – insect meal; n/s – not specified

al., 2019; Abdel-Ghany and Salem, 2020; Salam et al., 2021). In turn, the enrichment of gut microbiota communities with chitin-degrading or chitin-fermenting bacteria is crucial to increase carnivorous fish's ability to degrade chitin, as they are highly dependent on gut microbiota to degrade complex carbohydrates.

1.4.3 Fish gut microbiota roles in feed digestibility

The carbohydrolitic potential of the fish gut bacterial community has been extensively characterized (Nayak, 2010; Ray et al., 2012; Ringø et al., 2016). However, given the fish gut microbiota complexity and intra- and inter-specific variation, it is difficult to precisely estimate its contribution to host digestion (Ray et al., 2012). There is, however, unequivocal evidence hinting at a readjustment of the fish gut bacterial community towards organisms capable to degrade newly introduced carbohydrate substrates such as plant-derived NSPs (Askarian et al., 2013; Ringø et al., 2016; Serra et al., 2019; Serra et al., 2021), or chitin (Askarian et al., 2012; Antonopoulou et al., 2019; Józefiak et al., 2019; Li et al., 2021; Panteli et al., 2021; Rimoldi et al., 2021; Terova et al., 2021; Tran et al., 2022b).

1.4.3.1 Bacterial chitin utilization

Bacterial chitin digestion is a complex process. While most of the chitinolytic processes are transversal to several bacterial species, variations in the chitinolytic machinery and the chitin lysis process do occur depending on the environmental niche occupied by bacteria (Beier and Bertilsson, 2013; Wieczorek et al., 2019). As such, effective chitin digestion is reliant on a synergic action of the bacterial community, rather than on the action of a specific taxon (Datta et al., 2016; Wieczorek et al., 2019).

Bacterial chitin digestion usually starts with the adhesion of chitinolytic bacteria to chitin particles through chemotaxis, chemotropism, or random collision (Itoh and Kimoto, 2019). After adhesion to chitin particles, chitinases expression is usually increased, mostly as a response to the increased presence of COS (with a degree of polymerization between 2-6) in the vicinity of chitinolytic bacteria, and is followed by the secretion of multiple enzymes that, through synergistic action, will breakdown the chitin into monomers or small COS (Keyhani and Roseman, 1999; Itoh and Kimoto, 2019).

While chitinases can immediately start to break the chitin matrix, chitin catabolism usually starts with the action of chitin-oxidizing bacterial lytic polysaccharide monooxygenase

(LPMOs, E.C. 1.14.99.53; Figure 1.5) (Itoh and Kimoto, 2019). Discovered in 2010 (Vaaje-Kolstad et al., 2010), LPMOs catalyze the cleavage of 1,4-glycosidic bonds in crystalline regions of chitin (Courtade and Aachmann, 2019). This oxidative process disrupts the polymer crystallinity creating new open ends, thus increasing chitin's bioavailability for endo- (E.C. 3.2.1.14), and exo-chitinase (E.C. 3.2.1.200-201) action (CHIT; Figure 1.5 (Bissaro et al., 2018)). Endochitinase randomly cleave chitin internal sites generating non-reducing ends, and exochitinase cleave the chitin polymer either at reducing ends forming (GlcNAc)_n (n= 2 to 6) or at the novel non-reducing ends, forming (GlcNAc)₂ residues (Itoh and Kimoto, 2019; Shimoi et al., 2020). Finally, β -N-acetyl-hexosaminidases (CTB, E.C. 3.2.1.52; Figure 1.5), hydrolyze the GlcNAc dimers to GlcNAc monomers (Itoh et al., 2013).

Bacteria can also increase chitin digestion through an alternative route in which chitin deacetylases (CDA, E.C. 3.5.1.41) released into the extracellular space can convert chitin into chitosan releasing acetate in the process (Figure 1.5) (Tsigos et al., 2000). Chitosan can then be sequentially cleaved into GlcN, in a two-steps process: chitosanases (CSN; E.C. 3.2.1.132) catalyze the endohydrolysis of β (1 $\rightarrow$ 4)-linked GlcN residues; and exo- β -glucosaminidases (CSXA; E. C. 3.2.1.165) catalyze the hydrolysis of COS into GlcNAc and GlcN monomers at non-reducing ends (Figure 1.5) (Shinya and Fukamizo, 2017). Given chitosan's increased solubility compared to chitin, this increases enzymes' accessibility to the chitin/chitosan molecules or the entrapped proteins in the chitin matrix, thus potentially increasing protein digestion rates (Eijsink et al., 2008; Islam et al., 2017; Dawood and Koshio, 2020). Moreover, bacterial chitin fermentation can result in the formation of short-chain fatty acids (e.g., butyric, propionic, and acetic acid) and, given these molecules' ability to enhance intestinal health and nutrient absorption (Mao et al., 2013; Bhat et al., 2019; Tran et al., 2020), this process also contributes for the nutritional valorization of fish diets containing IM.

1.5 Potential strategies to solve the chitin conundrum

To increase chitin digestibility within the fish's gut, several strategies can be employed. For example, by applying a specific nutritional stimulus with chitin during a short, critical window, of fish development, long-term epigenetic or metabolic modifications can be triggered, improving the fish's future response to the same agent (e.g., IM or chitin) (Hou and Fuiman, 2020). Another possibility is to identify and selectively breed the best-performing individuals fed with a specific diet (insect-based diet) (Verdal et al., 2018). While, in theory, the application of nutritional programming techniques (first) and

selective breeding (later) can offer generational incremental gains, these techniques are still in their infancy (Verdal et al., 2018; Hou and Fuiman, 2020). Their applicability is extremely difficult as they rely on the maintenance of accurate feed efficiency and genetic record, as well as on selected strains productivity, viability, and reproduction (Lind et al., 2012). Moreover, the application of these techniques (alone or in tandem) is species-specific, thus decreasing its scope of application.

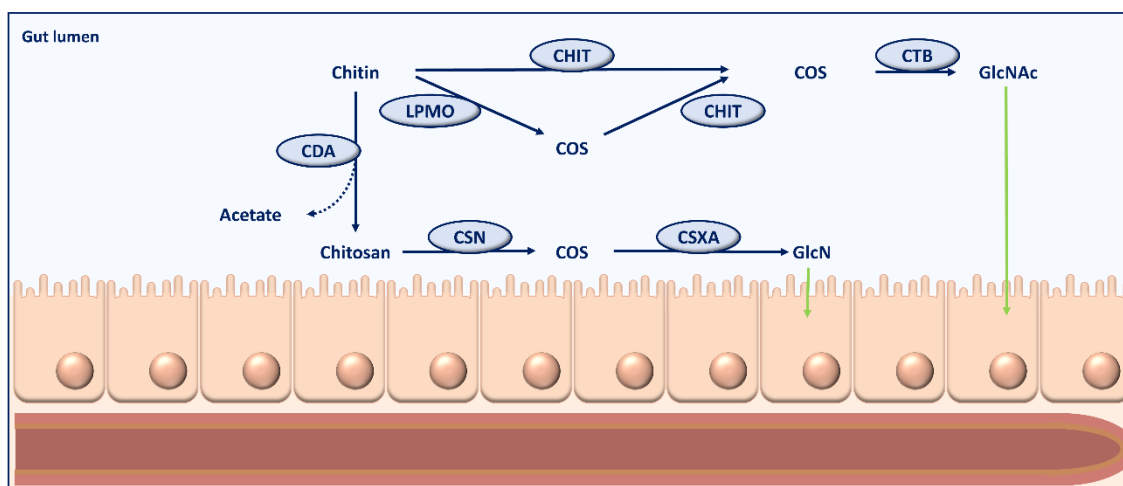


Figure 1.5 Schematic representation of the potential chitin digestion mechanisms occurring in the fish's intestine. Dark blue full arrows represent proposed pathways for both fish and bacteria. Dark blue dashed arrows represent pathways not described in fish and, thus, putatively belonging to bacteria only. Green arrows represent the potential uptake of N-acetylglucosamine (GlcNAc) and D-glucosamine (GlcN) by the fish cells. Abbreviations are as follows: COS –chitooligosaccharides; LPMO – chitin-oxidizing lytic polysaccharide monooxygenases (E.C. 1.14.99.53); CHIT – endo-chitinase (E.C. 3.2.1.14), and exochitinase (E.C. 3.2.1.200-201); CTB - β -N-acetyl-hexosaminidases (E.C. 3.2.1.52); CDA - chitin deacetylases (E.C. 3.5.1.41); CSN - chitosanases (E.C. 3.2.1.132); CSXA - exo- β -glucosaminidases (E.C. 3.2.1.165).

On the other hand, increasing chitin digestibility by modifying the chitinolytic activity from non-endogenous sources can be transversely applied and, thus, industrially appealing. Moreover, as these methodologies are essentially based on dietary strategies, they offer a flexible and sustainable approach as these do not require any technological or structural adaptation to be enforced (Herrera et al., 2019). Among these, enzymatic supplementation has been particularly effective to increase PF digestibility in fish feeds (Dalsgaard et al., 2012; Castillo and Gatlin III, 2015; Liang et al., 2022). However, to date, no successful application has been achieved when using IM in fish feeds. In fact, upon adding carbohydrases or proteases to diets containing 25% TM meal, Gasco et al. (2016) noted that not only the supplementation strategy failed to increase diets digestibility, but it also led to decreases in the digestibility of both proteins and

carbohydrates. As mentioned by the authors, failure to increase digestibility could result from the use of non-specific enzymes to target IM components (Gasco et al., 2016). On the other hand, as pointed out by several authors, detrimental results on digestibility upon enzymatic supplementation can be an outcome of improper supplementation strategies that negatively impact the residing gut microbiota (Ringø et al., 2015; Gasco et al., 2016; Liang et al., 2022).

Microbiota manipulation through dietary strategies is another potential alternative to increase chitin digestibility. As previously discussed in sub-chapter 1.4.2 (Table 1.3), the inclusion of either HI or TM meal into the fish diet can trigger modifications in fish gut microbiota resulting in a greater presence of chitin-degrading or chitin fermenting bacteria. However, as thoroughly demonstrated in chapter 1.2.2 (Table 1.1), the promoted changes do not seem to affect the overall diet digestibility. Moreover, dietary microbiota modulation is species-specific and can be outweighed by the pre-existing microbiota as well as several biotic (e.g., age and immune status) and abiotic (e.g., seasonality or water quality) stimuli (Kokou et al., 2019; Legrand et al., 2020; Minich et al., 2020; Keating et al., 2021). Further, this process can lack both reproducibility and/or replicability, as shown by the different outcomes described in Table 1.3 (sub-section 1.4.2). Alternatively, dietary probiotic supplementation could help to offset the natural gut microbiota variations and present itself as a valid alternative to improve chitin digestibility (Egerton et al., 2018; El-Saadony et al., 2021).

1.6 Probiotics

As defined by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), probiotics are “live micro-organisms which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). For animal feed, as stated in a 2016 FAO report, probiotics’ main function is to “...maintain and improve the performance (productivity and growth) of the animal and prevent and control enteric pathogens” (Bajagai et al., 2016). This definition is the most widely accepted, being embraced by the International Scientific Association for Probiotics and Prebiotics in 2014 (Hill et al., 2014).

The application of probiotics as a feed supplement with the aim of enhancing FCR and growth performance in carnivorous fish species has been widely successful, as demonstrated in rainbow trout fed diets supplemented with a mixture of *Bacillus subtilis* and *B. licheniformis* (1:1) (0.8×10^6 colony forming units (CFU) g⁻¹ feed; 1.6×10^6

CFU g⁻¹ feed, and 2.4×10^7 CFU g⁻¹ feed) (Naraghi et al., 2022); Atlantic salmon fed diets supplemented with a mixture of *B. velezensis* V4 plus (2.5×10^7 CFU g⁻¹ feed) and *Rhodotorula mucilaginosa* (2.5×10^8 CFU g⁻¹ feed) (Wang et al., 2019a); ESB fed diets supplemented with either *Virgibacillus proomii* or *B. mojavensis* (1×10^6 CFU g⁻¹ feed) (Hamza et al., 2016); gilthead seabream fed diets supplemented with *Lactococcus lactis* (5×10^6 CFU g⁻¹ feed) (Moroni et al., 2021); Asian seabass fed diets supplemented with *B. thuringiensis* (1×10^9 CFU g⁻¹ feed) (Ghanei-Motlagh et al., 2021b); and Siberian sturgeon fed diets supplemented with a probiotic mixture (3×10^6 CFU g⁻¹ feed) (Sayed Hassani et al., 2020).

While the underlining mechanisms resulting in increased feed utilization by fish are rarely identified, researchers postulate that this effect is the result of two main modes of action: i) production and secretion of a myriad of enzymes (e.g., trypsin, lipases, phytases, amylases, cellulases, and chitinases) that can digest otherwise indigestible components for the fish (Figure 1.6 A1) (Ray et al., 2012; Van Doan et al., 2020); ii) increase of the feed nutritional value through fermentation processes, releasing relevant secondary micro- and macro-nutrients such as fatty acids, amino acids, cofactors, minerals, or vitamins (Figure 1.6 A2) (Ringø et al., 2020; Wuertz et al., 2021). Improvement of feed conversion, however, is not limited to increased hydrolysis or fermentation, as probiotics can also engage with various fish endocrine processes that control appetite and growth regulation, or the fish gut morphology (Figure 1.6 A3 and 6 A4) (Wuertz et al., 2021).

Probiotics, when supplemented into fish diets, were also able to increase carnivorous fish survivability against several pathogens, as demonstrated in rainbow trout fed with diets supplemented with *Lactococcus lactis* subsp. *lactis* (1×10^9 CFU g⁻¹ feed), challenged with *Yersinia ruckeri* (Yeganeh et al., 2021); Atlantic salmon fed with diets supplemented with a mixture of *B. velezensis* V4 and *Rhodotorula mucilaginosa* (5×10^6 CFU g⁻¹ feed and 5×10^7 CFU g⁻¹ feed, respectively), challenged with *A. salmonicida* (Wang et al., 2019a); ESB fed diets supplemented with *B. velezensis* (1×10^6 CFU g⁻¹ feed) challenged with *V. anguillarum* (Monzón-Atienza et al., 2022); Asian seabass fed diets supplemented with *Lactobacillus fermentum* (2×10^6 CFU g⁻¹ feed) challenged with *A. hydrophila* (Lin et al., 2017a); and turbot fed diets supplemented with *Bacillus spp.* (5×10^8 g⁻¹ feed) challenged with *V. anguillarum* (Chen et al., 2016).

While the modes of action are not fully understood, probiotics' ability to increase fish disease resistance has been suggested to be an outcome of probiotics' immunostimulant action resulting from: i) probiotics' display of microbe-associated molecular patterns (MAMPs) and its subsequent recognition by pattern recognition receptors (PRRs) (e.g.,

Toll-like receptors (TLRs) and lectins) present in the gut epithelial surface or GALT leukocytes (Figure 1.6 B1) (Rakoff-Nahoum et al., 2004; Llewellyn et al., 2014); ii) the establishment of host-microbiota crosstalk mechanisms prompted by bacteria release of specific proteins or metabolites (Figure 1.6 B2) (Montalto et al., 2009; Kelly and Salinas, 2017; Feng et al., 2018). Moreover, gut microbiota can increase disease resistance by i) competing with pathogenic bacteria for adhesion sites (Figure 1.6 C1) or nutrients (Figure 1.6 C2); ii) producing natural antimicrobial compounds (NAC) that eliminate pathogenic bacteria (Figure 1.6 C3) or disrupt their growth, biofilm formation or quorum-sensing (Figure 1.6 C4) (Verschuere et al., 2000; Gómez and Balcázar, 2008).

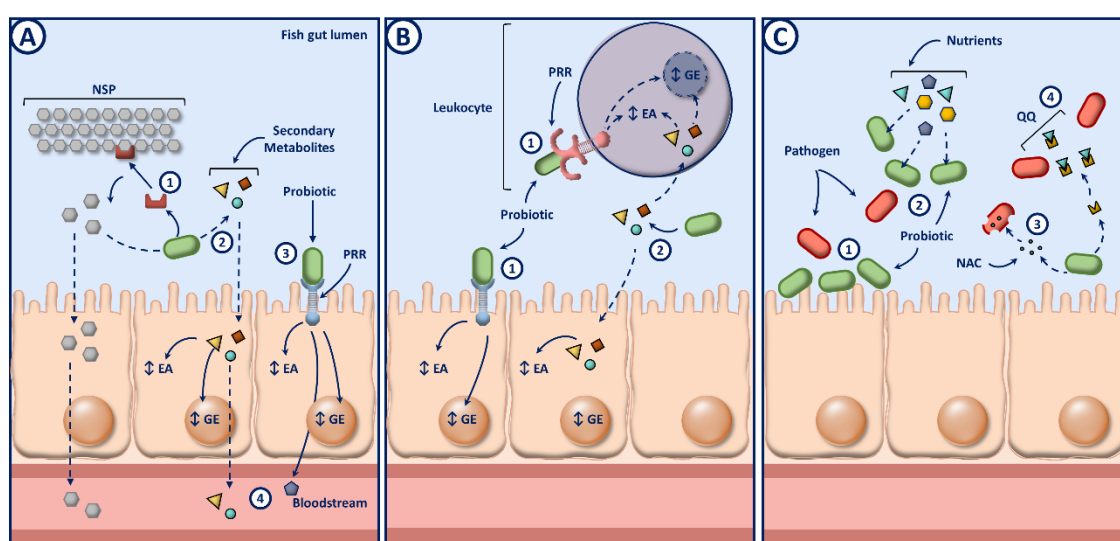


Figure 1.6 Potential modes of action of probiotics. A1: secretion of digestive enzymes; A2: nutritional valorization through the production of relevant secondary metabolites; A3: physiologic alterations triggered by probiotic recognition; A4: endocrine and neuro-endocrine signaling triggered by probiotic metabolites or probiotic recognition; B1: triggering of immunophysiological cues due to probiotic recognition; B2: triggering of immunophysiological cues as a response to probiotic metabolites; C1: competition for adhesion sites; C2: competition for nutrients; C3: production and secretion of natural antimicrobial compounds (NAC) with antibacterial action; C4: production and secretion of molecules with quorum-quenching activity. Abbreviations are as follow: NSP – non-starch polysaccharides; PRR – pattern recognition receptor; $\uparrow$ EA – Modulation of enzymatic activity; $\uparrow$ GE – Modulation of gene expression; NAC – natural antimicrobial compound; QQ – quorum-quenching.

1.6.1 *Bacillus* spp. as probiotics

Within the available array of known probiotic bacteria, *Bacillus* spp. are particularly interesting for aquaculture application (Kuebutornye et al., 2019; Soltani et al., 2019; Kuebutornye et al., 2020; Ringø et al., 2020). The *Bacillus* genus is comprised of aerobic or facultative anaerobic rod-shaped Gram-positive bacterial species, with the ability to

form spores (Kuebutornye et al., 2020). Sporulation, which starts with an asymmetric cell division, occurs when environmental conditions do not favor further population growth (e.g., starvation) (Setlow and Johnson, 2019). This division is followed by the engulfment of the smaller cell (forespore or prespore) by the larger cell (mother cell), which then mobilizes its resources to form a series of protective layers, including a thick layer of peptidoglycan (spore cortex) and two proteinaceous layers (inner and outer spore coat), all assembled around the prespore (Errington, 2003). Simultaneously, the prespore initiates the synthesis of novel small acid-soluble proteins (SASP) that protect the DNA located at the center of the prespore (spore core) (Setlow and Johnson, 2019). Additionally, the spores' core starts to accumulate pyridine-2,6 dicarboxylic acid (dipicolinic acid) as well a significant amount of divalent cations, such as Mg^{2+} , Mn^{2+} , and Ca^{2+} chelated with DPA (CaDPA), which result in a decrease of the spores core water content (Setlow and Johnson, 2019). Once the spore formation is completed, the mother cell produces autolysins that allow for the release of the mature spore into the environment (Setlow and Johnson, 2019).

Together these structural characteristics will equip the newly formed, metabolically dormant, spore with the ability to withstand extreme physicochemical insults (thus protecting DNA), including heat dehydration, high pressures, UV radiation, or several chemicals (Margosch et al., 2004; Cortesão et al., 2019; Setlow and Johnson, 2019; Christie and Setlow, 2020). Due to its remarkable resistance, *Bacillus* spp. spores are highly appealing for industrial applications as these thermo- and pressure-resistant structures can withstand cheaper and more efficient dehydration processes, such as spray-drying, or be included in the diets before feed extrusion without negative effects on their characteristics and viability (Setlow, 2006; Huang et al., 2017; Niu et al., 2018). Moreover, *Bacillus* spp. spores can also withstand harsh gastrointestinal conditions, thus being able to survive gut transit, ultimately germinating and colonizing within the host's gut (Tam et al., 2006; Nayak, 2021)

Given their physiological and metabolic plasticity, *Bacillus* spp. are practically ubiquitous, being commonly found in food, soil, aquatic environments, and in the gut of animals and humans (Iriarte et al., 2000; Barbosa et al., 2005; Stenfors Arnesen et al., 2008; Egan et al., 2021). *Bacillus* spp. are also routinely found within the fish gut, allowing for the selection of novel probiotics directly from autochthonous communities (Askarian et al., 2012; Ray et al., 2012; Serra et al., 2019; Soltani et al., 2019; Santos et al., 2021b). As microorganisms tend to present their highest survival and physiological activities in their natural habitat, this will ultimately result in better performance when compared to the

currently existing probiotics isolated from terrestrial sources (Wuertz et al., 2021). Moreover, although not all *Bacillus* spp. are considered safe for probiotic applications (e.g., *B. cereus* with toxin production abilities) (EFSA, 2014), several *Bacillus* spp., such as *B. subtilis* or *B. licheniformis*, are included in the qualified presumption of safety (QPS) list of the European Food Safety Authority (EFSA) and, thus, are generally regarded as safe for use in food and feeds (EFSA, 2022). While this status does not exclude the need for a comprehensible biosafety assessment before food and feed application (EFSA, 2012; 2014; 2018), it is a good indicator of the probability of finding an industrially applicable and safe probiotic.

When supplemented into carnivorous fish feeds, probiotic *Bacillus* spp. have been demonstrated to influence different fish physiologic functions, among which is the ability to enhance growth performance and feed utilization efficiency (Table 1.4). This ability was verified in several fish species, such as rainbow trout fed diets supplemented with *B. subtilis*, *B. licheniformis*, or a mixture (1:1) of both (2×10^6 CFU g⁻¹ feed) (Park et al., 2017); ESB fed diets supplemented with *B. mojavensis* (2×10^5 CFU g⁻¹ feed) (Hamza et al., 2016); Asian seabass fed diets supplemented with *B. licheniformis* and *B. subtilis* (1:1 mixture) (1×10^6 CFU g⁻¹ feed) (Adorian et al., 2019); grouper fed diets supplemented with *B. subtilis* (1×10^4 , 1×10^6 , and 1×10^8 CFU g⁻¹ feed) (Liu et al., 2012); hybrid sturgeon (*Acipenser schrenkii* ♂ and *A. baeri* ♀) fed diets supplemented with *B. amyloliquefaciens* (4×10^7 CFU g⁻¹ feed) (Fei et al., 2018); and Tongue sole (*Cynoglossus semilaevis*) fed diets supplemented with *B. subtilis* (1×10^7 CFU g⁻¹ feed) (Wang et al., 2021b).

While studies lack information on the contribution of probiotics on the diets' apparent digestibility coefficients, it has been postulated that probiotics contribute to the enhancement of fish FBW and FCR through the expression and secretion of several lytic enzymes that aid in the digestive process (Soltani et al., 2019; Nayak, 2021). While this hypothesis is mostly grounded on *in vitro* studies, which demonstrated that *Bacillus* spp. isolated from fish, such as *B. amyloliquefaciens*, *B. subtilis*, or *B. licheniformis*, can produce high-activity amylases, proteases, lipases, cellulases, phytases, xylanases, and chitinases (Askarian et al., 2012; Dutta et al., 2015; Ghosh et al., 2017; Dutta et al., 2018), several *in vivo* studies also support this hypothesis (Table 1.4) (López et al., 2016; Adorian et al., 2019; Wang et al., 2021b; Yang et al., 2021). Moreover, *Bacillus* spp. ability to positively modulate fish gut morphology must also be taken into account as increases in weight gain and feed efficiency were verified in tandem with positive gut morphology modulation, such as in golden pompano (*Trachinotus ovatus*) fed diets

supplemented with *B. pumilus* (1×10^8 CFU g⁻¹ feed) (Liu et al., 2020), or in rainbow trout fed diets supplemented with *B. subtilis* and *B. licheniformis* (mixture 1:1) (8×10^5 , 1.6×10^6 , and 2.4×10^6 CFU g⁻¹ feed) (Naraghi et al., 2022), which presented their intestinal villus length and width increased.

While no *Bacillus* spp. were used up to date as a tool to increase chitin digestibility, species from this genus have been demonstrated to possess all the needed chitinolytic machinery. Chitin-active LPMOs as well as endo- and exo-chitinases have been annotated in several *Bacillus* spp. such as *B. licheniformis* (Songsiriritthigul et al., 2010; Waghmare and Ghosh, 2010; Courtade et al., 2015; Laribi-Habchi et al., 2015), *B. thuringiensis* (Zhang et al., 2015; Saleem et al., 2016), and *B. amyloliquefaciens* (Das et al., 2012; Gregory et al., 2016; Guo et al., 2022). *B. licheniformis* were also demonstrated to have chitin-fermenting abilities, leading to the production of chitosan or short-chain fatty acids, such as acetate or butyrate (Mao et al., 2013; Bhat et al., 2019), further increasing chitin nutritional value.

When supplemented into carnivorous fish diets, *Bacillus* spp. probiotics were also found to enhance disease resistance (Table 1.4). For example, *B. subtilis* increased the survivability of rainbow trout to *A. salmonicida* (supplementation of 1×10^6 CFU g⁻¹ feed) (Park et al., 2017) and *Aeromonas* spp. (1×10^6 CFU g⁻¹ with 100% survival) (Newaj-Fyzul et al., 2007). *B. subtilis* was also able to increase the survivability of other fish species such as grouper (1×10^6 g⁻¹ feed and 1×10^9 g⁻¹ feed) to *Streptococcus* sp. (Liu et al., 2012) or turbot (5×10^8 g⁻¹ feed) to *V. anguillarum* (Chen et al., 2016). Other *Bacillus* spp. have been equally effective in increasing fish disease resistance when supplemented into fish feeds. *B. velezensis*, increased the survivability of ESB (supplementation of 1×10^6 CFU g⁻¹ feed) to *V. anguillarum* (Monzón-Atienza et al., 2022), pearl gentian grouper (1×10^7 CFU g⁻¹ feed) to *V. harveyi* (Li et al., 2019), and olive flounder (*Paralichthys olivaceus*) (1×10^7 CFU g⁻¹ feed) challenged with *Streptococcus iniae* (Cha et al., 2013) and *B. thuringiensis* increased the survivability of Asian seabass to either *V. harveyi* or *V. alginolyticus* (Ghanei-Motlagh et al., 2021b). Supplementation with multispecies probiotics such as *B. subtilis*:*B. licheniformis* mixture (1:1) tested by Park et al. (2017) in rainbow trout was also shown to effectively reduce the mortality of rainbow trout to *A. salmonicida*.

Bacillus spp. have several characteristics that can influence fish resistance to pathogens. For example, *Bacillus* spp. can either be recognized by the fish immune system or produce relevant metabolites (e.g., amino acids, fatty acids, vitamins, and cofactors) that generate immunophysiologic cues that enhance fish disease (Kuebutornye et al., 2020;

Ringø et al., 2020; Nayak, 2021). *Bacillus* spp. ability to augment fish disease resistance observed in several of the above-mentioned cases was preceded by an enhanced immune status (e.g., increased serum lysozyme, myeloperoxidase, bactericidal and phagocytic activity) (Table 1.4) (Newaj-Fyzul et al., 2007; Liu et al., 2012; Ghanei-Motlagh et al., 2021a; Ghanei-Motlagh et al., 2021b; Monzón-Atienza et al., 2022). *Bacillus* spp. are also known to have several natural antimicrobial compounds that can kill or interfere with Gram+ and Gram- pathogens growth (Kuebutornye et al., 2020; Nayak, 2021). Among these, bacteriocins are reported as one of the main antimicrobial compounds responsible for this action, as they are widely produced by several *Bacillus* species used in aquaculture as probiotics, such as *B. velezensis* (Yi et al., 2018), *B. amyloliquefaciens* (An et al., 2015), *B. subtilis* (Compaoré et al., 2013), and *B. licheniformis* (Teixeira et al., 2009). Additionally, the *Bacillus* genus is known to produce several quorum-quenching molecules, such as AHL-lactonases, which, by hindering pathogen communication mechanisms, limit bacteria growth and virulence, as demonstrated both *in vitro* (Zhou et al., 2016; Ghanei-Motlagh et al., 2020; Santos et al., 2021b), and *in vivo* (Zhou et al., 2016; Ghanei-Motlagh et al., 2021a; Ghanei-Motlagh et al., 2021b; Santos et al., 2021a).

1.7 European sea bass

In Portugal and, more broadly within the Mediterranean region, ESB stands out as a high-value species (INE, 2020; FAO, 2022c). Belonging to the Moronidae family (Teleostei: Perciformes), ESB is a euryhaline (0–40 ppt salinity) and eurythermal (2–32 °C) fish species. In nature, it usually inhabits coastal marine shallow waters with less than 100 meters depth, ranging from the Mediterranean to the north-eastern Atlantic Ocean, as well as the Black Sea, being regularly found, from spring to fall, in estuaries (FAO, 2022a).

ESB was the first non-salmonid marine species cultivated in Europe for commercial purposes. Today, it is mostly an aquaculture-produced species, with aquaculture production (276 422 tons, 97.9% of total ESB production) largely outweighing its capture (5998 tons, 2.1% of total ESB production) (FAO, 2022b). Out of the 276 422 tons of ESB produced worldwide, the EU is responsible for 81 369 tons (29.4%) with an estimated market value of 470 million euros, with Greece (50.1%, 40 763 tons) and Spain (28.0%, 22 765 tons) as its main producers (EUMOFA, 2022; FAO, 2022c). Adding Turkey, the largest ESB producer (148 907 thousand tons), and Egypt (32 555 thousand tons), the Mediterranean zone encompasses 95.1% of all ESB produced.

Table 1.4 Effect of dietary supplementation with *Bacillus* spp. on the growth performance, digestive enzymes activity, gut morphology, nonspecific innate immune response, and disease resistance of carnivorous fish species.

Fish species (Common name)	Probiotic species	Dietary inclusion level (x 10 ⁶ CFU g ⁻¹ feed)	Results [tissue]	References
<i>Dicentrarchus labrax</i> (European sea bass)	<i>B. amyloliquefaciens</i>	10	→ FBW and FCR ↑ Villi length and number	Chouayekh et al. (2022)
	<i>B. velezensis</i>	1	↑ Serum BA, LYZ, NO, and PA prior to challenge ↑ Survival when challenged with <i>V. anguillarum</i>	Monzón-Atienza et al. (2022)
	<i>B. mojavensis</i>	1	↑ FBW and ↓ FCR	Hamza et al. (2016)
<i>Sparus aurata</i> (Gilthead seabream)	<i>B. amyloliquefaciens</i>	n/s (0.1%)	→ FBW, SGR and FCR ↑ villus length	Simó-Mirabet et al. (2017)
<i>Oncorhynchus mykiss</i> (Rainbow trout)	<i>B. subtilis</i> and <i>B. licheniformis</i> (MIX)	0.8, 1.6 and 2.4	↑ FBW and SGR and ↓ FCR concomitantly with increasing probiotic supplementation ↑ Villus length, villus width, and crypt depth [distal intestine]	Naraghi et al. (2022)
	<i>Bacillus subtilis</i>	2	↑ WG, SGR, and FE ↑ Serum LYZ and → serum MPO ↑ Survival when challenged with <i>A. salmonicida</i>	Park et al. (2017)
	<i>B. licheniformis</i>	2	↑ WG and SGR and → FE ↑ Serum LYZ and → serum MPO ↑ Survival when challenged with <i>A. salmonicida</i>	Park et al. (2017)
	<i>B. subtilis</i> and <i>B. licheniformis</i> (MIX)	2	→ WB and FE, ↑ SGR ↑ Serum LYZ and → serum MPO ↑ Survival when challenged with <i>A. salmonicida</i>	Park et al. (2017)

Table 1.4 Effect of dietary supplementation with *Bacillus* spp. on the growth performance, digestive enzymes activity, gut morphology, nonspecific innate immune response, and disease resistance of carnivorous fish species. (Continued)

Fish species (Common name)	Probiotic species	Dietary inclusion level (x 10 ⁶ CFU g ⁻¹ feed)	Results [tissue]	References
<i>Oncorhynchus mykiss</i> (Rainbow trout)	<i>B. subtilis</i> and <i>B. cereus toyoi</i> (MIX)	0.006 and 1.5	→ FBW, SGR and FCR ↑ Lamina propria and submucosa width ↑ Number of inflammatory cell and enterocyte damage	Ramos et al. (2017)
	<i>Bacillus</i> spp.	0.1, 0.2, 0.3, and 0.4	↑ FBW, SGR, PER and ↓ FCR (0.2) → FBW, SGR, PER and FCR (0.1, 0.3, and 0.4)	Adineh et al. (2013)
	<i>B. subtilis</i>	0.01, 0.1, 1, 10, 100, 1000	↑ PA, RB [head kidney leukocytes] (10) ↑ Serum LYZ, MPO, total antiprotease activity and BA (10) ↑ Survival when challenged with <i>Aeromonas</i> sp. (best: 1, 100% survival)	Newaj-Fyzul et al. (2007)
<i>Salmo trutta</i> (Brown trout)	<i>B. subtilis</i> and <i>B. cereus toyoi</i> (MIX)	0.006 and 1.5	→ FBW, SGR and FCR ↑ Lamina propria and submucosa width ↑ Inflammatory cell and enterocyte damage	Ramos et al. (2017)
<i>Lates calcarifer</i> (Asian seabass)	<i>Bacillus</i> spp.	16	→ FBW, SGR, FCR, and PER → α-amylase, protease, and lipase	Salehi et al. (2022)
	<i>B. thuringiensis</i>	1000	↑ FBW, SGR, and PER and ↓ FCR ↑ α-amylase, protease, and lipase ↑ Total leukocyte count, serum RB ↑ Survival when challenged with <i>V. harveyi</i> and <i>V. alginolyticus</i>	Ghanei-Motlagh et al. (2021a); Ghanei-Motlagh et al. (2021b)
	<i>B. licheniformis</i> and <i>B. subtilis</i> (MIX)	0.001, 1, 1000	↑ FBW and SGR and ↓ FCR (best: 1) ↑ Protease and lipase (0.001, 1, 1000) ↑ Amylase (1 and 1000)	Adorian et al. (2019)

Table 1.4 Effect of dietary supplementation with *Bacillus* spp. on the growth performance, digestive enzymes activity, gut morphology, nonspecific innate immune response, and disease resistance of carnivorous fish species. (Continued)

Fish species (Common name)	Probiotic species	Dietary inclusion level (x 10 ⁶ CFU g ⁻¹ feed)	Results [tissue]	References
<i>Lates calcarifer</i> (Asian seabass)	<i>B. subtilis</i>		↑ FBW and FE ↓ The cumulative mortality when challenged with <i>A. hydrophila</i> (although not statistically significant)	Lin et al. (2017a)
<i>Lateolabrax japonicus</i> (Japanese seabass)	<i>B. siamensis</i>	100	↑ FBW and SGR → FCR and PER ↑ α-amylase and protease → lipase	Yang et al. (2021)
<i>Atractoscion nobilis</i> (White seabass)	<i>B. subtilis</i>	n/s (0.01%)	↑ FBW and → FE and PER	López et al. (2016)
<i>Epinephelus coioides</i> (Grouper)	<i>B. clausii</i>	100 (Live or heat-killed)	→ FBW and SGR, ↓ FCR and ↑ PER	Wang et al. (2018b)
	<i>B. subtilis</i>	0.01, 1, and 100	↑ WG and FE concomitantly with increasing probiotic supplementation ↑ Serum PA and RB (0.01, 1, and 100) in a dose-dependent manner ↑ Serum ACH ₅₀ , and LYZ (1, and 100) in a dose-dependent manner ↑ Survival when challenged with <i>Streptococcus</i> sp. (1 and 100)	Liu et al. (2012)
	<i>B. pumilus</i>	100	↑ FBW, → SGR, and ↓ FCR	Sun et al. (2010)
	<i>B. clausii</i>	100	→ FBW and SGR and ↓ FCR	Sun et al. (2010)
<i>Epinephelus fuscoguttatus</i> ♀ × <i>Epinephelus lanceolatus</i> ♂ (Pearl gentian grouper)	<i>B. velezensis</i>	10	→ FBW ↑ Survival when challenged with <i>V.</i> <i>harveyi</i>	Li et al. (2019)

Table 1.4 Effect of dietary supplementation with *Bacillus* spp. on the growth performance, digestive enzymes activity, gut morphology, nonspecific innate immune response, and disease resistance of carnivorous fish species. (Continued)

Fish species (Common name)	Probiotic species	Dietary inclusion level (x 10 ⁶ CFU g ⁻¹ feed)	Results [tissue]	References
<i>Acipenser schrenkii</i> ♂ and <i>Acipenser baeri</i> ♀ (Hybrid sturgeon)	<i>B. amyloliquefaciens</i>	40	↑ FBW and SGR and ↓ FCR	Fei et al. (2018)
<i>Paralichthys olivaceus</i> (Olive flounder)	<i>B. amyloliquefaciens</i>	1	→ FBW, SGR, FCR, and PER	Niu et al. (2021)
	<i>B. subtilis</i>	1	→ FBW, SGR, FCR, and PER	Niu et al. (2021)
	<i>B. amyloliquefaciens</i> , <i>B. subtilis</i> , and <i>B. licheniformis</i> (MIX)	1	→ FBW, SGR, FCR, and PER	Niu et al. (2021)
	<i>B. subtilis</i>	50	↑ FBW, ↓ FCR, and ↑PER ↑ Survival when challenged with <i>Streptococcus iniae</i> ↑ serum LYZ after the challenge	Cha et al. (2013)
	<i>B. pumilus</i>	50	→ FBW, FCR, and PER → Survival when challenged with <i>Streptococcus iniae</i>	Cha et al. (2013)
<i>Paralichthys olivaceus</i> (Olive flounder)	<i>B. licheniformis</i>	50	→ FBW, FCR, and PER ↑ Survival when challenged with <i>Streptococcus iniae</i>	Cha et al. (2013)
<i>Trachinotus ovatus</i> (Golden pompano)	<i>B. pumilus</i>	100	↑ WG, SGR and FE ↑ Villus length and width ↓mucosal width ↑ Survival when challenged with <i>V. ponticus</i>	Liu et al. (2020)

Table 1.4 Effect of dietary supplementation with *Bacillus* spp. on the growth performance, digestive enzymes activity, gut morphology, nonspecific innate immune response, and disease resistance of carnivorous fish species. (Continued)

Fish species (Common name)	Probiotic species	Dietary inclusion level 10 ⁶ CFU g ⁻¹ feed	Main effects (Inclusion level) [tissue]	Reference
<i>Cynoglossus semilaevis</i> (Tongue sole)	<i>B. subtilis</i>	10	↑ FBW and ↓ FCR ↑ Lipase and protease [foregut, midgut and, hindgut] ↑ α-amylase [foregut and midgut]	Wang et al. (2021b)
<i>Scophthalmus maximus</i> (Turbot)	<i>Bacillus</i> spp.	500	→ FBW, SGR and FCR ↑ Lipase [stomach] and protease [intestine] → Serum LYZ, NO, acid phosphatase, alkaline phosphatase ↑ Survival when challenged with <i>V.</i> <i>anguillarum</i>	Chen et al. (2016)
<i>Micropterus salmoides</i> (Largemouth bass)	<i>B. subtilis</i>	3 and 6	→ FBW, SGR, FE, and PER ↑ Villus length and muscular layer thickness	Du et al. (2021)

Probiotic mixtures containing other species than bacillus was not considered. Considerations on the *Bacillus* effect on the nonspecific innate immune response were only accounted when the sampling occurred prior to or after a bacterial challenge trial.

The symbols represent an increase (↑), decrease (↓), or no effect (→) on the tested parameters when compared to the control. n/s – value not specified in colony forming units g⁻¹ feed. FBW - Final body weight; FCR - Feed conversion ratio; BA - Bactericidal activity; LYZ - Lysozyme; NO - Nitric oxide; PA - Phagocytic activity; SGR - specific growth rate; WG - Weight gain; FE – Feed efficiency; MPO – myeloperoxidase; PER - Protein efficiency ratio; RB - Respiratory burst; ACH₅₀ - Alternative complement activity.

ESB production is usually carried out in two stages: the first stage, occurring mostly inland, is carried out in hatcheries with temperature-controlled systems, producing ESB up to 20 g, within 8 months; the second stage occurs mostly in cages (4 to over 10 m²) of 6-8 m depth, under an intensive regime; ESB is then grown up to 250-450 g within 12-20 months (Rad and Köksal, 2000; EUMOFA, 2022; FAO, 2022a). Recent years, however, have spurred a growing interest in larger fish (800 g to 1 kg) to be sold whole or as processed fillets (EUMOFA, 2022). Given ESB's status as a high-value species and the reliance on aquaculture for its production and commercialization, mainly under intensive conditions, research was quickly pointed toward the development of optimum feed formulations (FAO, 2022a).

ESB is a monogastric, carnivorous species that in the wild feed mainly on small fish, prawns, cuttlefish, and crabs (FAO, 2022a). In aquaculture, this translates to a high dietary protein requirement, which was traditionally reliant on FM, thus making feeds the main contributor to ESB production costs (>30%) (FAO, 2022c). To mitigate the usage of these commodities, researchers quickly tried to understand ESB nutritional requirements (Kousoulaki et al., 2015), allowing for a gradual FM replacement by PF (Kaushik et al., 2004; Enes et al., 2006a; b; Güroy et al., 2013; Castro et al., 2015b; Guerreiro et al., 2015a; Magalhães et al., 2015; Torrecillas et al., 2017a; b). More recently, novel strategies are being employed by using, for example, IM (Magalhães et al., 2017; Henry et al., 2018a; Basto et al., 2020; Mastoraki et al., 2020; Basto et al., 2021b; Basto et al., 2021c; Moutinho et al., 2021) or alternative circular economy feeds (Fernandes et al., 2021; Fernandes et al., 2022; Petereit et al., 2022). FM replacement strategies were followed by FO replacement by vegetable oils blends (Figueiredo-Silva et al., 2005; Castro et al., 2015a; Castro et al., 2015b; Torrecillas et al., 2017a; Torrecillas et al., 2017b), or animal fats (Monteiro et al., 2018; Campos et al., 2019), thus paving the way for the development of sustainable (both environmentally and economically), and efficient ESB feed formulation.

Over the last 25 years, understanding ESB stress and disease response was also a priority. While usually regarded as robust under intensive conditions, ESB was found to be affected by a wide range of pathogens, with *V. anguillarum* being one of the most important (Muniesa et al., 2020; FAO, 2022a; Kapetanović et al., 2022). Moreover, ESB production-related stressors (e.g., crowding, hypoxia, handling) were also verified to decrease growth performance and increase disease outbreaks, spurring researchers' interest to understand ESB acute and chronic stress responses (Di Marco et al., 2008; Petoichi et al., 2011; Samaras et al., 2018; Sadoul et al., 2021), as well as the immune

and antioxidant mechanisms governing its defenses (Chistiakov et al., 2007; Barroso et al., 2020; González-Fernández et al., 2020; Barroso et al., 2021; Picchietti et al., 2021).

Given ESB's relatively recent domestication (in the 1990s) and its maturation time (2-3 years males; 3-4 years females), the most distant ESB strains from wild populations were in 2016 only 8 generations away, and thus the selective breeding of phenotypes of interest is still on its infancy (Pérez-Ruzafa and Marcos, 2014; Chavanne et al., 2016). While several leaps were achieved, such as sex determination and differentiation mechanisms (Piferrer et al., 2005), genetic markers for lineage and phenotype analysis (Guyon et al., 2010), and whole genome assembly (Tine et al., 2014), ESB still lacks genetically optimized strains for improving feed efficiency and increased disease resistance. This leaves this species dependent on strategies such as vaccination and/or nutritional-based strategies to improve its disease resistance, stress response, growth performance, and feed utilization. Within these nutrient-driven strategies, the use of functional ingredients as dietary supplementation strategies have been applied, such as using amino acids (Machado et al., 2015; Coutinho et al., 2017; Martins et al., 2018; Martins et al., 2019; Martins et al., 2021), probiotics (Pereira et al., 2017; Pereira et al., 2018; Dawood et al., 2021; Monzón-Atienza et al., 2022), prebiotics (Guerreiro et al., 2015b; Azeredo et al., 2017; Torrecillas et al., 2018; Rimoldi et al., 2020), short-chain fatty acids (Rimoldi et al., 2016; Terova et al., 2016), and exogenous enzymes (Gasco et al., 2016; Magalhães et al., 2018).

Today, researchers have a better understanding of the ESB dietary requirements (Kousoulaki et al., 2015), immunity (Chistiakov et al., 2007; Barroso et al., 2020; González-Fernández et al., 2020; Barroso et al., 2021; Picchietti et al., 2021), and microbiota (Serra et al., 2019; Panteli et al., 2021; Serra et al., 2021) as well as a wide array of molecular resources available, such as the whole genome sequence (Tine et al., 2014) and transcriptomes (Geay et al., 2011; Caldach-Giner et al., 2016; Pinto et al., 2017). Altogether, ESB meets the conditions to be considered a key carnivorous marine fish model for the study of several of aquaculture's challenges (Vandeputte et al., 2019).

1.8 Objectives

To respond to the incremental fish feed demand, researchers and producers are doubling down on the development of species-specific feed formulations, that fulfill the species dietary requirements with optimal feed conversion ratios. Given the limited availability of several ingredients (due to scarcity or competition with other human sectors), as well as inadequate nutritional profiles, feed formulations for carnivorous

species are still seeking novel ingredients able to fulfill these gaps. As a result, IM, a novel promising ingredient for feed formulation, has been gradually included in fish feeds. While this inclusion has been mostly successful, it is far from optimized, being chitin, an indigestible carbohydrate for carnivorous fish, one of the main bottlenecks.

With the end goal of increasing IM inclusion in fish feeds, this work aimed to obtain an autochthonous chitinolytic bacteria isolated from the ESB gut microbiota able to increase chitin digestibility in fish feeds. Grounded on the high adaptability of ESB gut microbial communities to dietary variations, this study applied a dietary selective pressure to induce gut microbiota changes towards chitinolytic microorganisms.

Three main goals were defined: i) understand the gut bacterial microbiota modulation triggered by using different IM and how they affected the chitinolytic potential of the whole community (Chapter 2); ii) judiciously select among fish isolates obtained from ESB digesta fed different IM the best potential probiotic candidates according to their ability to form spores, degrade chitin, comply with EFSA safety guidelines, and survive passage through the fish gut (Chapter 3); iii) identify if the *in vitro* probiotic potential would be translated in an *in vivo* setting, by supplementing ESB diets containing HI meal (30%) with the selected isolates and evaluating fish performance and diets digestibility (Chapter 4). Finally, given the urgency to find safe alternatives to antibiotics able to prevent and combat bacterial pathogens outbreaks, the ability of the selected probiotics to increase ESB survivability to *V. anguillarum* was tested by performing an *in vivo* bacterial challenge trial (Chapter 4).

The successful completion of the proposed tasks will allow to i) increase the fundamental and practical knowledge on how dietary IM can influence fish gut bacterial communities; ii) obtaining a probiotic or a probiotic mixture with commercial potential for use in aquaculture, targeting a novel and most promising aquafeed commodity, the IM.

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Chapter 2.

Differential modulation of the European sea bass gut microbiota by distinct insect meals

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Abstract

The aquaculture industry is one of the fastest-growing sectors in animal food production. However, farming of carnivorous fish strongly relies on the use of wild fish-based meals, a practice that is environmentally and economically unsustainable. Insect-based diets constitute a strong candidate for fishmeal substitution, due to their high nutritional value and low environmental footprint. Nevertheless, data on the impact of insect meal (IM) on the gut microbiome of farmed fish is so far inconclusive, and very scarce in what the concerns modulation of microbial-mediated functions. Here we use high-throughput 16S rRNA gene amplicon sequencing and quantitative PCR to evaluate the impact of different IMs on the composition and chitinolytic potential of the European sea bass gut digesta- and mucosa-associated communities. Our results show that insect-based diets of distinct origins differently impact the gut microbiota of the European sea bass (*Dicentrarchus labrax*). We detected clear modulatory effects of IM on the gut microbiota, which were more pronounced in the digesta, where communities differed considerably among the diets tested. Major community shifts were associated with the use of black soldier fly larvae (*Hermetia illucens*, HM) and pupal exuviae (HEM) feeds and were characterized by an increase in the relative abundance of the Firmicutes families *Bacillaceae*, *Enterococcaceae*, and *Lachnospiraceae* and the Actinobacteria family *Actinomycetaceae*, which all include taxa considered beneficial for fish health. Modulation of the digesta community by HEM was characterized by a sharp increase in *Paenibacillus* and a decrease of several Gammaproteobacteria and Bacteroidota members. In turn, a mealworm larvae-based diet (*Tenebrio molitor*, TM) had only a modest impact on microbiota composition. Further, using quantitative PCR, we demonstrate that shifts induced by HEM were accompanied by an increase in copy number of chitinase ChiA-encoding genes, predominantly originating from *Paenibacillus* species with effective chitinolytic activity. Our study reveals an HEM-driven increase in chitin-degrading taxa and associated chitinolytic activity, uncovering potential benefits of adopting exuviae-supplemented diets, a waste product of insect rearing, as a functional ingredient.

Keywords: *Dicentrarchus labrax*, chitin, *Hermetia illucens*, *Tenebrio molitor*, feedstuff

2.1 Introduction

Human demand for fish protein has led to a large expansion of the aquaculture industry. The production of carnivorous species is heavily reliant on the use of fish meal (FM) as a major protein source for aquafeeds (Hoffmann et al., 2020). The reduced availability of FM and its subsequent increase in price due to depletion of wild fish stocks, together with the environmental impacts associated with FM use, is driving the sector to search for new sustainable feedstuffs (Gasco et al., 2020a).

Recently, insects started to be tested as a novel animal protein and lipid source with the potential to be consumed by humans, livestock, and fish (Tomberlin and van Huis, 2020; van Huis, 2020; van Huis et al., 2013). In Europe, interest in insect meal (IM) sparked upon implementation of a European Union (EU) Directive (Regulation No 2017/893), effective after July 2017, which authorized the use of seven insect species in aquafeeds. The nutritional value of insects can greatly vary depending on species, development stage, or processing method. Overall, IM protein content can vary between 30-68% dry matter (DM), having a more balanced amino acid profile than plant feedstuffs, and providing higher levels of essential amino acids (Gasco et al., 2020a; Nogales-Mérida et al., 2018). Lipid levels usually fluctuate between 10 to 30% DM (Gasco et al., 2020a; Nogales-Mérida et al., 2018), but can be reduced to 9–5% DM by defatting processes. Moreover, depending on the species and rearing material, IM presents a variable fatty acid profile, which can be modulated through the diets of the insects to meet the needs of a particular fish species (Barroso et al., 2014; Benzertiha et al., 2020; Danieli et al., 2019; Gasco et al., 2020b; Hoc et al., 2021; Liland et al., 2017; Nogales-Mérida et al., 2018; Pulido et al., 2022).

From the seven insect species authorized for use in aquafeeds in the EU, *Hermetia illucens* (HI) and *Tenebrio molitor* (TM) are among the most investigated (Gasco et al., 2019). Both are commonly used in their larval stage, characterized by a protein-rich content, and suitable amino acid and fatty acid profiles for inclusion in aquafeeds (Hawkey et al., 2021). Additionally, during the different life cycle stages, these insects go through metamorphosis, leaving behind chitin and protein-rich exoskeletons or shells, known as exuviae, an untapped valuable waste for fish feeds (Purkayastha and Sarkar, 2020). Data on fish growth performance are generally similar between diets containing HI or TM, with positive outcomes obtained when the IM inclusion levels are below 30% of the total meal (Caimi et al., 2021; Caimi et al., 2020; Hoffmann et al., 2020; Magalhães et al., 2017; Shafique et al., 2021; Stejskal et al., 2020; Terova et al., 2019). Dietary HI

or TM inclusions over 30% generally lead to detrimental effects in growth performance and are associated with decreases in the apparent digestibility coefficients of crude protein and/or lipids (Guerreiro et al., 2021; Piccolo et al., 2017). The lower digestibility of these ingredients has been proposed to be correlated with the increased dietary chitin content, a hypothesis supported by *in vitro* results (Marono et al., 2015) and *in vivo* trials (Guerreiro et al., 2021; Mastoraki et al., 2020; Piccolo et al., 2017). Chitin, through a process called sclerotization, can bind and entrap proteins and lipids in a structural matrix, such as the one that makes up the exoskeleton of Arthropoda (Resh and Cardé, 2009). This leads to reduced access of proteases and lipases to their substrates, leading to a lower IM digestibility (De Marco et al., 2015). This has been verified in digestibility trials with IM-containing diets (Chemello et al., 2020; Guerreiro et al., 2021; Piccolo et al., 2017). Therefore, for more efficient use of IM, chitinolytic activity is required. Endogenous chitinolytic activity has been detected in the digestive system of some marine fish such as juvenile cobia (*Rachycentron canadum*) and cod (*Gadus morhua*) (Danulat and Kausch, 1984; Fines and Holt, 2010), although it was found to be lacking in others including turbot (*Psetta maxima*) and meagre (*Argyrosomus regius*) (Guerreiro et al., 2021; Kroeckel et al., 2012). In European sea bass (*Dicentrarchus labrax*), although chitinase-encoding genes have been found (Calduch-Giner et al., 2016), to the best of the authors knowledge, no chitinolytic activity measurements have been made.

The gastrointestinal (GI) tract, however, depends not only on its endogenous enzymes for the digestive process. Fish and other animals rely on symbiotic relationships with their GI tract microbial community, which through enzymatic secretion, can breakdown specific, otherwise indigestible dietary components, such as chitin, and thereby help hosts to obtain energy from inaccessible nutrient sources (Ray et al., 2012; Roeselers et al., 2011; Tremaroli and Bäckhed, 2012). Dietary changes create different nutrient niches by promoting the use of novel substrates by different microbes (Ringø et al., 2015; Serra et al., 2021). Such alterations in fish gut microbiomes can alter the microbial digestive enzymatic contribution, and ultimately, lead to a better use of novel ingredients (Gasco et al., 2021).

Understanding the main modifications of fish gut microbiota due to dietary composition, as well as microbiota's role in digestion and host fitness, is important for the successful introduction of novel ingredients into aquafeeds. However, regarding IM in fish diets, data on the species-specific gut microbiota responses to chitin-containing diets and their mediated processes are still scarce, or non-existent for the case of exuviae diets (Perry et al., 2020). As such, this study aimed to assess the effect of three different IM, namely,

HI and TM larvae meals, and HI exuviae meal (HEM), on European sea bass gut microbiota using Illumina MiSeq 16S rRNA gene amplicon sequencing. To help elucidate the impact of chitin on the gut microbiota, a control FM-based diet supplemented with commercial chitin was also included. As previous studies have reported different responses of digesta- and mucosa-associated gut communities to dietary changes (Serra et al., 2021), we have here analyzed the impact of IM in these two intestinal sites separately. Additionally, using quantitative PCR targeting bacterial chitinase genes, we aimed to unveil the potential chitinolytic contribution of the microbiota under different diets.

2.2 Materials and Methods

2.2.1 Experimental diets and proximate analysis.

Five isoproteic (45%) and isolipidic (18%) diets were formulated: a control diet (CTR); a diet similar to the CTR with 5% commercial chitin (Sigma-Aldrich Química, S.L., Sintra, Portugal) supplementation (CHIT5); and three other diets with 25% inclusion of partially defatted black soldier fly (*Hermetia illucens*) larvae meal (HM25); 25% inclusion of partially defatted yellow mealworm (*Tenebrio molitor*) larvae meal (TM25); and 25% inclusion of *Hermetia illucens* exuviae meal (HEM25), respectively. The inclusion of the HM (crude protein: 60.3% dry matter (DM); gross lipid: 6.4% DM; chitin 6.8% DM), TM (crude protein: 69.5% DM; gross lipid: 14.1% DM; chitin 5.2% DM) and HEM (crude protein: 64.3% DM; gross lipid: 8.1% DM; chitin 7.2% DM) corresponded to a 39%, 51% and 46% of FM replacement, respectively. Diets were supplemented with monoammonium phosphate to avoid phosphorus imbalance. 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 then dried in an oven at 40 °C for 24 h and stored at -20 °C in airtight bags until used. Ingredients and proximate composition of the experimental diets are presented in Supplementary Table 2.1. Chemical analysis of the ingredients and experimental diets (dry matter, protein, lipid, and ash) were done following the Association of Official Analytical Chemists methods (AOAC, 2000). HM, TM, HEM and the final diets formulated with these meals were analyzed for chitin composition according to Guerreiro et al. (2020).

2.2.2 Animals and experimental conditions.

The trial was directed by accredited scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. European sea bass (*Dicentrarchus labrax*) were obtained from Sonrionansa (Cantabria, España), and transported to the Marine Zoology Station (Porto University, Porto, Portugal), where the experiment was conducted. After transport, fish were submitted to a quarantine period of 4 weeks. During this period, fish were fed a commercial diet (18% lipids and 44% protein, Aquasoja Sustainable Feed; Sorgal, Ovar, Portugal). Thereafter, 15 groups of 15 fish with an initial mean body weight of 53.7 ± 2.67 g were established. Experimental diets were randomly assigned to triplicate tanks. The trial was conducted in a recirculating aquaculture system equipped with 15 fiberglass tanks of 100 L water capacity, thermo-regulated to 22.7 ± 0.3 °C and supplied with a continuous flow of seawater (36.0 ± 0.5 g L⁻¹ salinity, circa 7 mg L⁻¹ oxygen). The photoperiod was set, with artificial illumination, to 12:12h light:dark. The trial lasted 8 weeks, and fish were fed by hand twice a day, 6 days per week, until apparent visual satiation. At the end of the feeding trial, all fish reached 2.1 to 2.2 times their initial body weight, as determined by randomly weighing one fish per tank.

2.2.3 Sampling.

Two fish per tank were randomly sacrificed with an anesthetic overdose (3.0 mL L⁻¹ ethylene glycol monophenyl ether), 4h after the first meal, to assure presence of digesta in the gut. Whole-gut (without pyloric caeca) was aseptically excised and squeezed to collect the digesta contents. Mucosa samples were obtained by scraping the internal intestinal mucosa after opening the intestines in their longitudinal axis. To overcome inter-fish variation, the resulting material from the two fish was pooled into one sample per tank, immediately frozen in liquid nitrogen and stored at -80°C until further analysis to assess differences between dietary groups.

2.2.4 DNA extraction.

DNA was extracted from 250 mg of either the collected gut contents or gut mucosa, which were transferred to 2 mL sterile tubes containing 400 mg of 425-600 µm glass beads (Sigma-Aldrich, Germany) and 500 µL of STE buffer. The samples were homogenized in a Precellys homogenizer (Bertin Instruments, France) using 3 cycles of

60 s at 4500 rpm with 60 s intervals on ice between cycles. The samples were incubated for 15 min at 75 °C, centrifuged (16000 g for 1 min at 4 °C) and 500 µL of supernatant was collected to a new 2mL sterile tube. DNA was extracted from the supernatant, using the GES buffer (5 M guanidine thiocyanate, 0.5 M EDTA, 10% N-lauroylsarcosine) and phenol methodology of Pitcher et al (1989), with the modifications described by Santos et al., (2021). Nucleic acid pellets were finally resuspended in 50 µL of nuclease free water. As a negative control for the extraction procedure, a sample with only the lysis buffer was processed in parallel with all samples.

2.2.5 Amplification and sequencing of the 16S rRNA genes

Amplification of bacterial and archaeal 16S rRNA genes from DNA extracts was performed with a two-step barcoding approach (UDB-H12, (Pjevac et al., 2021). In the first-step PCR, the primers 515F (5'-GTGYCAGCMGCCGCGGTAA-3') (Parada et al., 2016) and 806R (5'-GGACTACNVGGGTWTCTAAT-3') (Aprill et al., 2015), including a 5'-head sequence for 2-step PCR barcoding, were used. PCRs, barcoding, library preparation and Illumina MiSeq sequencing were performed by the Joint Microbiome Facility (Vienna, Austria) under project number JMF-2002-2. First-step PCRs were performed in triplicate (12.5 µL vol per reaction) with the following conditions: 1X DreamTaq Buffer (Thermo Fisher), 2 mM MgCl₂ (Thermo Fisher), 0.2 mM dNTP mix (Thermo Fisher), 0.2 µM of forward and reverse primer each, 0.08 mg mL⁻¹ Bovine Serum Albumin (Thermo Fisher), 0.02 U Dream Taq Polymerase (Thermo Fisher), and 0.5 µL of DNA template. Conditions for thermal cycling were: 95°C for 3 min, followed by 30 cycles of 30 s at 95°C, 30 s at 52°C and 50 s at 72°C, and finally 10 min at 72°C. Triplicates were combined for barcoding (with 8 PCR cycles). Barcoded samples were purified and normalized over a SequelPrep Normalization Plate Kit (Invitrogen) using a Biomek NXP Span-8 pipetting robot (Beckman Coulter), and pooled and concentrated on PCR purification columns (Analytik Jena). Indexed sequencing libraries were prepared with the Illumina TruSeq Nano Kit as described previously (Herbold et al., 2015), and sequenced in paired-end mode (2× 300 bp; v3 chemistry) on an Illumina MiSeq following the manufacturer's instructions. The workflow systematically included four negative controls (PCR blanks, i.e., PCR-grade water as template) for each 90 samples sequenced.

2.2.6 Analysis of 16S rRNA gene amplicon sequences

Amplicon pools were extracted from the raw sequencing data using the FASTQ workflow in BaseSpace (Illumina) with default parameters (Pjevac et al., 2021). Input data was filtered for PhiX contamination with BBDuk (BBTools, Bushnell B, sourceforge.net/projects/bbmap). Demultiplexing was performed with the python package demultiplex (Laros JFJ, github.com/jfjlaros/demultiplex) allowing one mismatch for barcodes and two mismatches for linkers and primers. DADA2 R package version 1.16.0 (<https://www.r-project.org/>, R 4.0.2) (Callahan, Benjamin J. et al., 2016) was used for demultiplexing amplicon sequencing variants (ASVs) using a previously described standard protocol (Callahan, Ben J. et al., 2016). FASTQ reads were trimmed at 150 nt with allowed expected errors of 2. Taxonomy was assigned to 16S rRNA gene sequences based on SILVA taxonomy (release 138) using the DADA2 classifier (Callahan, Benjamin J. et al., 2016). ASVs assigned as Chloroplast were considered dietary plant chloroplast contaminants and removed from the analysis. Sequencing of an extraction control yielded only 9 reads, part of these classified as a *Pseudomonas* sp. ASV (ASV_lwc_fxo), a common contaminant found in PCR reagents that was therefore also removed from analysis. In addition, we did find 5 reads belonging to ASV_tnz_8cx (average read abundance across non-control samples: 478 reads) and ASV_hek_q1g (average read abundance across non-control samples: 187 reads). As these ASVs are present in the samples in high abundance, we do believe that very few reads detected in the negative control may not be derived from contaminants (water or others) in reagents, but from the well-known low levels of cross-contamination that can be detected when handling multiple samples in parallel (Minich et al., 2019) and were therefore retained for further analysis. Amplicon sequence libraries were rarefied to 3100 reads per sample and analyzed using the vegan (v2.5-6) (Oksanen et al., 2020) and phyloseq (v1.30.0) (McMurdie and Holmes, 2013) packages of the software R (<https://www.r-project.org/>, R 4.0.2). A total of 740 unique ASVs were retained after filtering and rarefying. Sample coverage, alpha-diversity metrics, Bray-Curtis dissimilarity and non-metric multidimensional scaling (NMDS) were calculated using the phyloseq R package. Ellipses were drawn on NMDS plots using vegan's `veganCovEllipse()` function. Rarefaction curves were generated using the vegan's function `rarecurve()`. DESeq2 (v1.26.0) (Love et al., 2014) implemented in phyloseq was used to determine significant differences in ASV, genera and family abundances between diets and/or intestinal sampling site. Only ASVs that had in total ≥ 10 reads were considered for comparisons by DESeq2 analyses. All statistical analysis on microbiome data were carried out via the

software R (R 3.6.2), and statistical tests and P-values are indicated in the main text and figure legends.

2.2.7 Amplification of chitinase A genes

Forward (5'-GATATCGACTGGGAGTTCCC-3') and reverse (5'-CATAGAAGTCGTAGGTCATC-3') primers previously developed to target the Chitinase gene A (*chiA*) from bacteria of marine environments (Ramaiah et al., 2000) were used to detect and quantify the presence of bacterial ChiA-encoding genes in the digesta and mucosa of the European sea bass, as well as in the genomes of *Paenibacillus* spp. isolates. Initial PCR amplifications to check primer specificity were performed on DNA samples from the digesta of fish fed the HEM25 diet, using the following conditions: 1X DreamTaq Buffer (Thermo Fisher), 2 mM MgCl₂ (Thermo Fisher), 0.2 mM dNTP mix (Thermo Fisher), 0.2 µM of forward and reverse primer each, 0.08 mg mL⁻¹ Bovine Serum Albumin (Thermo Fisher), 0.02 U Dream Taq Polymerase (Thermo Fisher), and 2 µL of DNA template for a total 20 µL reaction volume. Conditions for thermal cycling were: 95°C for 4 min, followed by 30 cycles of 1 min each at 95°C, 55°C and 72°C, and finally 10 min at 72°C. PCR products were checked on a 0.8% agarose gel for any visible primer dimer or double bands. Triplicates were combined and purified with the innuPREP PCRpure Kit (Analytik Jena). Clean PCR products were pooled and ligated and transformed into vectors using the TOPO® TA Cloning® kit (Thermo Fisher) according to the manufacturer's protocol. Clones were checked using PCR with the universal M13 forward and reverse primers and the purified PCR product was sent for Sanger sequencing at the company Microsynth Austria GmbH. The sequencing results were analyzed by BLASTX against a database of non-redundant bacterial and archeal protein sequences from NCBI (<https://www.ncbi.nlm.nih.gov/>) to check for specificity of the primers (Supplementary Table 2.7).

2.2.8 Quantitative PCR of *chiA* gene copy number density

Extracted digesta and mucosa DNA (2 µL) was subjected to quantitative PCR using 0.2 µM of primers specifically targeting bacterial *chiA* (Ramaiah et al., 2000) and 1x SYBR green Master Mix (Bio-Rad) in a total reaction volume of 20 µL. Standard curves were generated from a 416 bp PCR fragment amplified from a clone containing a chitinase sequence 100% identical to a ChiA sequence from *Paenibacillus thiaminolyticus* (NCBI accession number: WP_143798588.1). The purified PCR product was quantified using a Qubit dsDNA BR Assay Kit Qubit in a Qubit 4 fluorometer (Thermo Fisher). For

standard preparation, a 10-fold dilution series was prepared from 10^8 to 10^0 copies per μL and checked for optimal efficiency. Amplification and detection were performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad) using the following cycling conditions: 95°C for 5 min, followed by 40 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 1 min. To determine the specificity of PCR reactions, melt curve analysis was carried out after amplification by slow cooling from 95 to 60°C, with fluorescence collection at 0.3°C intervals and a hold of 10 s at each decrement. Only assays with amplification efficiencies above 85% were considered for analysis.

2.2.9 Phylogenetic analysis

A reference tree was constructed using near full-length 16S rRNA gene reference sequences (>1200 bp) derived from selected isolates using IQ-TREE server (Trifinopoulos et al., 2016) with automatic model selection (TIM3+F+I+G4) and ultra-fast bootstrapping with 1000 replicates. Query sequences from ASVs and isolates obtained in this study were then added to the reference tree using the Evolutionary Placement Algorithm of RAxML (Stamatakis, 2014), using the GTRGAMMA model.

2.2.10 Bacterial isolation, identification and chitinolytic assays

Paenibacillus spp. were isolated from a 1:5 (wt:vol) dilution of each pooled digesta sample immediately before freezing. Serial dilutions (10^0 , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) in Bott & Wilson (B&W) salts were heat treated for 20 min at 65°C (Nicholson and Setlow, 1990), spread on Luria Bertani (LB) medium plates and incubated for up to 96 h at room temperature ($25 \pm 1^\circ\text{C}$). Colonies with the distinct morphologies were purified and stored at -80°C in 25% glycerol. Chitinolytic activity was evaluated by the presence of a chitin-hydrolysis halo around colonies of each isolate when grown up to 14 days at 28°C in a chemically defined media (w/v, 0.648% Na_2HPO_4 , 0.3% KH_2PO_4 , 0.05% NaCl , 1% NH_4Cl , 0.02% MgSO_4 , 0.01% Yeast extract, 1% colloidal chitin) containing 1.6% (w/v) agar. The *Chromobacterium violaceum* ATCC 12472, known to possess chitinolytic activity, was used as a positive control (Liu et al., 2013). The chromosomal DNA of each isolate was purified from a 2mL overnight LB culture (37°C, 120 rpm) using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research Corp., Irvine, CA, USA), according to the manufacturer instructions. Isolates identification was done by partially sequencing (STABVIDA, Caparica, Portugal) the 16S rRNA gene, previously amplified by PCR with oligonucleotide primers 16S-27F and 16S-1492R (Weisburg et al., 1991), and analyzed

by BLAST against the GenBank nonredundant (nr) nucleotide database (<https://www.ncbi.nlm.nih.gov>).

2.3 Results

To understand the impact of different IM types on the European sea bass gut microbiota, fish were fed five isoproteic and isolipidic diets, including: a control FM-based diet (CTR), a CTR diet supplemented with 5% of a commercial chitin from shrimp shells (CHIT5), and three diets including 25% of TM or HI larvae meals or HI exuviae meal (diets TM25, HM25 and HEM25, respectively) (Figure 2.1A-C, Supplementary Table 2.1). Dietary chitin content was similar among these diets (1.3% in TM25, 1.7% in HM25 and 1.8% in HEM25). Digesta (gut content) and mucosa samples (scraping of the mucosa from emptied gut) were collected and DNA was isolated and subjected to Illumina MiSeq sequencing of the V4 region of the 16S rRNA gene. After quality filtering, a total of 311,727 high-quality sequences were retained with an average of 10,390 high-quality sequences per sample. Sequencing of a DNA extraction control sample yielded a very low number of reads (<10 reads), partly belonging to a clear contaminant found in PCR reagents (*Pseudomonas* sp.). This ASV and all ASVs classified as chloroplast were removed from our analysis (see Materials and Methods). After rarefying, the ASV coverage was higher than 0.97 for all sequenced samples (Supplementary Table 2.2), indicating that sequencing depth was adequate to capture most of the diversity present (Supplementary Figure 2.2). Overall, the European sea bass gut microbiota was dominated by the phylum Proteobacteria (68.3% in relative abundance), followed by Firmicutes (17.2%), Actinobacteria (8.4%) and Bacteroidota (2.3%) (Figure 2.1D; Supplementary Table 2.3). Within the phylum Proteobacteria there is a dominance of organisms belonging to the Alphaproteobacteria orders Rhizobiales and Sphingomonadales, and Gammaproteobacteria orders Burkholderiales and Pseudomonadales (Figure 2.1D). The majority of Firmicutes belong to the order Lactobacillales (Figure 2.1D). Actinobacteria organisms mostly belonged to the order Corynebacteriales and to the potential pathogenic order Micrococcales.

2.3.1 Digesta and mucosa harbor distinct microbial communities

We found that there is a stronger impact of the intestinal site sampled (digesta versus mucosa) when compared with the impact of the tested diets on the beta-diversity variation of gut communities (Figure 2.2A; $p=0.031$, Wilcoxon test). Indeed, the beta-diversity analysis revealed a significant overall impact of the sampled intestinal site on

community composition, with microbial communities from digesta and mucosa clustering separately ($p=0.004$, $r^2=0.247$, PERMANOVA, Figure 2.2B). Additionally, we found that, on average, only 33% of the total ASVs detected for each pair of digesta and mucosa samples are shared between the two (Figure 2.2D), although no physical separation exists between them. Digesta communities are more diverse than their mucosal counterparts (Figure 2.2C, Supplementary Table 2.4), displaying significantly higher richness (199.2 ± 22.6 versus 181.3 ± 38.1 observed ASVs, $p=0.041$, paired Wilcoxon test) as well as a greater Inverse Simpson diversity index (11.4 ± 4.9 versus 7.5 ± 2.4 , $p=0.008$, paired Wilcoxon test). These results are in line with our analyses that show that higher percentages of total ASVs tend to be uniquely associated with the digesta compared with the mucosa (Figure 2.2D).

We next sought to investigate which species are preferentially found in each of the two intestinal sampling sites. Differential abundance analysis with DESeq2 revealed that the mucosa is significantly enriched for the genus *Granulicatella* of the family *Carnobacteriaceae* (Figure 2.2E; Log2 fold change=5.0, adjusted- $p=9.1E-6$, Wald test followed by Benjamini–Hochberg correction for multiple testing), as well as for several typically mucosal-associated Firmicutes and Proteobacteria genera such as *Vibrio*, *Veillonella*, *Neisseria* or *Haemophilus* (Figure 2.2E, Log2 fold change >2, adjusted- $p<0.05$). Digesta communities are in turn enriched in many *Bacillaceae* genera, *Pseudomonas*, *Legionella*, as well as Actinobacteria genera such as *Corynebacterium*, *Mycobacterium*, *Brevibacterium* or *Aeromicrobium* (Figure 2.2E, Log2 fold change <-2, adjusted- $p<0.05$).

2.3.2 Impact of distinct IM on the European sea bass gut microbiota

Beta-diversity analysis of samples from fish fed the experimental diets revealed an overall impact of diet on the composition of the European sea bass gut microbiota ($p=0.004$, $r^2=0.244$, PERMANOVA, Figure 2.3A). Among the tested diets, HI diets led to more pronounced shifts in microbial community composition, with major shifts detected in HEM25 ($p=0.041$, $r^2=0.199$, PERMANOVA; Supplementary Table 2.5). The shifts are accompanied by a slight increase in alpha diversity (Figure 2.3B), although this increase was not statistically significant. This is in line with other studies where IM-based diets were fed to the European sea bass (Antonopoulou et al., 2019; Panteli et al., 2021; Pérez-Pascual et al., 2020). Because the sampling habitat (mucosa versus digesta) strongly influences community composition, as shown above, the impact of the diets on

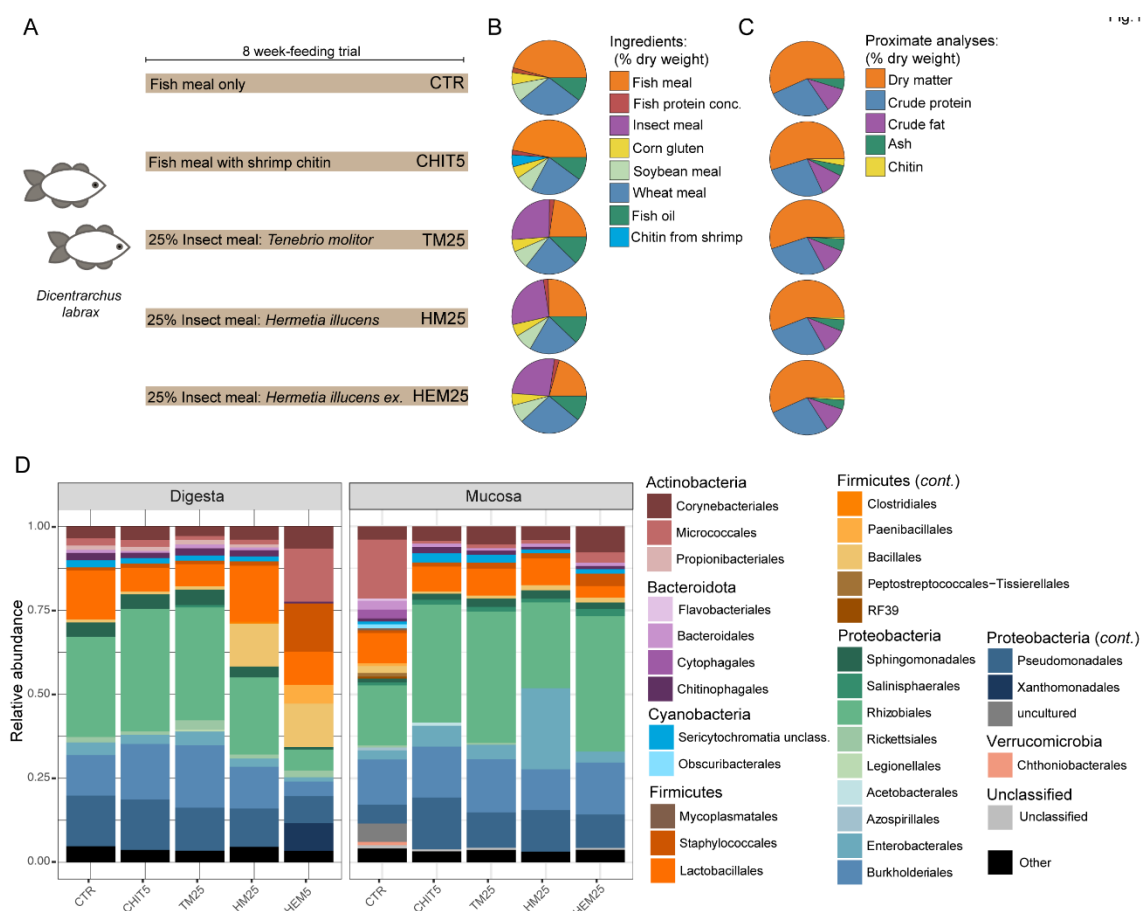


Figure 2.1 Feeding diets and gut microbiota composition of the European sea bass. (A) Three tanks containing 15 *Dicentrarchus labrax* fish each were fed with the five different depicted diets for 8 weeks (see Materials and Methods). ex, exuviae. Three different tanks per diet were included in the trial. (B) Percentages (dry weight basis) of the different ingredients in each diet. Only major ingredients (final percentage in diet >1.0%; Supplementary Table 2.1) are shown. (C) Despite different compositions, proximate analyses show that all diets contain similar amounts of dry matter, crude protein, crude fat and ash, while levels of chitin are rather more variable (Supplementary Table 2.1). (D) Phylum- and Order-level relative abundance profile of the gut microbiota of digesta and mucosa samples from fish fed the different diets depicted in (A). Orders with <1% relative abundance are collapsed into the category “Others.” Each bar represents the average of three different samples per diet for each habitat, each sample originating from a different tank

each of the two sampled habitats were also analyzed independently. We did not observe any diet-driven significant alterations in alpha diversity within samples coming from either habitat (Supplementary Figure 2.1). However, results show that the type of diet more strongly influences the digesta community than the mucosa community (Figure 2.3A, Supplementary Figure 2.1, Supplementary Table 2.5). Both HI-derived diets (HM25 and HEM25) lead to a digesta-specific increase in the relative abundance of the phylum Firmicutes, while the relative abundance of this phylum decreases in fish fed the TM diet

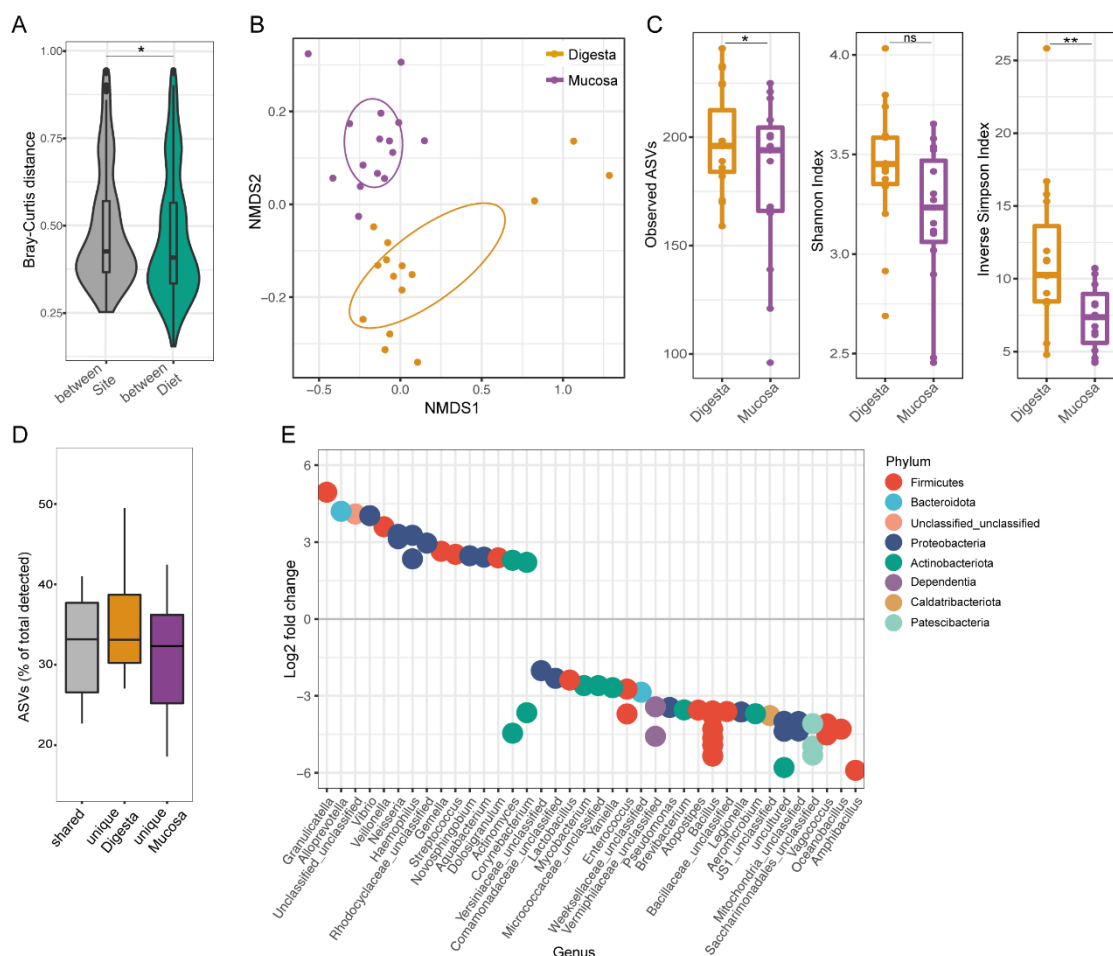


Figure 2.2 Sampling site as a driver of gut microbial community composition. (A) Violin plot representing the calculated Bray–Curtis distances between samples originating from different sampling intestinal sites (grey) or between samples originating from fish fed different diets (green). * $p = 0.031$; Wilcoxon test. (B) Ordination plot based on non-metric multidimensional scaling analysis of Bray–Curtis distances at the ASV level. SD ellipses and point dots representing each sample are depicted and are colored by sampling intestinal site. Stress = 0.057. (C) Alpha diversity metrics (number of Observed ASVs, Shannon Index, and Inverse Simpson Index) of the mucosa and digesta samples. Each point represents one sample ($n = 15$ paired mucosa and digesta samples). * $p = 0.041$; ** $p = 0.008$; ns = nonsignificant; paired Wilcoxon test. (D) Percentage of total ASVs detected in samples originating from the same tank that are shared between the mucosa and corresponding digesta sample, or unique to sampled site. (E) Differential abundant ASVs based on DESeq2 analysis (adjusted value of $p < 0.05$, Wald test followed by Benjamini–Hochberg correction for multiple testing) between mucosa and digesta samples (Log2 fold change >2 denotes enrichment in mucosa; Log2 fold change <-2 denotes enrichment in digesta). ASVs are assigned to genus (x-axis) and colored by phylum. In (A,C,D) boxes represent median, first and third quartile. Whiskers extend to the highest and lowest values that are within one and a half times the interquartile range.

(Figure 2.3C, Figure 2.1D). In contrast, both the phylum Bacteroidota and the class Alphaproteobacteria are significantly decreased in relative abundance in fish fed the HEM25 diet (Figure 2.3C). In the mucosa, the only major shift at a high taxonomic level was detected for the order Chitinophagales in fish fed a diet supplemented with the commercial shrimp chitin (CHIT5; Figure 2.3D). At lower taxonomic levels no significant

shifts were detected in fish fed a CHIT5 diet (adjusted- $p < 0.05$, DESeq2 analysis, Supplementary Table 2.6). Microbiota modulation by the mealworm-based diet (TM25) was modest and occurred only in the digesta, being characterized by an increase in the Betaproteobacteria genus *Cupriavidus* (from 0.3% to 2.3% increase in relative abundance), and by decreases in the family *Lactobacillaceae* and the genus *Ligilactobacillus*, which decreases from 11.2% to 4.9% in relative abundance (adjusted- $p < 0.05$, DESeq2 analysis, Supplementary Table 2.6).

To better understand the modulatory effect of HEM and HM, we proceeded to identify specific microbial families and genera that had altered abundances due to HEM or HM inclusion in the diets using DESeq2. A total of 50 taxa (22 families and 28 genera) and 12 taxa (5 families and 7 genera) were found to be differentially abundant in the digesta of fish fed the HEM25 or HM25 diets, respectively, when compared with the CTR diet (Figure 2.4; Supplementary Table 2.6: adjusted- $p < 0.05$, Wald test followed by Benjamini–Hochberg correction for multiple testing). Of these, the vast majority (40 out of 50 taxa for the HEM25 diet, and all 12 taxa of the HM25 diet) were shown to be more abundant in the HEM an HM diets compared with the CTR (Figure 2.4). These taxa largely belong to the phylum Firmicutes, and to a less extent to the phylum Actinobacteria, in line with the results presented above (Figure 2.1A and Figure 2.2C). Taxa who increase in abundance on both diets correspond to the genera *Oceanibacillus*, *Amphibacillus* and unclassified *Bacillaceae* (family *Bacillaceae*, which sharply increases from 1.0% to 12.8% and 12.4% in relative abundance for HM25 and HEM25 diets, respectively), *Actinomyces* (*Actinomycetaceae*), *Enterococcus* (*Enterococcaceae*) and *Lachnospiraceae*. Consumption of HEM25 diet, but not of HM25, leads to a further increase in the Firmicutes genera *Paenibacillus* (*Paenibacillaceae*; from not detected to 5.5% in relative abundance) and *Staphylococcus* (*Staphylococcaceae*, from 1.1% to 14.8% in relative abundance), *Planococcaceae*, as well as in several Actinobacteria families such as *Brevibacteriaceae* and *Micrococcaceae* (an increase from 0.2% to 6.0%, and from 0.5% to 3.4% in relative abundance, respectively) (Figure 2.4, panel “Dif.abundant HEM25”; Supplementary Table 2.6; Supplementary Table 2.3). In turn, organisms whose abundance decreases upon consumption of the HME25 diet belong predominantly to the class Alphaproteobacteria (*Legionellaceae*, *Vibrionaceae*, *Erwiniaceae*) (Figure 2.2C and Figure 2.4). Increase in the abundance of the *Staphylococcaceae* genus *Nosocomiicoccus* is only observed in fish fed the HM25 diet, but not the HEM25 diet (Figure 2.4; panel “Dif. abundant in HM25”). Shifts in abundance

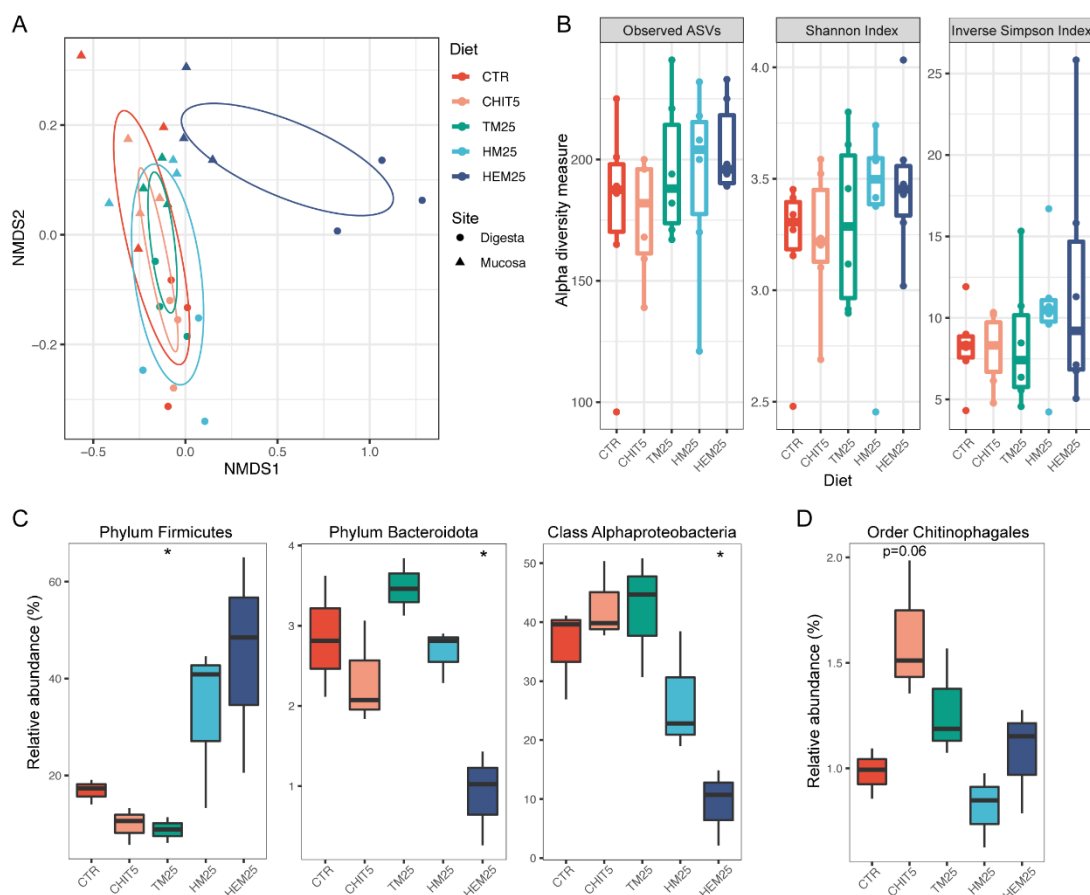


Figure 2.3 Insect meal-based diets induce major shifts in the European sea bass gut microbiome. (A) Ordination plot based on non-metric multidimensional scaling analysis of Bray–Curtis distances at the ASV level shown in Figure 2.2B, but now with individual data points colored by diet and shaped by sampling site. SD ellipses (colored by diet) are also depicted. (B) Alpha diversity metrics (number of Observed ASVs, Shannon Index, and Inverse Simpson Index) of samples from different diets. Each point represents one sample ($n = 6$ samples per diet). (C,D) Relative abundances of major taxa present in the fish digesta (C) or mucosa (D) which are significantly altered according to diet ($n = 3$). Phylum Firmicutes: $*p = 0.020$; Phylum Bacteroidota: $*p = 0.027$; Class Alphaproteobacteria: $*p = 0.011$; Welch two-sample t test. In (B–D), boxes represent median, first and third quartile. Whiskers extend to the highest and lowest values that are within one and a half times the interquartile range.

of many of these taxa were also detected for mucosal-associated communities and tended to follow the same trends as for the digesta communities (with few exceptions), although these were found not to be statistically significant (Figure 2.4; adjusted- $p > 0.05$, Wald test followed by Benjamini–Hochberg correction for multiple testing). The only taxa that significantly changed in abundance in the mucosa are the family *Vibrionaceae* and the *Vibrio* genus, which increases in abundance upon consumption of the HM25 diet, and the genus *Ligilactobacillus* (family *Lactobacillaceae*), which decreases upon consumption of the HEM25 diet (Figure 2.4, bottom panel).

2.3.3 HI exuviae diet drives an increase in abundance of chitinase-encoding *Paenibacillus* species with effective chitinolytic activity

Chitin degradation has been shown to enhance host and microbiota utilization of otherwise inaccessible proteins and lipids present in IM-based diets (De Marco et al., 2015). Genomic potential for chitin degradation and/or proven chitinolytic activity has been demonstrated for many bacteria of marine environments, including Gamma- and Beta-proteobacteria, Cyanobacteria, Bacteroidetes, Actinobacteria and Firmicutes (Enke et al., 2018; Hobel et al., 2005; Kawase et al., 2004; Paulsen et al., 2016; Ramaiah et al., 2000). Among the different reported types of bacterial chitinases (Martínez-Zavala et al., 2020; Orikoshi et al., 2005), ChiA has proven useful as a molecular marker for chitinase presence due to its high degree of sequence conservation and occurrence in a large array of phylogenetically distant organisms (Hobel et al., 2005; Ramaiah et al., 2000). To determine whether the dramatic shifts in microbial community structure and composition observed for the HM25 and HEM25 diets were accompanied by an increase in microbial chitinolytic potential, we screened samples for the presence of *chiA* using primers targeting a conserved region of the gene (Ramaiah et al., 2000). To confirm the selectivity of the primers, amplicon products were cloned and sequenced. All sequenced clones ($n=15$) contained sequences matching chitinases (Supplementary Table 2.7). The vast majority (73%) of the chitinase sequences display high similarity (67.16% to 98.78%) with chitinases originating from *Paenibacillus* sp. (*Paenibacillus thiaminolyticus* and *Paenibacillus solanis* or closely related organisms), followed by *Streptomyces* sp. (27%), with a single clone containing a sequence identical to a chitinase from the *Pelomonas puraquae* (Figure 2.5A, Supplementary Table 2.7). Using quantitative PCR we show that *chiA* is detected in the digesta of fish on all diets, but fish fed the HEM25 diet have significantly higher copy numbers of *chiA* compared to fish fed the CTR or any of the other diets ($2.4 \pm 0.2E04$ versus $3.7 \pm 1.7E03$ copies per gram of digesta; Figure 2.5B, $p=0.0077$, ANOVA). Levels of *chiA* were below the limit of detection (10 copies per μL of DNA or $2E03$ copies per gram of digesta) for all mucosal samples tested. These results show an increased *chiA*-driven chitinolytic potential of the European sea bass digesta microbiota fed a HEM25 diet.

Further, phylogenetic analysis of *Paenibacillus* 16S rRNA gene amplicon sequences revealed that two of the ASVs identified in our study (ASV_eox_xvi and ASV_jbc_4sr) are highly related to *P. thiaminolyticus* (100% identity) and *P. solanis* (97.2% identity), respectively (Figure 2.5C). Interestingly, both of these ASVs increased in relative

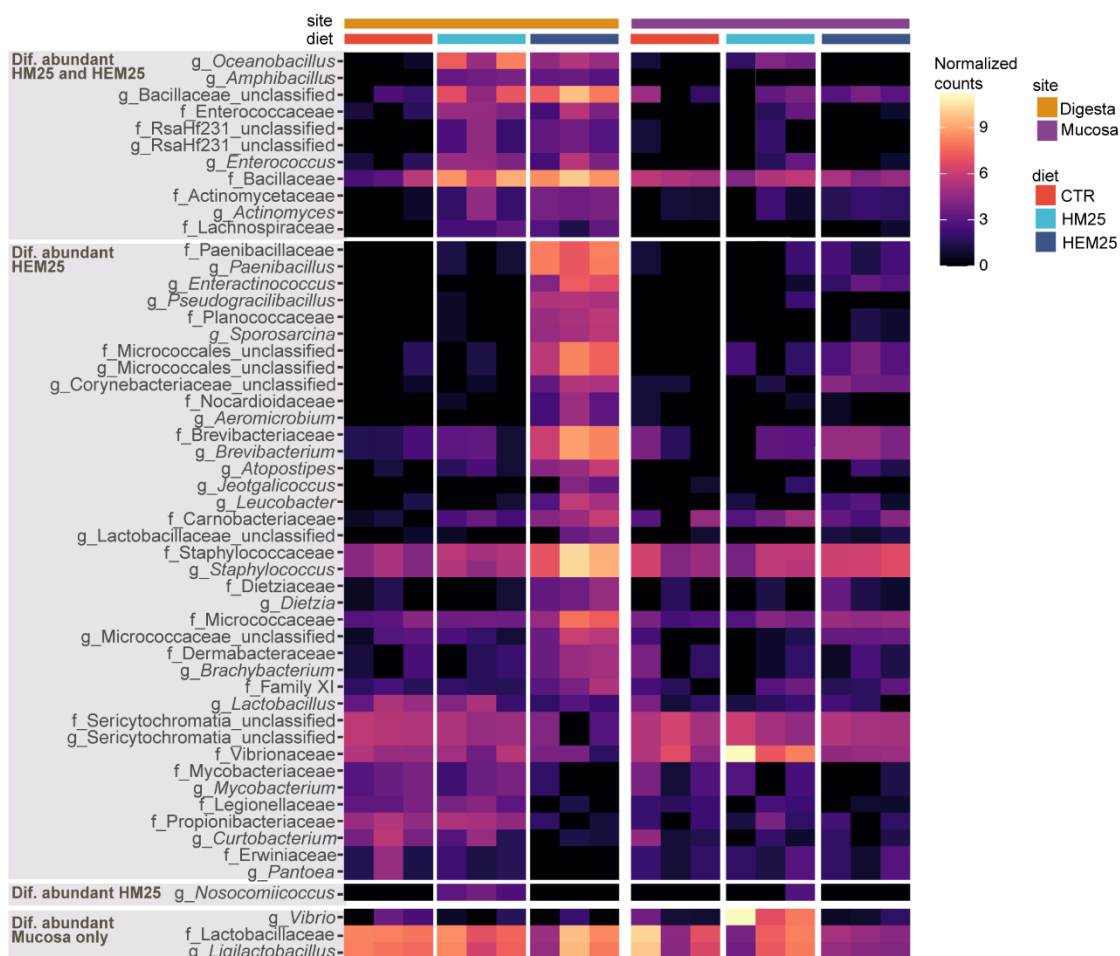


Figure 2.4 Genus and Family-level shifts induced by HI-based diets. Heat map showing transformed (log2 base) normalized read counts of genus and family-level taxa found to be differentially abundant in the guts of fish fed HM25 vs. a CTR diet, or fed a HME25 vs. CTR diet, based on DESeq2 analysis (adjusted value of $p < 0.05$, Wald test followed by Benjamini–Hochberg correction for multiple testing). Top three panels show taxa differentially abundant in digesta-originating samples or in both digesta and mucosa samples (marked with an asterisk), while the bottom panel shows taxa differentially abundant in mucosa-originating samples only.

abundance in fish fed the HEM25 diet (Figure 2.5D and 2.5E), thus potentially explaining the increased *chiA* levels associated with the HEM25 microbiota. To confirm that the increase in *chiA* copy number could be translated into an increase in effective microbiota-driven chitinolytic activity, we screened a collection of spore-forming organisms isolated from the guts of these fish for the presence of *Paenibacillus* isolates with chitinolytic capacity (Rangel et al., manuscript in preparation). All recovered *Paenibacillus* isolates displayed chitinolytic activity (Figure 2.5C, Isolates FI698, FI620, FI622 and FI710; see Materials and Methods). Based on phylogenetic analyses we confirm that two of these isolates (FI698 and FI710) are highly related (>98% identity) to the identified *Paenibacillus* ASVs mentioned above, and at least one of these isolates (FI698)

contains a copy of *chiA*. Together, these results demonstrate that the HEM25 diet, but not any of the other IM diets, is able to modulate the fish digesta microbiota leading to an increase in the abundance of several chitinolytic *Paenibacillus* sp., and to overall microbiota-associated chitinolytic potential.

2.4 Discussion

The rapid expansion of aquaculture dictates that, for a sustainable industry, aquafeeds must be adapted to the available resources. Changes in fish dietary regime, with the inclusion of novel ingredients such as IM, could however, reshape the microbial communities present in the digestive tract. As aquaculture turns to the inclusion of insect protein in fish diets, understanding microbial community responses to these ingredients is paramount. Several studies have evaluated the effects of IM-based diets on fish growth and digestibility (Nogales-Mérida et al., 2018), however, studies on its impact on the gut microbiota of marine fish are still in low number and in certain cases, inconclusive (Antonopoulou et al., 2019; Józefiak et al., 2019; Li et al., 2021; Panteli et al., 2021; Rimoldi et al., 2021; Terova et al., 2021; Tran et al., 2022). In this study, we unveil shifts occurring on European sea bass digesta- and mucosa-associated gut microbial communities in response to three different IM including, for the first time, an IM based on pupae exuviae.

Microbial composition analysis of the mucosa and digesta communities revealed high abundance of Proteobacteria, Firmicutes and Actinobacteria in the guts of the European sea bass in aquaculture, in agreement with previous reports (Kokou et al., 2019; Rimoldi et al., 2020; Serra et al., 2021). In this study we observed that mucosal communities are less diverse than digesta communities and are more resilient to changes induced by IM-based diets. These findings demonstrate that the intestinal sampling site acts as a filter, enabling the establishment of distinct microbial communities along the cross-sectional axis of the gut, similarly to what has been documented longitudinally along the different compartments of the European sea bass gastrointestinal tract (Kokou et al., 2019). The resilience of mucosa-associated communities of marine fish to dietary changes has been reported in other studies (Li et al., 2021; Serra et al., 2021), and is likely to be the result of stable symbiotic relationships established with the host and other microorganisms upon adhesion to the mucosal surface. Within the intestinal lumen, however, constant renovation of dietary nutrients and associated niches might allow the co-existence of otherwise competitive exclusive bacteria and, thus, greater variability (Pereira and Berry, 2017). Additionally, the lower richness and diversity of the autochthonous microbiota

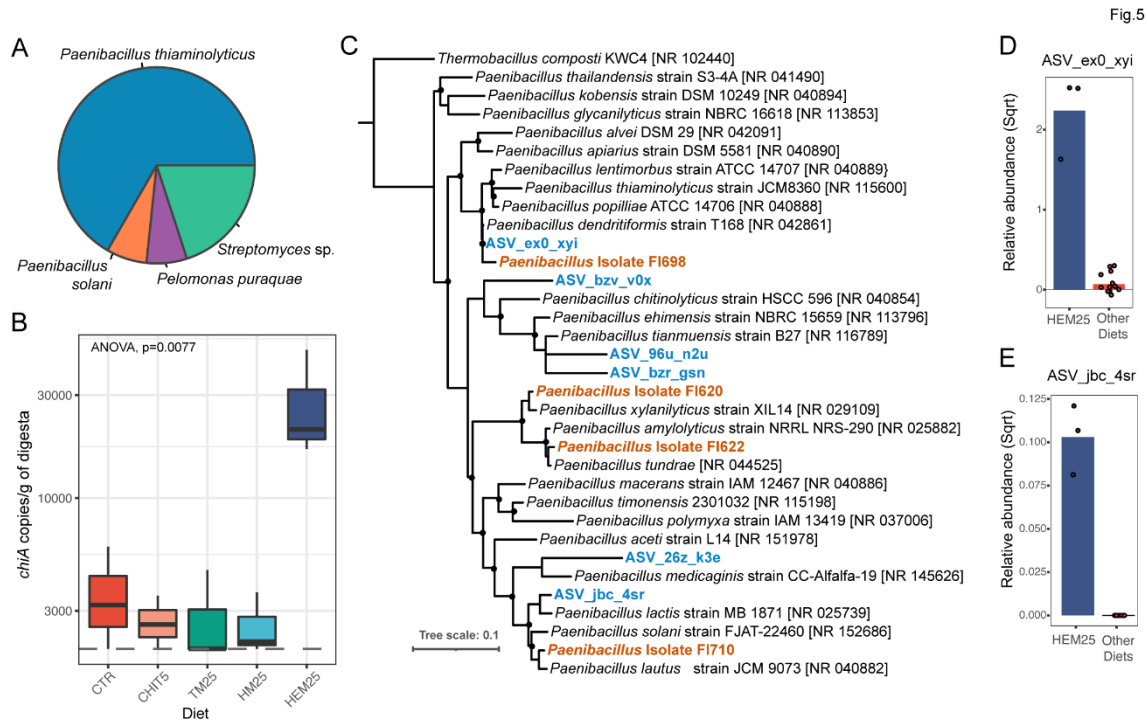


Figure 2.5 HI exuviae diet increases the abundance of Chitinase A-encoding taxa. (A) Taxonomic affiliation of cloned chitinase genes (*chiA*) amplified from digesta samples ($n = 15$ sequenced clones). (B) Copy number (determined by qPCR) of *chiA* in DNA samples obtained from the digesta of fish fed different diets. For some of the CHIT5, TM25 and HM25 diet samples, the presence of *chiA* was determined to be below the limit of detection of 10 copies per μ l of DNA (equivalent to 2,000 copies per gram of digesta), represented by a dashed line. (C) Tree showing phylogenetic affiliations of ASVs and *Paenibacillus* sp. isolates with chitinolytic activity (recovered from the fish intestines) in relation to reference *Paenibacillus* isolate sequences. ASVs are presented in blue and bold, and isolates are highlighted in orange and bold. Bootstrap values >50% are presented as black filled circles on nodes. Scale bar represents 10% sequence divergence. (D,E) Relative abundance of ASV_ex0_xyi (D) and ASV_jbc_4sr (E) (square root normalized) in digesta of fish fed HEM25 diet or all other diets combined. Bars represent mean values, and each point represents an individual sample ($n = 3$ for HEM25 diet and $n = 12$ for other diets).

could also indicate that several allochthonous microorganisms do not possess the necessary characteristics to colonize the mucosal surface of the host (Gajardo et al., 2016; Serra et al., 2021).

Dietary components promoted pronounced digesta-associated microbial community shifts (Figure 2.3). When compared to the control diet, both HM25 and HEM25 lead to an increase in the relative abundance of the phylum Firmicutes in the digesta-associated microbial communities, whereas TM25 lead to a decrease. In fact, the increase of Firmicutes, generally thought to be advantageous due to the multiple beneficial bacteria known within this phylum (Perry et al., 2020; Ringø, Einar et al., 2020; Ringø, E. et al., 2020; Van Doan et al., 2020), appears to be consistent independently of the fish species

fed HM (Foyosal and Gupta, 2022). At the family level, both HM diets lead to an increase of the families *Bacillaceae*, *Enterococcaceae*, and *Lachnospiraceae* (all Firmicutes), all of which contain species with beneficial potential: the *Bacillaceae* family is known to possess species with identified probiotic abilities such as the *Bacillus subtilis* (Butt and Volkoff, 2019; Ringø, E. et al., 2020; Van Doan et al., 2020); *Enterococcaceae* are known to produce antibacterial compounds and to display probiotic abilities, increasing the fish immunologic response and resistance to disease, as well as improving growth (Ringø, Einar et al., 2020; Ringø et al., 2018); and *Lachnospiraceae* has members capable of producing butyrate, a highly important short-chain fatty acid (SCFA), known to act as an anti-inflammatory agent and to play a key role in the fish immune system regulation (Terova et al., 2016; Zhang and Davies, 2016). Further, the HEM diet had an even stronger influence over the digesta-microbiota, increasing the abundance of additional families of the Firmicutes phylum such as *Planococcaceae*, *Staphylococcaceae* and *Brevibacteriaceae*. Increases in these families due to dietary HM inclusion has also been shown by other authors (Panteli et al., 2021; Terova et al., 2019), although its function on the fish gut microbiota has been poorly discussed. Our data also revealed that HEM25 promoted an increase in *Paenibacillaceae* (Figure 2.4). This family, which was also shown to increase upon a dietary inclusion of 15% HM in rainbow trout (*Oncorhynchus mykiss*) (Rimoldi et al., 2021), contains the genus *Paenibacillus*, known to have probiotic abilities when added to the fish feed (Chen et al., 2019; Gupta et al., 2014, 2016), as well as chitinolytic capabilities (discussed below).

Interestingly, we observed distinct changes in the gut microbiota community in response to the different diets used in this study, despite their similar crude protein, lipid, and chitin content (Figure 2.1). Inclusion of dietary fibers, such as chitin, generally leads to gut microbiota shifts towards Firmicutes and/or Actinobacteria (Huyben et al., 2019; Panteli et al., 2021; Rimoldi et al., 2019; Terova et al., 2019). However, similar dietary chitin contents (1.7% in HM25 vs 1.8% in HEM25 vs 1.3% in TM25) led to different levels in both Firmicutes (increased in HM25 and HEM25 vs decrease in TM25), and Actinobacteria (increase in HM25 and HEM25 vs no-effect in TM25), as well as to disparate shifts at low taxonomic levels (Figure 2.4). As with Firmicutes, selective increase of Actinobacteria in the guts of fish fed diets containing HM, appears to be transversal independently of the fish species (Foyosal and Gupta, 2022). Moreover, dietary inclusion of 5% of commercial chitin did not result in the increase of bacteria of either of those phyla. This can be due to different physicochemical characteristics of chitin present in the different diets. Varying degrees of acetylation, chitin-associated

proteins and insects' life cycle stages can change, among others, chitin's surface, porosity, or solubility thus affecting its bioaccessibility (Muthukrishnan et al., 2019; Purkayastha and Sarkar, 2020; Waśko et al., 2016; Zargar et al., 2015). Furthermore, we also hypothesize that dietary components other than chitin are behind some of the modulatory effects observed. Indeed, the constitution of macromolecules such as lipids and proteins differ according to the insect's species, developmental stage and rearing substrate, which can result in different modulatory effects of the microbiota (Bruno et al., 2019; Gołębiowski et al., 2014; Nogales-Mérida et al., 2018; Vogel et al., 2018). As an example, fatty acid profile in lipids has been shown to differently impact gut microbial communities, with diets supplemented with medium chain fatty acids (MCFA) favoring an increase of the Firmicutes phylum (Rimoldi et al., 2018). The latter might help to explain the observed increase in the Firmicutes phylum in HM-based diets as HM, in contrast with TM, are rich in the MCFA lauric acid (C12:0) (Guerreiro et al., 2020). Thus, diet-induced alterations in the gut microbiota may occur due to IM-specific fatty acid, amino acid, or even simple-sugar profiles, potentially creating micro-niches that promote the establishment of organisms with selective nutritional preferences (Pereira and Berry, 2017). This could also help explain the absence of microbiota shifts in response to a CHIT5 diet, which shares the same overall crude macromolecular composition as the control diet, except for the presence of chitin (Figure 2.1). Finally, we cannot exclude that some of the differences among diets originate from amplification of DNA from dead vegetative cells or from spores that were present in the own insect's gut or cuticle. Other approaches such as transcriptomics or activity-based methods would be necessary to fully unravel the contribution of food-borne microorganisms to the overall fish gut microbiota profiles (Emerson et al., 2017).

The presence of chitin is a negative aspect of IM because it is difficult to breakdown and because it can entrap other nutrients, like lipids and proteins, thereby limiting their digestibility (De Marco et al., 2015; Guerreiro et al., 2021). Upon HM inclusion in diets, we observed an increase in Firmicutes families *Bacillaceae*, *Enterococcaceae* and *Actinomycetaceae*, and a HEM-specific increase in the families *Planococcaceae*, *Brevibacteriaceae* and *Paenibacillaceae*, all of which contain species with chitinolytic activity (Callegari et al., 2020; Shimoi et al., 2020). Indeed, we detected an increase in the copy number of genes encoding ChiA, used here as a marker for chitinolytic potential, in fish fed the HEM25 diet (Figure 2.5). However, we detected no increase in *chiA* copies in fish fed the CHIT5 diet, despite an increase of the order Chitinophagales which contains species that can degrade chitin (Rosenberg, 2014; Wieczorek et al., 2019), nor

in fish fed the HM25 diet, despite an increase in *Bacillaceae* and *Actinomycetaceae* (Shimoi et al., 2020). Chitinolytic *Chitinopagaceae* species, along with Actinobacteria species, have been shown to employ chitinases other than ChiA, such as exochitinases or endochitinases belonging to other glycoside hydrolase families, for chitin breakdown (Lacombe-Harvey et al., 2018; Ramakrishna et al., 2018). As these chitinases share no sequence similarity with ChiA (Lacombe-Harvey et al., 2018), they may have been missed in our study. Apart from DNA-based evidence, future investigation of chitin-degradation capacity should include evaluation of the effective chitinolytic enzymatic activity of the digesta. Together, increases in abundance of 16S rRNA and *chiA* gene sequences belonging to *Paenibacillus* species in HEM25 diet, as well as recovery of *Paenibacillus* isolates with chitinolytic activity, support an HEM25-driven increase in chitinolytic potential that is likely translated into chitin degradation in the fish lumen. A detailed evaluation of the possible impact of HEM on fish fitness, at the inclusion levels here used or at higher inclusion levels, should be performed in the future.

2.5 Conclusion

In summary, the data reported here provides a detailed characterization of microbiota shifts induced by two commonly used IM (TM and HM) and one underexplored insect-based ingredient (HEM) on the European sea bass gut microbiota. Overall, the data suggests that IM diets of different origins differently impact the gut microbiota of the European sea bass digesta-associated communities, while mucosal-associated communities are more resilient to diet-induced alterations. The inclusion of HEM in the diet increased the digesta bacterial-associated chitinolytic potential, likely resulting in enhanced utilization of otherwise inaccessible proteins, lipids, and energy present in this diet, which ultimately can lead to better fish growth performance. Our study uncovers possible advantages of incorporating exuviae in fish diets, an economically attractive product that may be key to raise levels of IM inclusion in aquafeeds.

Declarations

Ethics approval and consent to participate

Animal experiments were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) and carried out in a registered installation (N16091.UDER). The trial was performed by trained scientists (following FELASA category C recommendations) in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Availability of data and Materials

The 16S rRNA gene sequences were deposited in the NCBI Sequence Read Archive (SRA) as BioProject Accession PRJNA767204. The datasets generated and/or analyzed during the current study are available in the github repository, under the link https://github.com/fatimapereira454/Amplicon-sequencing_Seabass_IMdiets (metadata table, read count table).

Authors' contributions

C.R.S., P.E., F.C.P. and A.O.T. designed and conceived the study. F.R. and F.C.P. performed the experiments. F.C.P. and B.H. generated and analyzed the sequencing data. L.G. and F.G. formulated and provided the diets. F.R., F.C.P., C.R.S. and P.E. wrote the manuscript with contributions from A.O.T. and D.B.. All authors read and approved the final version of the manuscript.

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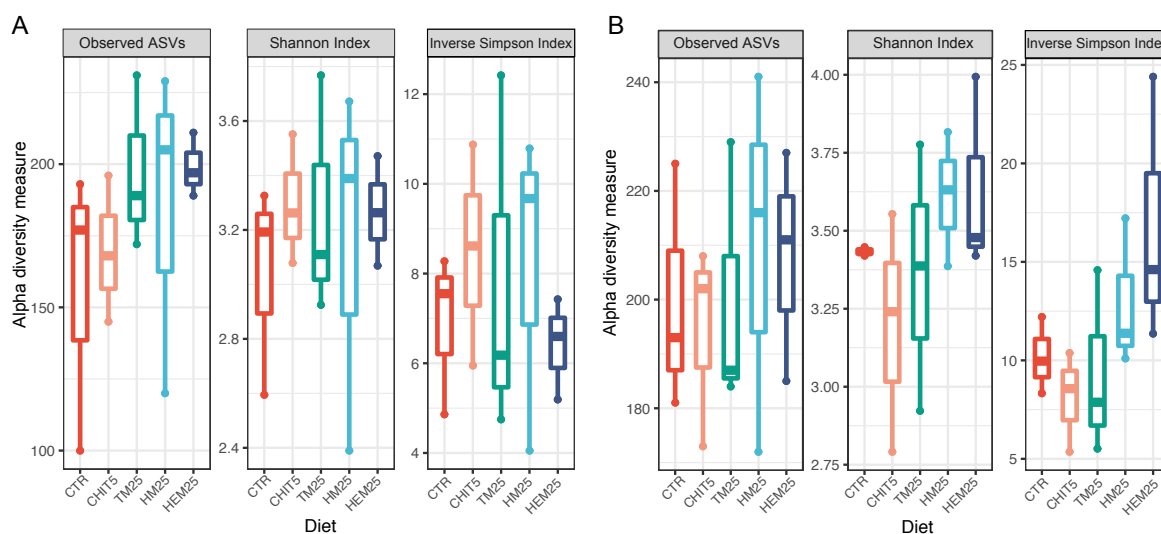
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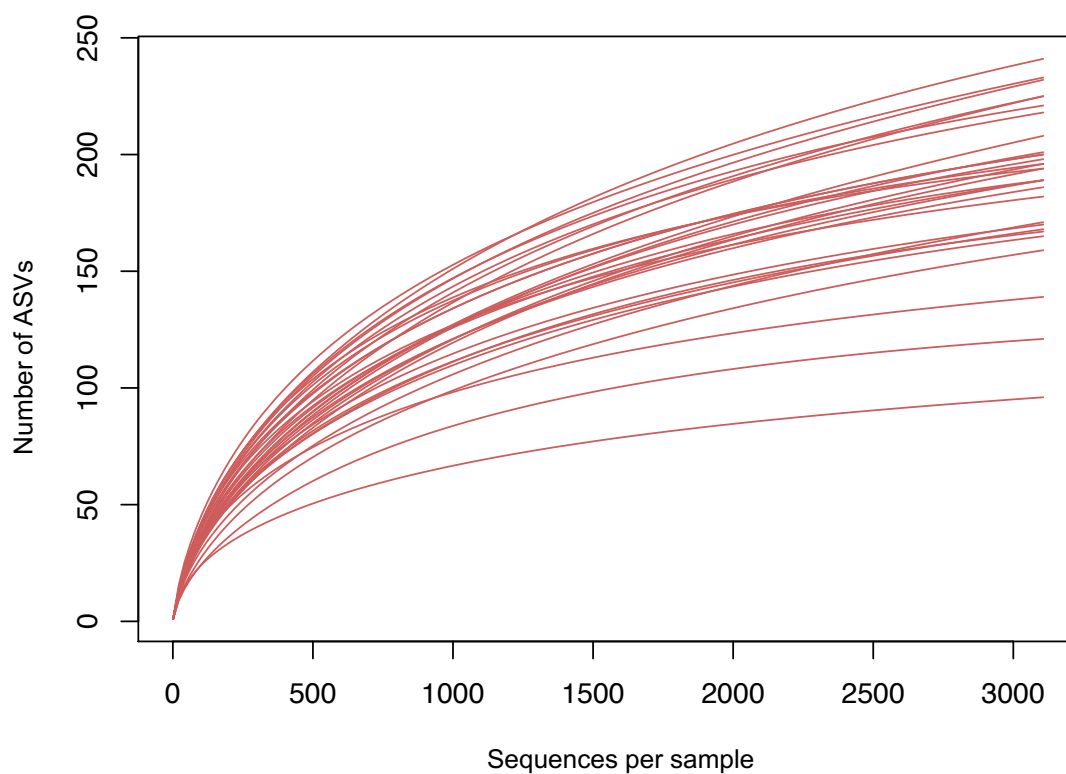
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2.7 Supplementary material



Supplementary figure 2.1 Alpha diversity metrics (number of Observed ASVs, Shannon Index and Inverse Simpson Index) of the mucosa (A) and digesta samples (B) from fish fed indicated diets. Each point represents one sample ($n=3$ samples per diet per site; each sample was obtained after pooling material from 2 fish from the same tank). Boxes represent median, first and third quartile. Whiskers extend to the highest and lowest values that are within one and a half times the interquartile range. No significant differences between diets were detected ($p>0.05$, Wilcoxon test).



Supplementary figure 2.2 Rarefaction curves for the different samples, after rarefying.

Supplementary table 2.1 Ingredient composition and proximate analysis of the experimental diets.

	Diets				
	CTR	HM25	HEM25	TM25	CHIT5
<i>Ingredients (% dry weight basis)</i>					
Fish meal ^a	44.0	24.4	21.6	19.7	45.1
Soluble fish protein concentrate ^b	2.0	2.0	2.0	2.0	2.0
<i>Hermetia illucens</i> larvae meal ^c	-	25.0	-	-	-
<i>Hermetia illucens</i> exuvia meal ^d	-	-	25.0	-	-
<i>Tenebrio molitor</i> larvae meal ^e	-	-	-	25.0	-
Corn gluten ^f	5.0	5.0	5.0	5.0	5.0
Soybean meal ^g	7.5	7.5	7.5	7.5	7.5
Wheat meal ^h	27.9	20.4	22.1	25.9	21.9
Fish oil	9.9	11.7	11.7	10.5	9.7
Vitamin premix ⁱ	1.0	1.0	1.0	1.0	1.0
Mineral premix ^j	1.0	1.0	1.0	1.0	1.0
Choline chloride (50%)	0.5	0.5	0.5	0.5	0.5
Binder ^k	1.0	1.0	1.0	1.0	1.0
Taurine ^l	0.2	0.2	0.2	0.2	0.2
Dibasic calcium phosphate	-	0.3	1.4	0.7	-
Chitin ^m	-	-	-	-	5.0
<i>Proximate analyses (% dry weight basis)</i>					
Dry matter	93.6	92.8	89.7	93.4	93.6
Crude protein	46.0	45.4	45.2	45.3	46.3
Crude fat	17.8	17.9	18.1	17.8	18.2
Ash	7.8	8.3	8.6	6.7	7.7
Chitin	-	1.7	1.8	1.3	5.0

^aSorgal, S.A. Ovar, Portugal (CP: 72.4 % DM; GL: 17.0 % DM).

^bSorgal, S.A. Ovar, Portugal (CP: 79.7 % DM; GL: 7.14 % DM).

^cBlack soldier fly larvae meal (CP: 55.4 % DM; GL: 10.9 % DM; chitin 6.8 % DM).

^dBlack soldier fly exuvia meal (CP: 64.3 % DM; GL: 8.1 % DM; chitin 7.2 % DM).

^eYellow mealworm larvae meal (CP: 69.5 % DM; GL: 14.1 % DM; chitin 5.2 % DM).

^fSorgal, S.A. Ovar, Portugal (CP: 69.9 % DM; GL: 3.3 % DM).

^gSorgal, S.A. Ovar, Portugal (CP: 54.2 % DM; GL: 1.8 % DM).

^hSorgal, S.A. Ovar, Portugal (CP: 13.8 % DM; GL: 1.1 % DM).

ⁱVitamins (mg kg⁻¹ diet): retinol, 18 000 (IU kg⁻¹ diet); cholecalciferol, 2 000 (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.

^jMinerals (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).

^kAquacube. Agil, UK.

^lFeed-grade taurine, Sorgal, S.A. Ovar, Portugal.

^mChitin from shrimp shells, practical grade powder, Sigma-Aldrich

Supplementary table 2.2 Coverage (as a measure of sample completeness) for each analysed sample.

Sample ID	Sample type	Diet	Sample Coverage
D1	Digesta	CTR	0.98
D2	Digesta	TM25	0.98
D3	Digesta	HEM25	0.97
D4	Digesta	HM25	0.98
D5	Digesta	TM25	0.98
D6	Digesta	CHIT5	0.98
D7	Digesta	CTR	0.98
D8	Digesta	CHIT5	0.98
D9	Digesta	HEM25	0.98
D10	Digesta	CHIT5	0.98
D11	Digesta	HM25	0.98
D12	Digesta	CTR	0.98
D13	Digesta	HEM25	0.98
D14	Digesta	TM25	0.98
D15	Digesta	HM25	0.98
M1	Mucosa	CTR	0.98
M2	Mucosa	TM25	0.98
M3	Mucosa	HEM25	0.98
M4	Mucosa	HM25	0.99
M5	Mucosa	TM25	0.98
M6	Mucosa	CHIT5	0.99
M7	Mucosa	CTR	0.99
M8	Mucosa	CHIT5	0.99
M9	Mucosa	HEM25	0.98
M10	Mucosa	CHIT5	0.98
M11	Mucosa	HM25	0.98
M12	Mucosa	CTR	0.98
M13	Mucosa	HEM25	0.98
M14	Mucosa	TM25	0.98
M15	Mucosa	HM25	0.98

Supplementary table 2.3 Relative abundances of all taxa (Kingdom, Phylum, Class, Order, Family and Genus level)

(Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9041418/>).

Supplementary table 2.4 Alpha diversity metrics.

Sample_ID	tank	site	diet	Observed ASVs	Shannon	InvSimpson
<i>Digesta samples</i>						
D1	1	Digesta	CTR	193	3.45	12.21
D7	7	Digesta	CTR	177	3.40	9.83
D12	12	Digesta	CTR	217	3.37	8.38
D3	3	Digesta	HE25	211	3.48	12.24
D9	9	Digesta	HE25	198	3.55	14.65
D13	13	Digesta	HE25	225	4.03	25.46
D2	2	Digesta	TM25	232	3.78	14.88
D5	5	Digesta	TM25	173	2.97	5.91
D14	14	Digesta	TM25	183	3.32	7.48
D4	4	Digesta	HM25	208	3.74	15.94
D11	11	Digesta	HM25	241	3.63	10.10
D15	15	Digesta	HM25	170	3.36	10.83
D6	6	Digesta	CHT	197	3.56	10.10
D8	8	Digesta	CHT	163	2.75	5.09
D10	10	Digesta	CHT	200	3.22	8.35
<i>Mucosal samples</i>						
M1	1	Mucosa	CTR	177	3.19	8.27
M7	7	Mucosa	CTR	92	2.52	4.70
M12	12	Mucosa	CTR	192	3.32	7.52
M3	3	Mucosa	HE25	194	3.50	7.72
M9	9	Mucosa	HE25	190	3.06	5.21
M13	13	Mucosa	HE25	231	3.29	6.57
M2	2	Mucosa	TM25	229	3.73	12.16
M5	5	Mucosa	TM25	162	3.08	6.09
M14	14	Mucosa	TM25	184	2.91	4.66
M4	4	Mucosa	HM25	117	2.39	4.17
M11	11	Mucosa	HM25	229	3.67	10.79
M15	15	Mucosa	HM25	203	3.39	9.92
M6	6	Mucosa	CHT	152	3.28	8.34
M8	8	Mucosa	CHT	166	3.08	6.01
M10	10	Mucosa	CHT	202	3.59	10.69

Supplementary table 2.5 PERMANOVA analysis.

adonis ^a	Digesta and mucosa		Digesta		Mucosa	
	p-value	R ²	p-value	R ²	p-value	R ²
All diets	0.004	0.247	0.002	0.580	0.396	0.291
CHIT5 vs CTR	0.682	0.070	0.700	0.161	1.000	0.128
TM25 vs CTR	0.548	0.079	0.300	0.234	1.000	0.078
HM25 vs CTR	0.378	0.094	0.200	0.259	0.900	0.163
HEM25 vs CTR	0.041	0.199	0.100	0.567	0.600	0.181

^aadonis() function in R for analysis of variance using distance matrices.

Supplementary table 2.6 DESeq2 analysis of differentially abundant Genera and Families for the tested diets TM25, HM25 and HEM25 versus control (CTR) diet, for either digesta (TM25) or digesta and mucosal communities (HM25 and HEM25). For diet CHIT5 no significant differences were detected.

Taxa ^a	base Mean ^b	log2_Fold Change	lfcSE ^c	p-value	adjusted p-value
TM25 versus CTR-Digesta					
g_Cupriavidus	41.9694	2.5804	0.6260	0.0000	0.0103
f_Lactobacillaceae	291.0094	-1.1312	0.3028	0.0002	0.0259
g_Ligilactobacillus	247.2979	-1.1327	0.3152	0.0003	0.0301
HEM25 versus CTR-Digesta					
f_Paenibacillaceae	109.5574	10.6415	1.4788	0.0000	0.0000
g_Paenibacillus	107.2806	10.6112	1.4780	0.0000	0.0000
f_Brevibacteriaceae	135.0298	6.4369	1.0134	0.0000	0.0000
g_Brevibacterium	135.0298	6.4369	1.0134	0.0000	0.0000
g_Bacillaceae_unclassified	190.0325	6.9860	1.1096	0.0000	0.0000
g_Enteractinococcus	42.7933	9.2848	1.4941	0.0000	0.0000
f_Micrococcales_unclassified	82.5225	7.6867	1.2744	0.0000	0.0000
g_Micrococcales_unclassified	82.5225	7.6867	1.2744	0.0000	0.0000
g_Pseudogracilibacillus	18.5842	8.0806	1.3574	0.0000	0.0000
f_Planococcaceae	18.0009	8.0390	1.3671	0.0000	0.0000
g_Sporosarcina	16.9006	7.9483	1.3699	0.0000	0.0000
f_Carnobacteriaceae	17.0636	5.5299	1.1101	0.0000	0.0000
g_Oceanobacillus	14.1811	6.7114	1.3700	0.0000	0.0000
g_Atopostipes	16.7539	6.0937	1.2661	0.0000	0.0000
f_Staphylococcaceae	323.8769	4.5873	0.9609	0.0000	0.0000
g_Staphylococcus	320.0448	4.5694	0.9598	0.0000	0.0000
g_Corynebacteriaceae_unclassified	13.6506	6.6594	1.4110	0.0000	0.0000
g_Leucobacter	14.6242	5.7962	1.3043	0.0000	0.0001
f_Bacillaceae	278.0321	4.5757	1.0328	0.0000	0.0001
f_Propionibacteriaceae	16.4044	-4.5670	1.0716	0.0000	0.0002
f_Nocardioidaceae	6.3181	6.5159	1.5800	0.0000	0.0003
g_Aeromicrobium	6.3181	6.5159	1.5800	0.0000	0.0003
g_Curtobacterium	13.7607	-4.9123	1.2760	0.0001	0.0009
f_Micrococcaceae	69.6549	3.5101	0.9400	0.0002	0.0013
f_Actinomycetaceae	6.1038	5.4803	1.5026	0.0003	0.0017
g_Actinomyces	6.1038	5.4803	1.5026	0.0003	0.0017
f_Enterococcaceae	10.4342	3.9177	1.1038	0.0004	0.0023
f_RsaHf231_unclassified	4.0979	5.8955	1.6662	0.0004	0.0023
g_Enterococcus	10.4342	3.9177	1.1038	0.0004	0.0023
g_RsaHf231_unclassified	4.0979	5.8955	1.6662	0.0004	0.0023
f_Dermabacteraceae	11.5417	3.3097	0.9632	0.0006	0.0029

Taxa ^a	base Mean ^b	log2_Fold Change	lfcSE ^c	p-value	adjusted p-value
HEM25 versus CTR-Digesta					
g_Amphibacillus	3.8502	5.8066	1.6905	0.0006	0.0029
g_Brachy bacterium	11.5417	3.3097	0.9632	0.0006	0.0029
g_Micrococcaceae_unclassified	22.2801	3.0326	0.8827	0.0006	0.0029
f_Dietziaceae	7.4570	3.8792	1.1541	0.0008	0.0036
g_Dietzia	7.4570	3.8792	1.1541	0.0008	0.0036
f_Sericytochromatia_unclassified	28.9179	-2.8432	0.8520	0.0008	0.0038
g_Sericytochromatia_unclassified	28.9179	-2.8432	0.8520	0.0008	0.0038
f_Demequinaceae	3.6187	5.7296	1.7784	0.0013	0.0054
g_Demequinaceae_unclassified	3.6187	5.7296	1.7784	0.0013	0.0054
g_Lactobacillus	15.2746	-2.7128	0.8615	0.0016	0.0068
f_Erwinia	4.8241	-5.2970	1.7485	0.0025	0.0096
g_Pantoea	4.8241	-5.2970	1.7485	0.0025	0.0096
f_Legionellaceae	5.6015	-4.4736	1.5195	0.0032	0.0124
f_Family XI	10.8410	2.4355	0.8825	0.0058	0.0217
f_Lachnospiraceae	2.5335	5.2174	1.9346	0.0070	0.0257
g_Lactobacillaceae_unclassified	3.9555	4.8413	1.8042	0.0073	0.0262
f_Mycobacteriaceae	5.9562	-3.5990	1.3971	0.0100	0.0345
g_Mycobacterium	5.9562	-3.5990	1.3971	0.0100	0.0345
g_Family XI_unclassified	3.2372	4.5181	1.8004	0.0121	0.0409
HM25 versus CTR-Digesta					
g_Oceanobacillus	65.6323	9.2829	1.4807	0.0000	0.0000
g_Amphibacillus	6.8354	6.4464	1.3973	0.0000	0.0004
g_Bacillaceae_unclassified	174.5504	4.9680	1.1015	0.0000	0.0005
f_Enterococcaceae	15.3439	4.1707	0.9978	0.0000	0.0010
f_RsaHf231_unclassified	6.8103	6.3838	1.5353	0.0000	0.0010
g_Enterococcus	15.3439	4.1707	0.9978	0.0000	0.0010
g_RsaHf231_unclassified	6.8103	6.3838	1.5353	0.0000	0.0010
f_Bacillaceae	330.9931	4.0901	0.9914	0.0000	0.0010
f_Actinomycetaceae	7.8338	5.2402	1.5037	0.0005	0.0107
f_Lachnospiraceae	3.9602	5.5091	1.5913	0.0005	0.0107
g_Actinomyces	7.8338	5.2402	1.5037	0.0005	0.0107
g_Nosocomiicoccus	2.8863	5.9964	1.8674	0.0013	0.0242
HEM25 versus CTR-Mucosa					
f_Lactobacillaceae	240.3031	-3.9755	0.9790	0.0000	0.0067
g_Ligilactobacillus	187.3716	-3.9730	0.9658	0.0000	0.0067
HM25 versus CTR-Mucosa					
g_Vibrio	412.4551	7.5674	1.2042	0.0000	0.0000
f_Vibrionaceae	457.5447	3.9646	1.0089	0.0001	0.0113

^a The prefix “f_” denotes family and “g_” denotes genus.

^b Base count mean.

^c Standard error value returned by DESeq2.~

Supplementary table 2.7 DESeq2 analysis of differentially abundant Genera and Families in diet HEM25 versus control and H;25 versus control, for both digesta and mucosal communities.

clone ID ^a	BLAST NCBI ^b			
	Identity	Sequence ID	Identity %	coverage
ChiA_A1	chitinase [Paenibacillus solani]	WP_054405196.1	67.24	94
ChiA_A2	hypothetical protein [Pelomonas puraquae] (GH18)	WP_158218444.1	98.15	98
ChiA_A3	chitinase [Paenibacillus thiaminolyticus]	WP_143798588.1	98.72	97
ChiA_A4	family 18 chitinase, Partial [Streptomyces coelicoflavus]	SNU02820.1	67.16	98
ChiA_A5	chitinase [Paenibacillus thiaminolyticus]	WP_119791674.1	97.26	98
ChiA_B1	chitinase [Paenibacillus thiaminolyticus]	WP_119791674.1	98.78	98
ChiA_B2	chitinase [Paenibacillus thiaminolyticus]	WP_119791674.1	98.78	98
ChiA_B3	chitinase [Paenibacillus thiaminolyticus]	WP_119791674.1	96.72	98
ChiA_B4	chitinase [Paenibacillus thiaminolyticus]	WP_119791674.1	98.63	84
ChiA_B5	chitinase [Paenibacillus thiaminolyticus]	WP_143798588.1	98.63	84
ChiA_B6	family 18 chitinase, Partial [Streptomyces lasiicapitis]	SNU02819.1	68.75	94
ChiA_B7	MULTISPECIES: chitinase [Streptomyces]	WP_030882014.1	71.64	98
ChiA_C1	chitinase [Paenibacillus thiaminolyticus]	WP_087440603.1	98.18	71
ChiA_C2	chitinase [Paenibacillus thiaminolyticus]	WP_143798588.1	98.78	98
ChiA_C3	chitinase [Paenibacillus thiaminolyticus]	WP_119791674.1	96.72	97

^a Clone highlighted in bold (ChiA_B2) was used as template for qPCR standards.

^b <https://blast.ncbi.nlm.nih.gov/> (Blastx option was used).

Chapter 3.

Isolation of chitinolytic bacteria from European sea bass gut microbiota fed diets with distinct insect meals

Work published in:

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Abstract

Insect meal (IM), recently authorized for use in aquafeeds, positions itself as a promising commodity for aquafeed inclusion. However, insects are also rich in chitin, a structural polysaccharide present in the exoskeleton, that is not digested by fish, resulting in lower fish performance. Through the application of a dietary pressure, this study aimed to modulate European sea bass gut microbiota towards the enrichment of chitinolytic bacteria, to allow the isolation of novel probiotics capable of improving the use of IM-containing diets, overcoming chitin drawbacks. Five isoproteic (44%) and isolipidic (18%) diets were used: a control FM-based diet (diet CTR), a chitin-supplemented diet (diet CHIT5), and 3 diets with either 25% of *Hermetia illucens* and *Tenebrio molitor* larvae meals (HM25 and TM25, respectively), or *H. illucens* exuviae meal (diet HEM25) as partial FM substitutes. After 8 weeks feeding trial, the results showed a clear modulatory effect towards spore-forming bacteria by HM25 and HEM25 diets with the latter being responsible for the majority of the chitinolytic fish isolates (FI) obtained. Sequential evaluation of the FI hemolytic activity, antibiotic resistance, total chitinolytic activity, sporulation, and survival in gastrointestinal-like conditions identified FI645 and FI658 as the most promising chitinolytic probiotics for *in vivo* application.

Keywords: *Hermetia illucens*, Exuviae, *Tenebrio molitor*, Spore-formers, *Bacillus*, Probiotic, Aquafeeds, Chitinase, *Bacillus licheniformis*

3.1 Introduction

Incremental demand for aquaculture products, coupled with the unsustainable use of fisheries products as feed ingredients, has driven the aquafeeds industry towards the use of sustainable fish meal (FM) alternatives. Insect meal (IM) has shown great potential as a novel protein and lipid source for fish farming (Gasco et al., 2019). IM nutritional value can vary with the insect species, development stage, or processing method (Gasco et al., 2018). Overall, IM has a high protein content (30-68% dry matter, DM), with suitable amino acid profiles (Hawkey et al., 2021). Moreover, lipid content (10-30% DM) can be reduced through defatting processes and its fatty acid profile modulated (Benzertiha et al., 2020; Gasco et al., 2020; Nogales-Mérida et al., 2018).

To date, IM inclusion in aquafeeds has been successfully tested in several fish species, and using different insect species, to a maximum of 25% inclusion, replacing up to 100% of FM (Belghit et al., 2019; Caimi et al., 2020; Chemello et al., 2020; Magalhães et al., 2017; Rema et al., 2019; Stejskal et al., 2020; Zhou et al., 2017). However, dietary IM inclusion higher than 25% tends to decrease protein and lipid digestibility (Guerreiro et al., 2021; Mastoraki et al., 2020; Piccolo et al., 2017; Renna et al., 2017), mainly due to high chitin content (Gasco et al., 2019; Lock et al., 2018). Morono, et al. (2015) supported this claim by demonstrating, *in vitro*, that crude protein digestion is mostly affected by dietary chitin levels. Moreover, when the insects' exoskeleton is removed and, with it, the majority of the chitin content, dietary IM inclusions up to 60% can be achieved without a negative impact on fish performance (Belghit et al., 2018).

Chitin is a structural polysaccharide composed of covalent β (1 $\rightarrow$ 4)- linkages between N-acetyl glucosamine (Resh and Cardé, 2009). In the insect's exoskeleton, chitin forms a tightly packed matrix by bonding itself with structural molecules, such as proteins and lipids (Resh and Cardé, 2009). In the fish gastrointestinal (GI) tract, the disruption of these chitinous exoskeletons into N-acetylglucosamine monomers is mainly attributed to chitinolytic enzymes (chitinase and chitobiase) (Danulat, 1987; Fines and Holt, 2010; Gasco et al., 2020). As such, the lack of these enzymes in the fish GI tract, whether by the inability of fish to produce them or an inefficient production by gut microbiota renders chitin indigestible. This limits its potential use as an energy source and the digestive enzymes' access to the entrapped proteins or lipids, thus decreasing IM and diets digestibility (De Marco et al., 2015; Guerreiro et al., 2021; Mastoraki et al., 2020; Piccolo et al., 2017).

Microbiota's role in the fish performance and health has become increasingly acknowledged, playing key roles in the fish's immune and digestive functions (Butt and Volkoff, 2019; Egerton et al., 2018; Wang, A.R. et al., 2018). The fish gut microbiota is highly variable as its establishment is influenced by several host-related characteristics (e.g. genetic information, development state, immunologic response, the physicochemical profile of the different regions of the GI tract, stress) (Wang, A.R. et al., 2018), as well as environmental and dietary factors (Ringø et al., 2016). Identifying and characterizing these microbial communities, and understanding their contribution to nutrients digestion is of special importance, as through enzymatic secretion gut microbes can breakdown otherwise indigestible dietary components such as chitin (Perry et al., 2020; Ray et al., 2012; Roeselers et al., 2011; Tremaroli and Bäckhed, 2012; Wu et al., 2015). Moreover, dietary inclusion of specific microorganisms as probiotics can be a strategy to potentiate nutrient digestion, and ultimately improve aquaculture production (Llewellyn et al., 2014; Serra et al., 2019).

Based on the high adaptability of gut microbial communities, and the already demonstrated European sea bass (ESB) gut microbiota sensitivity to dietary modulation (Carda-Diéguez et al., 2014; Serra et al., 2019; Serra et al., 2021), this study applied a dietary pressure to selectively induce gut microbiota changes towards chitinolytic microorganisms. Using different insect meals and a chitin-enriched diet we aimed to isolate aerobic spore-forming chitinolytic bacteria. Aerobic sporeformers were target due to their advantageous resistance characteristics and well documented probiotic properties. We comprehensively screened the isolates concerning important probiotic and biosafety traits, namely the absence of antibiotic resistance and hemolytic ability and gut-survival performance, and aptitude. The successful isolation of bacteria able to digest chitin may allow obtaining a probiotic or a probiotic mix with potential interest for the aquaculture industry, capable of improving the use of IM-containing diets.

3.2 Materials and Methods

3.2.1 Animals and experimental conditions

European sea bass (*Dicentrarchus labrax*) juveniles were acquired from Sonrionansa (Cantabria, Spain), and transported to the Marine Zoology Station (Porto University, Porto, Portugal). Following their arrival, the fish were submitted to a 4-week quarantine period and fed with a commercial diet (18% lipids and 44% protein, Aquasoja Sustainable Feed, Sorgal, Ovar, Portugal). After the quarantine, the fish were divided into 15 groups

of 15 fish with an initial mean body weight of 53.7 ± 2.7 g, and the experimental diets were arbitrarily allocated to triplicate groups. The fish were hand-fed to apparent visual satiation twice daily, 6 days per week. The trial lasted 8 weeks and was performed in a recirculating aquaculture system composed of 15 fiberglass tanks of 100 L capacity, thermo-regulated to 22.7 ± 0.3 °C and supplied with a continuous flow of seawater (36.0 ± 0.5 g L⁻¹ salinity, circa 7 mg L⁻¹ oxygen), with an artificial illumination photoperiod set to 12:12 h light:dark.

3.2.2 Experimental diets

Five isoproteic (44%) and isolipidic (18%) diets were formulated: a control FM-based diet without IM inclusion (diet CTR); three diets with 25% inclusion of IM, namely, partially-defatted black soldier fly (*Hermetia illucens*) larvae meal (diet HM25), black soldier fly exuviae meal (remains of the chitinous husk left behind during insect molts) (diet HEM25), or 25% partially-defatted yellow mealworm (*Tenebrio molitor*) larvae meal (diet TM25); and, a diet similar to the CTR but including 5% commercial chitin (practical grade powder, Sigma, Química, S.L., Sintra, Portugal) (diet CHIT5). The IM composition was as follow, on a DM basis: HM (protein: 60.3% lipid: 6.4%; chitin 6.8%), TM (protein: 69.5%; lipid: 14.1% chitin 5.2%), and HEM meal (protein: 64.3%; lipid: 8.1%; chitin 7.2%). 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 resulting pellets were dried in an oven at 40 °C for 24 h and stored in airtight bags at -20 °C until used. The ingredients and proximate composition of the experimental diets are detailed in (Rangel et al., 2022). The DM, protein, lipid, and ash analysis for all the ingredients and experimental diets were carried out according to the Association of Official Analytical Chemists methods (AOAC, 2000). The chitin contents of HM, TM, HEM, and the experimental diets were analyzed according to Guerreiro, *et al.* (2020).

3.2.3 Sampling

To guarantee that fish guts were full, fish were fed 4 h before sampling. Two fish were randomly selected per tank and sacrificed with an anesthetic overdose (3.0 mL⁻¹ ethylene glycol monophenyl ether). The whole gut (without pyloric caeca) was aseptically excised and carefully squeezed to collect the digesta content. To overcome inter-fish variations, digests were pooled into one sample per tank.

3.2.4 Isolation of spore-forming bacteria from the European sea bass gut microbiota

Digesta aliquots containing the pooled material were diluted 1:5 (wt:vol) in Bott & Wilson (B&W) salts (1.24% K_2HPO_4 , 0.76% H_2PO_4 , 0.1% trisodium citrate, 0.6% $[NH_4]_2SO_4$, pH 6.7), and resuspended by vortexing thoroughly. Serial dilutions (10^{-0} , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}) of the homogenized samples were heat-treated at 65 °C for 20 min (Nicholson and Setlow, 1990), and spread on the surface of Luria Bertani (LB) agar plates, to select aerobic spore-forming bacterial isolates. Samples were then incubated for up to 96 h at room temperature (25 ± 1 °C) and colonies obtained from each experimental group were selected based on their different morphologies. All selected isolates were purified by sequential re-streaking on LB agar plates until pure colonies were obtained. Colonies were then numbered and stored at -80 °C in 25% glycerol. All isolates were tested for catalase activity, through colony resuspension in a 3% hydrogen peroxide solution (Sigma, Química, S.L., Sintra, Portugal), and for spore production ability, induced by nutrient exhaustion on Difco Sporulation Medium (DSM) (Becton, Dickinson and Company, USA). Sporulation was confirmed by phase-contrast microscopy (Nicholson and Setlow, 1990; Serra et al., 2019).

3.2.5 Colloidal chitin preparation

Colloidal chitin preparation was performed according to (Hsu and Lockwood, 1975), with minor modifications. Briefly, powdered chitin from shrimp shells (practical grade powder, Sigma-Aldrich Química, S.L., Sintra, Portugal) was homogenized in concentrated HCl (40g/600 mL, w/v) with vigorous stirring for 60 min at 25 °C. The colloidal chitin suspension was then precipitated by slowly adding 2 L of deionized water at 4 °C. Colloidal chitin was sedimented by centrifugation (15 900g, 10 min at 4 °C) and resuspended in 3 L of deionized water. The process was repeated until the suspension pH was 3.5, and then the colloidal chitin was freeze-dried and stored until further use.

3.2.6 Selection of isolates with chitinolytic activity

To select bacteria capable of hydrolyzing chitin, each isolate was grown on a chemically defined media, hereafter designated ChitMedia (w/v, 0.65% Na_2HPO_4 , 0.3% KH_2PO_4 , 0.05% NaCl, 1% NH_4Cl , 0.02% $MgSO_4$, 0.01% yeast extract, 1% colloidal chitin), containing 1.6% (w/v) Agar, hereafter referred as ChitAgar. Isolates were grown up to 14 days at 28 °C and selected based on their chitinolytic ability, identified by the presence

of a hydrolysis halo. *Chromobacterium violaceum* ATCC 12472, known to possess chitinolytic activity, was used as a positive control (Liu et al., 2013).

The chitinolytic activity was determined indirectly, by measuring the N-acetylglucosamine produced by the bacterial culture supernatant. To do so, each isolate was grown in ChitMedia for 6 days at 37 °C at 150 rpm. Then, 2 mL of each culture was centrifuged at 4 °C for 10 min at 13 600g, and the supernatant was collected. A reaction mixture composed of 250 µL of the supernatant, 250 µL of citrate-phosphate buffer (0.15 M citric acid, 0.3 M sodium phosphate dibasic, pH 7), and 250 µL of chitin suspension (5mg mL⁻¹), was incubated for 2 h at 37 °C in a rocking shaker. A sample blank (250 µL supernatant + 500 µL distilled water), a substrate blank (250 µL citrate-phosphate buffer + 250 µL chitin suspension + 250 µL distilled water), and a reagent blank (250 µL citrate-phosphate buffer + 500 µL distilled water) were also prepared. Duplicates were carried out for each sample and blanks. After incubation, the reaction was stopped by heating at 100 °C for 10 min, and buffered with 0.1 mL of 0.8 M borate buffer (K₂B₄O₇) at pH 9.3, with the remaining protocol being carried out as described by Guerreiro, et al. (2021). N-acetylglucosamine concentration was determined by measuring the sample's absorbance at 585 nm. An N-acetylglucosamine standard curve was carried out (65.5-1000 µM). Enzymatic activity was conveyed in µg of N-acetylglucosamine mL⁻¹ of culture supernatant min⁻¹. All readings were carried out at room temperature in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). All reagents used to perform the enzymatic analysis were purchased from Sigma-Aldrich (Química, S.L., Sintra, Portugal). The fish isolates (FI) presenting the highest chitinolytic activity were tested under conditions that mimic the ESB stomach or intestinal pH. For that purpose, the citrate-phosphate buffer was adjusted to pH 5 or pH 7, respectively (Nikolopoulou et al., 2011). The reaction mixtures were then incubated for 2 h in a rocking shaker at 24 °C, which is within the optimum growth temperature for ESB (Dülger et al., 2012). Following enzymes inactivation at 100 °C for 10 min, to maintain a final reaction pH of 9.8 in the solution, the reaction mixtures were buffered with 0.1 mL of 0.8 M borate buffer (K₂B₄O₇), at either pH 10.8 or pH 9.3, respectively for the reactions carried out at pH 5 or pH 7. The remaining process was carried out as described above.

3.2.7 Taxonomic identification of isolates with extracellular chitinolytic activity

The chromosomal DNA of each isolate was purified from a 2 mL overnight culture grown in LB at 37 °C, 120 rpm, using the ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research

Corp., Irvine, CA, USA), according to the manufacturer instructions. PCR amplification of the 16S rRNA gene was carried out using the 27F and 1492R oligonucleotide primers (Weisburg et al., 1991), at an annealing temperature of 55 °C. Each 25 µL reaction was composed of 1 × x MyTaq Reaction Buffer (Bioline, BIOPORTUGAL, Lda., Lisboa, Portugal), 0.2 µM of each primer (STAB Vida, Lisboa, Portugal), 1.25 U of MyTaq™ DNA Polymerase (Bioline, BIOPORTUGAL, Lda., Lisboa, Portugal) and 2.5 µL of DNA template. Sequencing of the amplified products was carried out using the primer 27F (Weisburg et al., 1991) by STABVIDA (Caparica, Portugal). The sequencing data was analyzed by BLASTn against a database of non-redundant bacterial sequences from NCBI (<https://www.ncbi.nlm.nih.gov>).

3.2.8 Hemolytic activity and antibiotic resistance

Hemolytic activity was determined using freshly grown LB colonies cultured on Columbia Agar with 5% Sheep Blood (Becton Dickinson GmbH) at 37 °C for 24, 48, and 72 h. The isolates were identified as β-hemolytic if the bacterial hemolytic enzymes completely break down the blood cells, α-hemolytic if the bacterial hemolytic enzymes only partially break down the blood cells, or γ-hemolytic if no hemolytic activity was detected.

Antibiotic susceptibility was carried out by determining minimal inhibitory concentrations (MIC) using Etest® (bioMérieux, Inc. Marcy-l'Étoile, France). Following the recommendations of the European Food Safety Authority Panel on Additives and Products or Substances used in Animal Feed (EFSA-FEEDAP) (EFSA, 2018), the isolates were tested against 7 antibiotics belonging to 4 different antibiotic classes: Erythromycin (E), which belong to the Macrolides class; Kanamycin (K), Streptomycin (S), and Gentamycin (G), which belong to the Aminoglycosides class; Tetracycline (T), which belong to the Tetracyclines class; and Vancomycin (V) and Chloramphenicol (C), which belong to the Glycopeptides class. Overnight grown bacteria were adjusted to an optical density of 0.5 McFarland standard units (OD₆₀₀ ~ 0.1) in sterile saline solution and homogeneously spread in 20 mL MH (Mueller-Hinton, Oxoid, Fisher Scientific, Oeiras, Portugal) agar plates. Two Etest® strips were used per plate. The MIC was determined after 24 h incubation at 37 °C.

3.2.9 Screening for putative chiA

To tentatively confirm the presence of the chitinolytic domain ChiA, the FI were screened for the presence of chitinase A- encoding gene (*chiA*) using two sets of primers. Based

on the already demonstrated ability to amplify *chiA* from other *Bacillus* species, the first set of primers was used to target a conserved region of ChiA as designed by Ramaiah et al. (2000). Based on the taxonomic proximity of our FI to *Bacillus licheniformis*, a second set of primers was used. For that purpose, multiple *chiA* sequences belonging to *B. licheniformis* and identified with the accession numbers GQ899144, FJ465148, KJ017976, CP042252, CP041154, CP023729 were retrieved from the NCBI database (<https://blast.ncbi.nlm.nih.gov>) and aligned (Supplementary Figure 3.1) using ClustalW algorithm with Molecular Evolutionary Genetics Analysis (MEGA) software version 10.2.6 (Kumar et al., 2018). Conserved regions of aligned sequences were chosen for primer design using Primer3 software (Rozen and Skaletsky, 2000). Primer sequences and amplicon size can be found in Supplementary Table 3.1. PCR amplification was done as described for the 16S rRNA gene (section 2.7), with the annealing temperature of 55 °C and 54 °C, for the first and second set of primers, respectively.

3.2.10 Spore concentration, sporulation efficiency, and spore viability after gut environment simulation

Sporulation induction was carried out by nutrient exhaustion in DSM for 48 h at 37 °C in an orbital shaker at 150 rpm, with sporulation efficiency being determined as described by (Serra et al., 2019). The remainder of the media containing each isolate culture was collected, submitted to heat treatment at 80 °C for 20 min to eliminate vegetative cells, and the cells were harvested by centrifugation at 13 600g for 10 min. Evaluation of the FI resistance to gastrointestinal tract transit was performed by sequentially exposing the heat-resistant cells of the FI to stomach-like and intestine-like conditions as described by (Serra et al., 2019). Determination of the colony-forming units (CFU mL⁻¹) was carried out at each evaluation point. Triplicates were used for each isolate. All reagents used were purchased from Sigma-Aldrich (Química, S.L., Sintra, Portugal).

3.2.11 Statistical analysis

All data were checked for normality and homogeneity of variances before analysis and transformed if necessary. The isolates' total chitinolytic activity at pH 7, 37 °C was analyzed by one-way Analysis of Variance (ANOVA). The isolates' total chitinolytic activity in gut-like conditions was analyzed by two-way ANOVA with the isolates and pH as the main factors. To identify differences between means Tukey multiple range tests were performed. Differences were considered statistically significant at $p < 0.05$. All

statistical analyses were performed using the software IBM SPSS 27.0 software package for Windows (IBM® SPSS® Statistics).

3.3 Results

3.3.1 Isolation, identification, and characterization of putative probiotics with chitinolytic potential

A total of 366 heat-resistant colonies with different morphologies were isolated. Of these, 310 isolates were obtained from fish fed the HM-based diets, namely 107 isolates (29.2% of the total) from the HM25 group, and 203 isolates (55.5% of the total) from the HEM25 group. The remaining isolates were obtained from CTR, TM25, and CHIT5 diets corresponded to, respectively, 18 (4.9% of the total), 23 (6.3% of the total), and 15 (4.1% of the total) isolates.

Following colony purification (Figures 1A and 1D), sporulation was induced by nutrient exhaustion in DSM, and spores morphology was characterized by phase-contrast microscopy (Nicholson and Setlow, 1990) (Figures 1B and 1E). Results showed that 353 of the 366 isolates (96.4% of total isolates) produced spores. Of these, 346 isolates (98.0% of total isolates) were positive for the catalase testing and were subsequently inoculated into ChitAgar for chitinolytic activity screening. Colonies presenting a hydrolysis halo (Figures 1C and 1F) were selected, resulting in 27 spore-forming isolates with chitinolytic potential (7.6% of the catalase-positive isolates). These chitin-degrading microorganisms were mainly found in samples from fish fed the HM-based diets (23 out of 27, corresponding to 85.2% of the catalase-positive isolates) with HEM25 having the highest contribution (20 out of 27, corresponding to 74.1%) (Table 3.1). No spore-forming chitinolytic isolates were identified from fish fed the TM25 diet. Taxonomic identification of the 27 chitinolytic spore-forming isolates by partially sequencing the 16S rRNA gene revealed a predominance of *Bacillus* species (23 out of 27, corresponding to 85.2% of the total), followed by *Paenibacillus* species (4 out of 27, corresponding to 14.8% of the total) (Table 3.1). The majority of isolates (13 out of 27, corresponding to 48.1% of the total) were closely related to *B. licheniformis*, with the remaining isolates being closely related to *B. tropicus* (1), *B. cereus* (3), *B. paranthracis* (2), *B. clausii* (1), *Bacillus* spp. (3), *P. xylanilyticus* (1), *P. tundrae* (1), *P. dendritiformis* (1), and *P. lautus* (1) (Table 3.1).

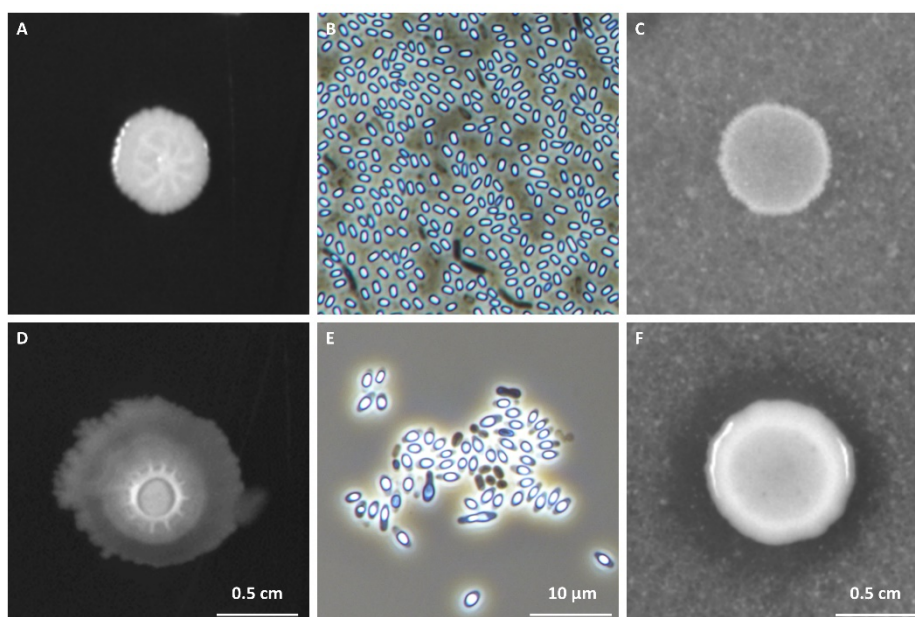


Figure 3.1 Morphological diversity (Panels A and D) of colonies of representative spore-forming fish isolates obtained from European sea bass intestinal contents after a 96 h at 25 °C growth in Luria Bertani (LB) media. Panels B and E depict phase-contrast microscopy images of sporulating cells and free spores of the same 2 representative isolates. Sporulation was induced by nutrient exhaustion in solid Difco Sporulation Medium (DSM). Panels C and F illustrate the criteria for the determination of chitinolytic ability of the isolates in ChitAgar media, with E representing a negative result (no chitin- degradation halo) and F representing a positive result (visible chitin-degradation halo).

3.3.2 Hemolytic activity and antibiotic resistance

According to EFSA-FEEDAP recommendations, the 27 isolates selected as putative chitinolytic probiotics were tested for minimal biosafety requirements, namely for hemolytic activity and antibiotic resistance. Most isolates did not present hemolytic activity when cultivated on 5% sheep blood agar, thus being classified as γ hemolytic (20 out of 27, corresponding to 74.1% of the total). The 7 isolates presenting β hemolysis activity were excluded from further analysis. These corresponded to FI568 (closely related to *B. tropicus*), FI633, FI635 and FI670 (closely related to *B. cereus*), FI652 (closely related to *B. clausii*), and FI640 and FI805 (closely related to *B. paranthracis*) (Table 3.1). The 20 isolates with no hemolytic activity were tested for antimicrobial resistance to 7 antibiotics for *Bacillus* and other Gram-positive bacteria (to test isolates closely related to *Paenibacillus*) recommended by the EFSA Panel on Additives Products or Substances used in Animal Feed (EFSA, 2018). Of the 20 isolates, only FI832 presented no antibiotic resistance. Fifteen isolates, closely related to the *Bacillus* genera,

Table 3.1 Characterization and identification of the 27 isolates displaying chitinolytic activity.

Isolates ^a	Diet ^b	Spores ^c	Catalase ^d	Hemolysis ^e	AbR ^f	16S rRNA sequence analysis	
						Closest known species ^g	% ID
FI540	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	91.4
FI542	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	93.7
FI546	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	91.2
FI564	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	94.7
FI568	HEM25	+	+	β	n/d	<i>Bacillus tropicus</i>	100.0
FI590	HM25	+	+	γ	C	<i>Bacillus licheniformis</i>	94.5
FI620	CHT5	+	+	γ	KS	<i>Paenibacillus</i>	98.8
FI622	CTR	+	+	γ	SC	<i>Paenibacillus tundrae</i>	99.3
FI633	HEM25	+	+	β	n/d	<i>Bacillus cereus</i>	98.3
FI635	HEM25	+	+	β	n/d	<i>Bacillus cereus</i>	99.9
FI640	HEM25	+	+	β	n/d	<i>Bacillus paranthracis</i>	99.9
FI645	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	99.7
FI650	HEM25	+	+	γ	EC	<i>Bacillus spp.</i>	91.5
FI652	HEM25	+	+	β	n/d	<i>Bacillus clausii</i>	99.5
FI657	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	99.1
FI658	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	98.5
FI662	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	90.0
FI669	HEM25	+	+	γ	C	<i>Bacillus licheniformis</i>	92.1
FI670	HEM25	+	+	β	n/d	<i>Bacillus cereus</i>	99.0
FI677	HEM25	+	+	γ	EC	<i>Bacillus licheniformis</i>	93.3
FI698	CHT5	+	+	γ	KTV	<i>Paenibacillus</i>	92.3
FI699	CHT5	+	+	γ	EC	<i>Bacillus licheniformis</i>	98.0
FI710	HM25	+	+	γ	EKSC	<i>Paenibacillus lautus</i>	99.4
FI760	HEM25	+	+	γ	C	<i>Bacillus spp.</i>	92.3
FI779	HEM25	+	+	γ	EC	<i>Bacillus spp.</i>	94.4
FI805	HEM25	+	+	β	n/d	<i>Bacillus paranthracis</i>	99.9
FI832	HM25	+	+	γ	-	<i>Bacillus licheniformis</i>	99.7

^aIsolates showing strong hemolytic activity are displayed in bold. ^bCTR, control fishmeal-based diet; HM, *Hermetia illucens* larvae meal-based diet; HEM, *Hermetia illucens* exuviae meal-based diet; CHT5, control fishmeal-based diet with 5% chitin supplementation. ^cSpores detected by phase-contrast microscopy of 24 h cultures in Difco Sporulation Medium (DSM) agar. ^dCatalase activity tested by resuspending a colony in a 3% solution of hydrogen peroxide (Sigma). ^eHemolysis determined on Columbia 5% sheep blood agar plates after incubation at 37 °C for 72 h. β-hemolysis, the bacterial hemolytic enzymes completely break down the blood cells; α-hemolysis, the bacterial hemolytic enzymes only partially break down the blood cells; γ-hemolysis corresponds to essentially no hemolytic activity detected. ^fAntimicrobial resistance determined by the E-test method against several antibiotics namely: Erythromycin (E), Kanamycin (K), Tetracycline (T), Vancomycin (V), Streptomycin (S), Gentamycin (G), Chloramphenicol (C), No Resistances (-), Not determined (n/d). Resistances to a specific antibiotic are illustrated by the corresponding letters. Resistance was defined according to the EFSA's breakpoints (EFSA, 2012) for *Bacillus* sp. or other Gram+ bacteria in accordance with each isolate identification. ^gClosest known species found using BLASTn of the 16S rRNA gene against a database of non-redundant bacterial sequences from NCBI.

were resistant to chloramphenicol, with 12 also being resistant to erythromycin (Table 3.1). Four isolates, namely FI620, FI622, FI698, and FI710, all closely related to the genera *Paenibacillus*, were resistant to two or more of the following antibiotics: kanamycin, streptomycin, chloramphenicol, tetracycline, vancomycin, or erythromycin (Table 3.1). Of the isolates tested for antimicrobial resistance, 80% presented resistance against 2 or more antibiotics, and 15% presented resistance against 3 or more antibiotics. Erythromycin (65%) and chloramphenicol (85%) were the antibiotics against which most of the isolates presented resistance. The minimum inhibitory concentrations are presented in Supplementary Table 3.2. Due to the multiple resistances detected, and their antibiotic dissemination ability Eduardo-Correia et al. (2020), all *Paenibacillus* sp. isolates were excluded from further testing. As such, we proceeded with a strict group of 16 isolates, all belonging to the genus *Bacillus*.

3.3.3 Chitinolytic activity of gut spore-formers

The 16 isolates selected above were tested for their ability to degrade chitin into N-acetylglucosamine at 37 °C, pH7. All but one isolate, FI658, presented significantly lower activities than the control (Figure 3.2). Additionally, isolates FI645, FI699 and FI832 presented a similar chitinolytic activity to that of isolate FI658 and were also selected to proceed.

These isolates were further evaluated regarding their *in vitro* potential to degrade chitin into N-acetylglucosamine at 24 °C and at pH 5 and 7, to mimic ESB stomach and intestine conditions, respectively (Figure 3.3). In identical growth and enzymatic activity assay conditions, at pH7, a temperature decrease from 37 °C to 24 °C lead to an increased chitinolytic activity of isolates FI645 (3.82-fold), FI658 (1.76-fold), and FI699 (1.69-fold), while it remained unchanged in isolate FI832 (1.09-fold) and decreased in *B. licheniformis* (0.68-fold) (Figures 2 and 3). On the other hand, pH variation at 24 °C had no significant impact on the chitinolytic activity of the isolates (Figure 3.3). Overall, under gut-like conditions, FI645 presented the highest chitinolytic activity, followed by FI658, with *B. licheniformis*, FI699, and FI832 presenting similar results.

Isolates were also tested for the presence of *chiA*, a gene coding for the catalytic ChiA domain of bacterial chitinases. For this purpose, we first used a pair of oligonucleotide primers designed by Ramaiah, et al. (2000), which were previously

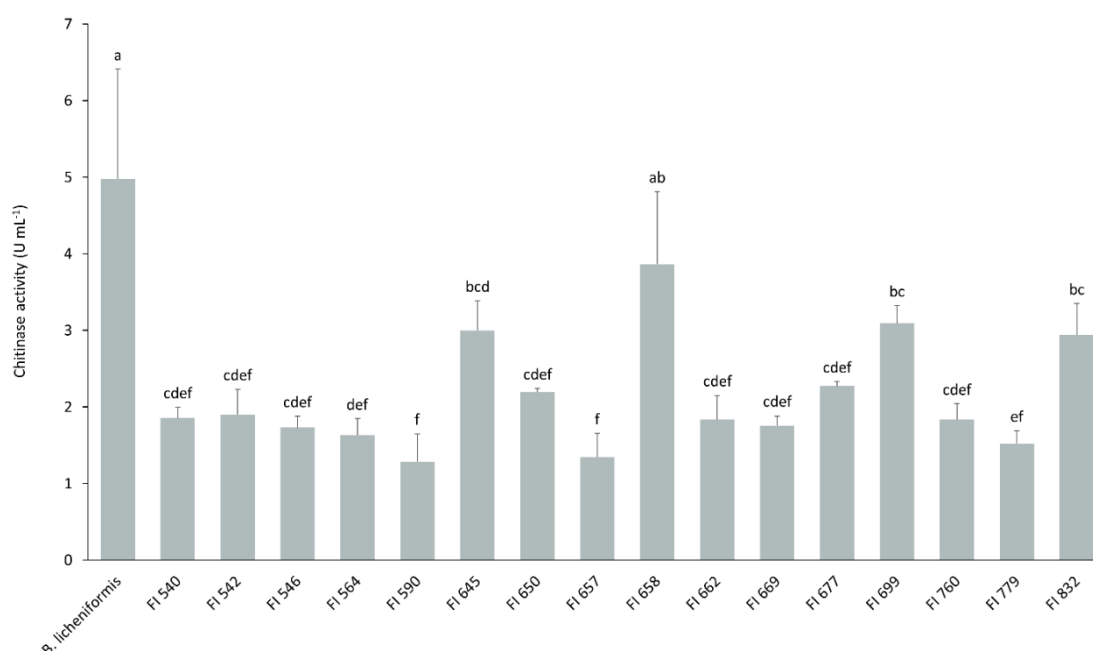


Figure 3.2 Chitinolytic profile of the selected 16 fish isolates (FI codes in the x axis) obtained from the intestines of European sea bass. Total chitinolytic activity was determined at pH 7 and 37 °C after culturing for 6 days in ChitAgar at 37 °C, 150 rpm. 1U corresponds to the production of 1 µg min⁻¹ of N-acetylglucosamine. *B. licheniformis* BGSC 9945A was used as control. The results are the average of three independent experiments and were analyzed by a one-way Analysis of Variance (ANOVA), followed by a Tukey multiple range test. Error bars represent the standard deviation. Statistical significance was considered at $p < 0.05$

demonstrated to detect *chiA* in a wide array of phylogenetically distant microorganisms, including *Bacillus*. As these primers were not able to detect *chiA* presence in our isolates (Figure 3.4A), a new set of primers was designed using known *chiA* sequences of closely related microorganisms, namely *B. licheniformis* (Supplementary Table 3.1 and Supplementary Figure 3.1). With this new set of primers, a DNA fragment of the expected size was obtained, thus demonstrating the presence of *chiA* gene in our isolates (Figure 3.4B). Sequencing of the corresponding DNA fragments revealed an identity >98.9% with previously described chitinase sequences of *B. licheniformis* (data not shown).

3.3.4 Sporulation efficiency and resistance to the gut environment simulation

Isolates FI645, FI658, FI699, and FI832 were characterized for their sporulation efficiency and potential to survive passage through the gastrointestinal tract, to establish their suitability for industrial production and feed incorporation. After a 48h sporulation

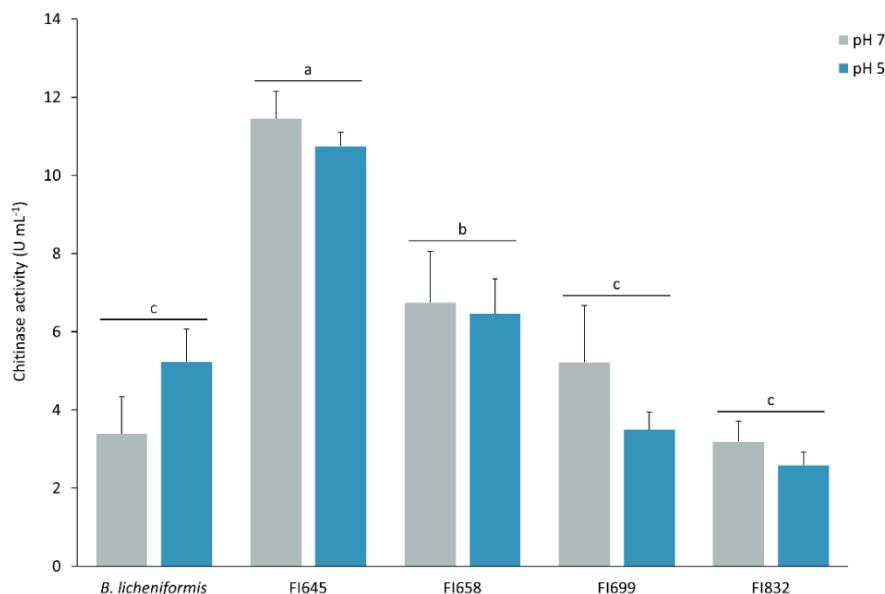


Figure 3.3 Chitinolytic profile of the best 4 fish isolates (FI codes in the x axis) obtained from the intestines of European sea bass. Total chitinolytic activity of the FI was determined at pH 5 (blue) and pH 7 (grey) at 24 °C after bacterial culturing for 6 days in ChitAgar at 37 °C, 150 rpm. 1U corresponds to the production of 1 $\mu\text{g min}^{-1}$ of N-acetylglucosamine. *B. licheniformis* BGSC 9945A was used as control. The results are the average of three independent experiments and were analyzed by a two-way ANOVA, followed by a Tukey multiple range test. No differences were found regarding the two pH conditions tested. Independently of the pH, differences between the FI are represented by small letters. Error bars represent the standard deviation. Statistical significance was considered at $p < 0.05$.

induction by nutrient exhaustion in liquid DSM medium, all isolates yielded more than 10^8 heat-resistant cells mL^{-1} (Figure 3.5), corresponding to sporulation efficiency values higher than 45%. These spores were then subjected to stomach-like conditions followed by intestine-like conditions. After 3 h in simulated stomach conditions, all isolates maintained over 10^8 CFU mL^{-1} viable spores, with FI645 presenting the highest spore count, when compared to the other isolates and the control. When exposed to intestine-like conditions, the control *B. licheniformis* spore count decreased from 10^8 mL^{-1} to 10^7 mL^{-1} , being significantly lower than that of all isolates, which maintained a titer $> 10^8$ CFU mL^{-1} (Figure 3.6).

3.4 Discussion

The microbiota's role in fish performance and health is nowadays well established (Butt and Volkoff, 2019; Egerton et al., 2018; Wang, A.R. et al., 2018). While imbalances in the gut microbiota community can lead to detrimental effects on the host, probiotic

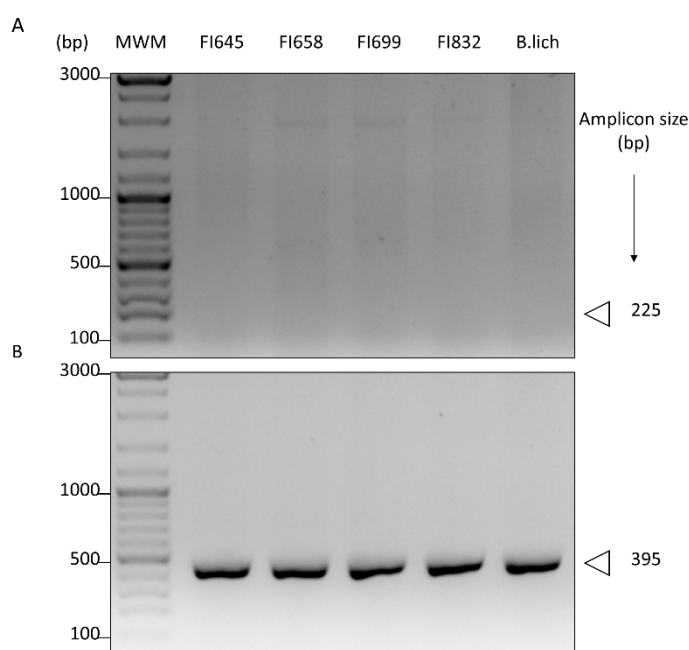


Figure 3.4 PCR detection of genes coding for the chitinase ChiA protein domain in the genome of fish isolates (FI codes on top of the figure) using **(A)** oligonucleotide primers targeting a conserved region of ChiA, previously designed by Ramaiah et al. (2000) or **(B)** primers targeting conserved regions of *B. licheniformis chiA* designed in this study. Genomic DNA of *B. licheniformis* BGSC 9945A (*B. lich*) was used as control. The amplicon size, in base pairs (bp) is depicted on the right.

administration can enhance fish health and growth performance by promoting a balanced microbiota (Butt and Volkoff, 2019). To date, several species such as *Bacillus* spp., *Lactobacillus* spp., *Enterococcus* spp., *Streptomyces* spp., and *Carnobacterium* spp., have been successfully used as probiotics in aquaculture (Van Doan et al., 2020). Within these, spore-forming microorganisms such as *Bacillus* spp. are particularly attractive. Their robustness and survival ability in extreme conditions, conveys a clear advantage for industrial applications, namely for inclusion in animal feeds and for survival inside the animal gut (Bajagai et al., 2016; Cutting, 2011; Nicholson et al., 2000; Setlow, 2006).

In this study, dietary inclusion of different IM and commercial chitin prompted differential shifts in the digesta-associated culturable spore-forming community, with HM25 and HEM25 diets aggregating most of the FIs obtained. These results are in line with the accentuated increase of Firmicutes detected in HM25 and HEM25 diets, as demonstrated in our previous work (Rangel et al., 2022). The increase in the Firmicutes phylum has been verified in different fish species by the dietary inclusion of *H. illucens* meal (Huyben et al., 2019; Panteli et al., 2021; Rimoldi et al., 2021; Terova et al., 2019).

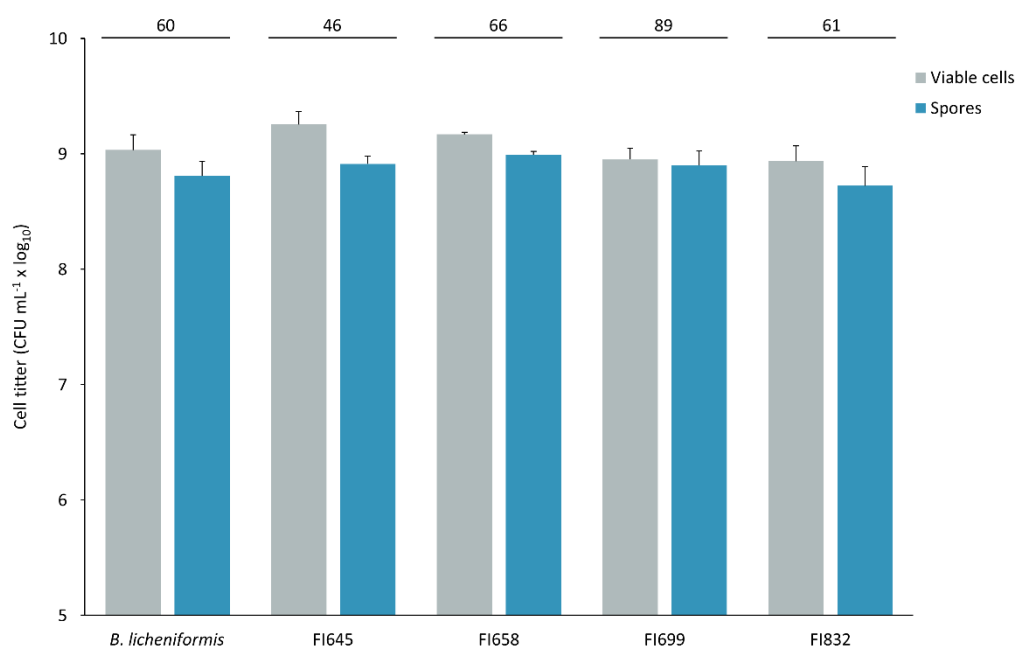


Figure 3.5 Titer of viable cells of each fish isolate (FI codes in x axis) present in 48 h DSM cultures before (grey, total cells) and after (blue, sporulating or heat resistant cells) a 20 min heat treatment at 80 °C. Sporulation was produced by nutrient exhaustion in liquid Difco Sporulation Medium (DSM) at 37 °C, 150 rpm. The numbers displayed on top of the panel represent the percentages (%) of sporulation calculated as the ratio between sporulating cells and total cells. *B. licheniformis* BGSC 9945A was used as control. The results are the average of three independent experiments. Numbers on top correspond to the percentages (%) of sporulation calculated as the ratio between sporulating cells and total cells. The error bars represent the standard deviation.

To date, no single component has been identified as responsible for these microbiota shifts. Several hypotheses are being postulated, namely: insect species (Panteli et al., 2021); insect microbiota profile (Osimani et al., 2018); insect development stage (Huyben et al., 2019); rearing substrate (Danieli et al., 2019; Liland et al., 2017); medium-chain fatty acids profiles present in IM (e.g. lauric acid, C12:0) (Rimoldi et al., 2018); insects antimicrobial peptides, which can vary between species and life stages (Vogel et al., 2018); and chitin (Huyben et al., 2019; Panteli et al., 2021; Rimoldi et al., 2019; Terova et al., 2019). Nonetheless, the increase in Firmicutes upon including HM in fish diets is hypothesized to be attended by an increase in chitinolytic bacteria, which would use chitin as a substrate to grow (Antonopoulou et al., 2019; Bruni et al., 2018; Huyben et al., 2019).

The emergence of chitinolytic bacteria is particularly important when feeding IM to fish, as it will promote chitin digestion. Due to its indigestibility in some economically

valuable fish species, chitin is often pointed out as the main obstacle to the effective use of IM in diets (Gasco et al., 2019). However, the Firmicutes increase previously verified in fish fed the HM25 diet (Rangel et al., 2022) was not concomitant with an increase in cultivable aerobic spore-forming chitinolytic bacteria, as verified in this work. On the other hand, diet HEM25, which presents a similar chitin content to diet HM25 (1.8% vs 1.7%), led to the majority of the chitinolytic FI obtained. A potential explanation for the emergence of chitinolytic bacteria in fish fed with the HEM25 diet might be the result of the molting processes that lead to exuviae detachment. Secretion of chitinase and peptidase-rich molting fluids during apolysis can lead to higher amounts of N-acetylglucosamine monomers and dimers in HEM25 (Belles, 2020; Muthukrishnan et al., 2019; Zhu et al., 2008), which can prompt the proliferation of known chitinolytic families of the Firmicutes phylum, such as *Bacillaceae* and *Planococcaceae* (Hui et al., 2020; Shimoi et al., 2020). Moreover, during ecdysis, the loosening and splitting of the insect's old cuticle leads to the disruption of the chitin matrix and, thus, increases enzymes and/or bacteria access to this polymer, potentially facilitating its colonization and/or use (Belles, 2020; Purkayastha and Sarkar, 2020; Resh and Cardé, 2009). Independently of the underlining mechanisms of microbiota modulation, the increase in cultivable aerobic spore-forming chitinolytic bacteria coupled with the increased chitinolytic genomic potential previously demonstrated (Rangel et al., 2022), establish HEM potential to modulate the fish gut microbiota toward chitinolytic microorganisms.

Bacillus, the most predominant genus among our FI (85.2%), has been extensively explored for probiotics supplementation in aquaculture (Ringø et al., 2020; Soltani et al., 2019), with several species being listed in the EFSA list of Qualified Presumption of Safety (QPS) (Koutsoumanis et al., 2019). On the other hand, *Paenibacillus* application in aquaculture is still limited. Both genera share characteristics that make them good probiotic candidates for feed inclusion as they can produce antimicrobial compounds capable of disrupting pathogen's growth and/or communication (Abriouel et al., 2011; Grady et al., 2016; Santos et al., 2021; Soltani et al., 2019; Sumi et al., 2014); can enhance fish growth performance and immune status (Amoah et al., 2020; Chen et al., 2019; Liu et al., 2012; Midhun et al., 2019; Newaj-Fyzul et al., 2007; Ringø et al., 2020), and can decompose non-starch polysaccharides (NSPs) and chitin (Askarian et al., 2012; Grady et al., 2016; Saleem et al., 2016; Serra et al., 2019).

Within the European Union, potential probiotics must comply with the guidelines of the EFSA Panel on Additives Products or Substances used in Animal Feed before approval

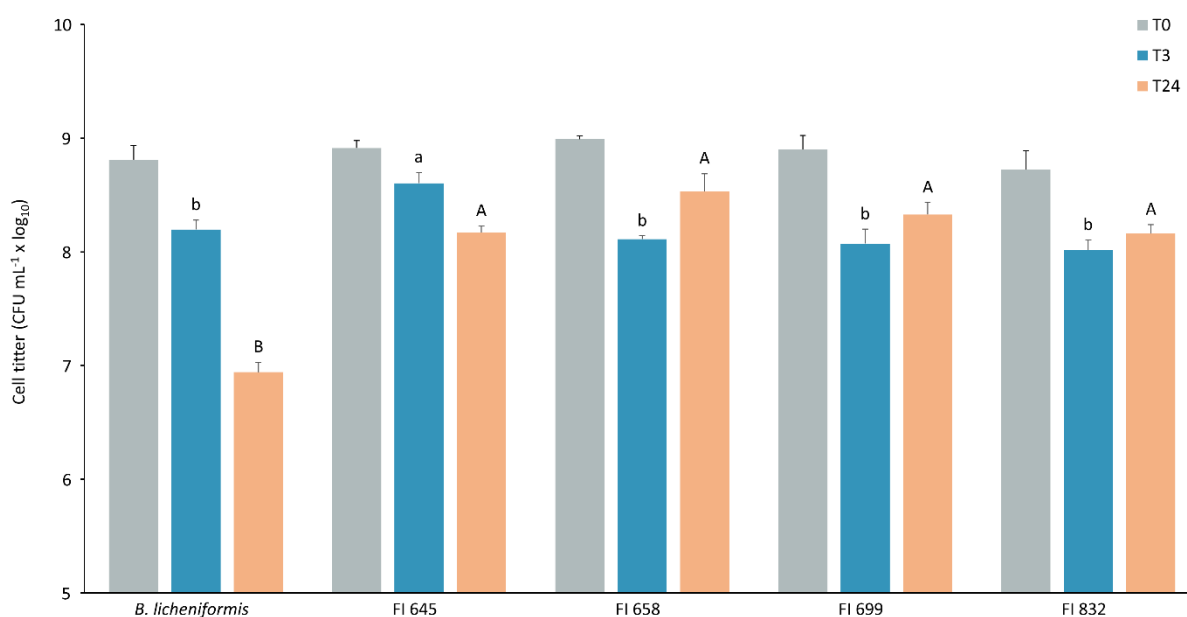


Figure 3.6 Viability of spores from each fish isolate (FI codes in x axis) before (T0, grey) and after exposure to simulated stomach conditions for 3h (T3, blue) followed by simulated intestinal conditions for 24h (T24, orange). *B. licheniformis* BGSC 9945A was used as control. The results are the average of three independent experiments. Data was analyzed by a one-way Analysis of Variance (ANOVA), followed by a Tukey multiple range test. Error bars represent the standard deviation. Differences of spore's viability between strains at T3 are represented by small letters, while capital letters represent differences between strains at T24. Statistical significance was considered at $p < 0.05$.

for inclusion in aquafeeds (EFSA, 2018). Safety-associated criteria must be fulfilled, including the absence of toxigenic activity. While in *Paenibacillus*, information regarding toxicity and endotoxins production is scarce (Grady et al., 2016), in *Bacillus*, a comparative pathogenomics analysis using the Virulence Factor Database (VDFB) (Liu, et al., 2019), demonstrated that its endotoxin production might lead to hemolysis. Thus, determination of hemolytic activity allowed to exclude all potentially harmful species such as *B. cereus* and *B. paranthracis*, reducing the putative probiotic candidates to 20 FI. Absence of acquired antibiotic resistance is another safety-associated criterion required by EFSA for probiotics approval. Using EFSA-established epidemiological cutoff values (ECOFF) (EFSA, 2018), antibiotic resistance was found in 19 of the 20 FI, with only FI832 being susceptible to all tested antibiotics. Bacteria belonging to the *Paenibacillus* genus, to which no genus-specific ECOFF values are available, were readily excluded from further testing, as members of this genus were recently indicated as potential disseminators of antibiotic resistance (Eduardo-Correia et al., 2020). On the other hand, the genus *Bacillus* has several species with QPS status (e.g. *B. subtilis*, *B. licheniformis*), as well as established ECOFF values defined by EFSA (Koutsoumanis et al., 2021). These ECOFF values are, however, defined at the genus

level and not species-specific, thus providing limited information for the distinction of harmful strains with acquired resistance, from strains with intrinsic resistances (Agersø et al., 2018; Turnidge et al., 2006). Recently, Agersø et al. (2019) determined that erythromycin and chloramphenicol resistance in *B. licheniformis* was associated with an ancient resistome with no mobile elements in the vicinity, suggesting novel species-specific tentative ECOFF values for *B. licheniformis* (Agersø et al., 2018). Re-evaluating our FI isolates with these novel ECOFF values would classify almost all of them as susceptible to chloramphenicol (of 32mg L⁻¹). Therefore, and assuming that also in our FI isolates, resistance might be intrinsic rather than horizontally acquired, we have decided to move on with their characterization for potential application as chitinolytic probiotics. Notwithstanding this assumption, in the future, for EFSA approval as probiotic, the genetic basis behind the resistance observed will have to be determined for the final selected FI isolate with most promising chitinolytic activity.

Aiming to maximize chitin digestion in IM and, thus, its bioavailability, FI were further selected based on their extracellular ability to degrade chitin into N-acetylglucosamine monomers. This selection criterion was chosen as in teleost fish, intestinal uptake of N-acetylglucosamine, or its oligomers, is poorly understood being hypothesized that it is the monomeric form that enters the fish enterocytes through diffusion (Krogdahl et al., 2005). The ability of bacteria to reduce chitin into its monomers in the extracellular space occurs due to the synergetic action of lytic chitin monooxygenase and endo- and exo-chitinases (Itoh and Kimoto, 2019). These enzymes have been identified in several members of the *Bacillus* genera, such as *B. thuringiensis* (Saleem et al., 2016; Zhang et al., 2015), *B. subtilis* (Wang, D. et al., 2018; Yu et al., 2016), and *B. licheniformis* (Courtade et al., 2015; Laribi-Habchi et al., 2015; Songsiriritthigul et al., 2010), the closest known species of the 4 selected isolates (FI645, FI658, FI699, and FI832). ChiA-type chitinases are key players in the chitin degradation process, being the predominant chitinase type in both cultured and uncultured bacteria (Beier and Bertilsson, 2013; Beier et al., 2011; Paulsen et al., 2016). As such, *chiA* has been used as the target gene to correlate the chitinolytic phenotype with chitinase presence (Hobel et al., 2005; Ramaiah et al., 2000). In marine environments, primers designed by Ramaiah, et al. (2000) were shown to be highly effective in amplifying the *chiA* gene of a large array of phylogenetically distant bacteria, including *Bacillus* spp. (Hobel et al., 2005; Ramaiah et al., 2000). However, these primers were ineffective for the determination of *chiA* in our 4 FI. Confirmation of *chiA* presence was only possible by using primers specifically designed in this work to target the *chiA* gene of *B. licheniformis*. Variations of the *chiA*

gene are known to occur in response to the microenvironments occupied by bacteria, potentially altering the protein structure to be better suited to the conditions found (Kielak et al., 2013). As such, the ineffectiveness of the primers designed by Ramaiah, *et al.* (2000) can be the result of a selective pressure resulting from the particular conditions found within ESB gut, such as temperature and pH. When exposed to ESB gut-like environments regarding temperature and pH, namely 24 °C and pH 7 (Dülger et al., 2012; Nikolopoulou et al., 2011), the FI either increased their activity (FI645, FI658, FI699) or maintained it (FI832) when compared to their activity at 37 °C, differing from other *Bacillus* chitinases which have been shown to decrease their activity at lower temperatures (Gomaa, 2012; Songsiriritthigul et al., 2010; Wang, D. et al., 2018). Among these FI645 and FI658 were shown to have the highest chitinolytic activities in ESB gut-like conditions, thus being particularly promising for aquaculture applications. Additionally, increased chitinolytic activity at lower temperatures (close to room temperature) opens novel biotechnological possibilities for these FI and/or their chitinases as they offer significant advantages, for instance, for the chitin bioconversion industry and fungal control (Liu, K. et al., 2019; Ramli et al., 2011).

Finally, the 4 putative FI probiotics, should possess essential traits such as high sporulation yields and survivability to the gastro-intestinal environment, characteristics that ascertain their economic viability, industrial scaling and oral application (Elisashvili et al., 2019). At a laboratory scale, all isolates presented high sporulation yields, between 5.25×10^8 cells mL⁻¹ and 9.77×10^8 cells mL⁻¹, and good sporulation efficiencies, between 46% and 89%, thus anticipating a cost-effective industrial scaling process. Moreover, when sequentially exposed to simulated gastric and intestinal conditions, they also presented high survival performances, thus demonstrating the ability to survive passage through the harsh gastrointestinal environment. These are promising results for in vivo application, as the survival observed after gastro-intestinal environment simulations indicate that the 4 FI isolates will be able to potentially maintain their viability and desirable characteristics which are critical for germination, adhesion, and colonization within the fish intestine, where they will exert their desired probiotic abilities (Guarner, 1998; Meng et al., 2008), namely chitin degradation.

3.5 Conclusions

The data gathered in the present study demonstrate that *H. illucens* derived meals exert a selective pressure on fish gut microbiota towards an increase of bacteria belonging to the Firmicutes phylum, being in line with our previous findings and the literature. In

particular, *H. illucens exuviae*, a novel ingredient, greatly impacted the digesta-associated fish gut microbiota increasing the number of chitin-metabolizing bacteria. This opens encouraging prospects for the use of an unexplored by-product of insect production as a functional ingredient. The application of a series of consecutive selection criteria to the initial pool of aerobic chitinolytic spore-forming bacteria obtained in this study, allowed the identification of 4 isolates (FI645, FI658, FI699, and FI832) out of 366, as the most promising to increase chitin digestibility. Among these, FI645 and FI658 produced the best results regarding chitinolytic activity in gut-like conditions, sporulation yield and efficiency, and spore survival after exposure to gut-like conditions. Given the in vitro potential demonstrated by these isolates, in future works they will be evaluated in vivo, in IM-containing diets, to confirm their probiotic potential.

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3.7 Supplementary material



Supplementary figure 3.1 Alignment of *chiA* sequences from *B. licheniformis* strains identified with the accession numbers GQ899144, FJ465148, KJ017976, CP042252, CP041154, CP023729. Sequences were retrieved from NCBI database (<https://blast.ncbi.nlm.nih.gov>) and aligned using ClustalW algorithm with Molecular Evolutionary Genetics Analysis (MEGA) software version 10.2.6 (Kumar et al. 2018). Conserved regions of aligned sequences highlighted in blue were chosen to design oligonucleotide primers Chit_Blic_FW and Chit_Blic_REV design using Primer3 software (Rozen and Skaletsky 2000).

Supplementary table 3.1 *chiA* -specific oligonucleotide primer-pairs used in this study

Primer Name	Primer Sequence (5' - 3')	Amplicon size (bp)	Reference
Chit_sm_FW	GATATCGACTGGGAGTTCCC	225 bp	Ramaiah et al. (2000)
Chit_sm_REV	CATAGAAGTCGTAGGTCATC		
Chit_Blic-FW	ACGTTTCGAAAGTCAGCCAC	395 bp	This study
Chit_Blic-REV	CCCGTCAAACCCGTATTTCC		

Supplementary table 3.2 Antibiotic resistance of the tested strains. Two different sets of cut-off values were used. *Bacillus* were evaluated using the *Bacillus* sp. cut-off values: Erythromycin (E) (4mg/L), Kanamycin (K) (8mg/L), Tetracycline (T) (8mg/L), Vancomycin (V) (4mg/L), Streptomycin (S) (8mg/L), Gentamycin (G) (4mg/L), Chloramphenicol (C) (8mg/L). *Paenibacillus* sp. were evaluated with the cut-off values attributed to other Gram + bacteria: E (1mg/L), K (16mg/L), T (2mg/L), V (4mg/L), S (8mg/L), G (4mg/L), C (4mg/L).

Isolate	Diet ^a	Closest known species ^b	MIC ^c						
			E	K	T	V	S	G	C
FI540	HEM25	<i>Bacillus licheniformis</i>	>256 ^R	0.5	0.19	0.75	3	0.094	16 ^R
FI542	HEM25	<i>Bacillus licheniformis</i>	>256 ^R	0.5	0.19	0.75	3	0.094	24 ^R
FI546	HEM25	<i>Bacillus licheniformis</i>	>256 ^R	0.5	0.19	0.5	2	0.094	24 ^R
FI564	HEM25	<i>Bacillus licheniformis</i>	>256 ^R	0.5	0.19	0.5	3	0.094	12 ^R
FI590	HM25	<i>Bacillus licheniformis</i>	0.25	1	0.5	1	6	0.19	32 ^R
FI620	CHT5	<i>Paenibacillus xylanilyticus</i>	0.125	16 ^R	0.125	1.5	>1024 ^R	0.5	2
FI622	CTR	<i>Paenibacillus tundrae</i>	1 ^R	2	0.094	1	>1024 ^R	0.125	16 ^R
FI645	HEM25	<i>Bacillus licheniformis</i>	>256 ^R	4	4	2	6	0.75	48 ^R
FI650	HEM25	<i>Bacillus</i> spp.	16 ^R	0.5	0.5	0.75	2	0.064	16 ^R
FI657	HEM25	<i>Bacillus licheniformis</i>	>256 ^R	0.75	0.5	1	3	0.19	16 ^R
FI658	HEM25	<i>Bacillus licheniformis</i>	3 ^H	1.5	0.75	3	4	1	16 ^R
FI662	HEM25	<i>Bacillus licheniformis</i>	8 ^R	1.5	0.5	0.5	2	0.094	16 ^R
FI669	HEM25	<i>Bacillus licheniformis</i>	3	0.5	0.38	0.5	3	0.094	16 ^R
FI677	HEM25	<i>Bacillus licheniformis</i>	>256 ^R	0.5	0.5	0.5	2	0.064	16 ^R
FI698	CHT5	<i>Paenibacillus dendritiformis</i>	0.125	32 ^R	12 ^R	32 ^R	4	1	1.5
FI699	CHT5	<i>Bacillus licheniformis</i>	16 ^R	1	0.5	2	4	0.19	8 ^R
FI710	HM25	<i>Paenibacillus lautus</i>	16 ^R	64 ^R	4 ^R	1	384 ^R	1	48 ^R
FI760	HEM25	<i>Bacillus</i> spp.	3	0.5	0.38	0.5	3	0.094	16 ^R
FI779	HEM25	<i>Bacillus</i> spp.	6 ^R	0.38	0.19	0.5	2	0.25	16 ^R
FI832	HM25	<i>Bacillus licheniformis</i>	0.19	0.05	0.05	1	2	0.19	3

^aCTR, control fishmeal-based diet; HM, *Hermetia illucens* larvae meal-based diet; HEM, *Hermetia illucens* exuviae meal-based diet; CHT5, control fishmeal-based diet with 5% chitin supplementation. ^bClosest known species found using BLASTn of the 16S rRNA gene against a database of non-redundant bacterial sequences from NCBI. ^cMIC, minimum inhibitory concentration ^RResistant according to the EFSA's breakpoints (EFSA 2012). ^HPresents heteroresistant colonies

Chapter 4.

**Unlocking insect meal potential through
probiotics: novel chitinolytic *Bacillus* spp.
increase feed efficiency, digestibility, and
survivability of European seabass fed with
Hermetia illucens-based diets**

Abstract

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 (FI645 and 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, digestibility, and ability to protect from a *Vibrio anguillarum* infection. The spores were able to maintain their viability throughout the 180 days of diet storage at room temperature. Dietary inclusion of FI645 increased the intestinal total chitinolytic activity, leading to significantly higher chitin digestibility. Concomitantly, the digestibility of dry matter, protein, and energy, overall leading to higher feed efficiency and protein efficiency ratio. The increased probiotic-mediated chitin digestibility also led to increased plasma N-acetylglucosamine (GlcNAc). 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. Finally, when challenged with *Vibrio anguillarum*, FI645 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.

Keywords: Chitin, Insect meal, Hexosamine biosynthetic pathway, N-acetylglucosamine, *Vibrio anguillarum*, Probiotics

4.1 Introduction

Insect meals (IM), due to their suitable protein contents and amino acid profiles, modulable lipid profile and content, are in a prime spot to be used as an ingredient in aquafeeds (Hua et al., 2019; 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; Magalhães et al., 2017; Melenchón et al., 2021). However, high HI meal inclusion (>25%) in fish diets usually leads to poorer protein and/or lipid apparent digestibility coefficients (ADC) and growth performance (Dumas et al., 2018; Fisher et al., 2020; Guerreiro et al., 2020; Guerreiro et al., 2021; 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; Guerreiro et al., 2021; Karlsen et al., 2017; Mastoraki et al., 2020; Piccolo et al., 2017; Renna 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 & Holt, 2010; Henry et al., 2015). To date, in fish, little is known about the extent of the potential endogenous or microbiota enzymatic contribution to chitin breakdown (Eggink et al., 2022; Gasco et al., 2016; Guerreiro et al., 2021; 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, though 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 and Gerald, 2014; Holt et al., 1987).

Chitin digestion can be particularly challenging for carnivorous species (NRC, 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, the apparent inability of HI meal to promote the emergence of chitinolytic bacteria in the fish gut (Rangel et al., 2022a; Rangel et al., 2022b), supports the need for alternative strategies 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 European sea bass 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.

4.2 Materials and Methods

4.2.1 Probiotic spores' production and purification

Each fish isolate (FI645 and FI658) 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% K₂HPO₄, 0.76% H₂PO₄, 0.1% trisodium citrate, 0.6% [NH₄]₂SO₄, 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 3x 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.

4.2.2 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 × 10⁹ CFU kg⁻¹ of either FI645 (D645 diet) or FI558 (D658 diet); and a CTR-like diet supplemented with 1 × 10⁹ 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 (Cr₂O₃) was added to the diets as an inert marker. Ingredients and proximate composition of the experimental diets are presented in Table 4.1. 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 time points (days 1, 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.

Table 4.1 Ingredient composition and proximate analysis of the experimental diets.

Diets	Growth			
	CTR	D645	D658	MIX
<i>Ingredients (% dry weight basis)</i>				
Fish meal ^a	20.0	20.0	20.0	20.0
Soluble fish protein concentrate ^b	2.0	2.0	2.0	2.0
<i>Hermetia illucens</i> 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
Chromium oxide	-	-	-	-
<i>Probiotic inclusion (10⁹ CFU Kg⁻¹)</i>				
FI645	-	2	-	1
FI658	-	-	2	1
<i>Proximate analyses (% dry weight basis)</i>				
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

^aSorgal, S.A. Ovar, Portugal (CP: 70.3 % DM; GL: 12.0 %DM).

^bSorgal, S.A. Ovar, Portugal (CP: 79.7 % DM; GL: 7.14 % DM).

^cBlack soldier fly larvae meal (CP: 62.4 % DM; GL: 6.3 % DM).

^dSorgal, S.A. Ovar, Portugal (CP: 62.8 % DM; GL: 1.2 % DM).

^eSorgal, S.A. Ovar, Portugal (CP: 55.4 % DM; GL: 1.9% DM).

^fSorgal, S.A. Ovar, Portugal (CP: 15.5 % DM; GL: 1.9 % DM).

^gVitamins (mg kg⁻¹ diet): retinol, 18 000 (IU kg⁻¹ diet); cholecalciferol, 2 000 (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.

^hMinerals (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).

ⁱAquacube. Agil, UK.

^jFeed-grade taurine, Sorgal, S.A. Ovar, Portugal

4.2.3 Animals and growth trial conditions

European sea bass (*Dicentrarchus labrax*) (ESB) juveniles were obtained from Instituto Português do Mar e da Atmosfera (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 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 12:12h light: dark), and water parameters maintained at 35.6 ± 0.6 g L⁻¹ salinity, 7.6 ± 0.4 mg L⁻¹ O₂ saturation, 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.

4.2.4 Digestibility trial

At the end of the growth trial, 8 fish from each tank with a mean body weight of 75 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) thermo-regulated 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 $3,000 \times g$ 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.

4.2.5 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 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 Lateral for 24 h at 4°C , being subsequently transferred at -80°C until further processing for gene expression analyses.

4.2.6 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 $33,000 \times g$ 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 $5,000 \times g$ and the supernatants were aliquoted and stored at -80° until further processing. Total chitinase activity was measured as described by Guerreiro et al. (2020). All enzyme assays were performed at 37°C with Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China).

4.2.7 Plasma metabolites and innate immune parameters

Plasma glucose and cholesterol were determined using Spinreact (Girona, Spain) commercial kits (glucose kit, ref. 1001191; cholesterol kit, ref. 1001091). 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) 12N hydrochloric acid), and incubated at 37 °C for 20 min. A GlcNAc standard curve ranging from 0.4-250 μM was prepared. Plasma total proteins and total immunoglobulins were quantified using Pierce™ BCA Protein Assay Kit (ref. 23225; 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).

4.2.8 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. 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).

4.2.9 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 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 4.2). 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 with elongation-factor 1-alpha gene (*ef1a*), previously described by Geay et al. (2010), due to its expression stability in the intestine, and calculated using the Pfaffl method (Pfaffl, 2001).

Table 4.2 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	TGCCAGGCTTTTGTGTTAGC		
nagk_esb_FW	ATCAGGGACCGGCTCTAACTG	103 bp	This study
nagk_esb_REV	CCAGAATGCTGAGCCCTCAT		
naga_esb_FW	TCTGGAGGGTCCGTTTCATC	219 bp	This study
naga_esb_REV	GGCCACAGAGTGACCTAAGGAC		
pgm3_esb_FW	AGCTCAAAGTCAAGGTGTCCG	176 bp	This study
pgm3_esb_REV	CCTCTGCGTAACTCTCACGA		
ogt_esb_FW	ATGCCCTGAAGGAGAAAG	190 bp	This study
ogt_esb_REV	AACTCTGGGAACACCTCTAGT		
ef1 α _esb_FW	GCTTCGAGGAAATCACCAAG	153 bp	Geay et al. (2010)
ef1 α _esb_REV	CAACCTTCCATCCCTTGAAC		

4.2.10 Bacterial challenge trial

The challenge trial was performed using 10 groups of 20 fish with a mean body weight of 54 g, in a recirculating aquaculture system equipped with 100 L fiberglass tanks. Four groups were fed the CTR diet and two groups each the tested diets. Fish were maintained with an artificial illumination photoperiod set to 12:12h light:dark and controlled water parameters (35.6 ± 0.6 g L⁻¹ salinity, 7.6 ± 0.4 mg L⁻¹ O₂, 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 μ L 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.

4.2.11 Statistical analysis

Data are represented as means $\pm$ 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 as 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).

4.3 Results

Fish isolates (FI) spore quantification at time 1, immediately after diet manufacture, showed that the isolates were within the expected concentrations and spores' viability was maintained up to 180 days (Figure 4.1).

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 4.3). In contrast, dietary supplementation with FI645 significantly increased feed efficiency. 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 4.4). No effect of dietary probiotic supplementation was observed in trypsin, lipase, and α -amylase activities. Total chitinolytic activity was higher in fish fed the FI645 supplemented diets (D645 and MIX diets) than the other diets ($p = 0.052$).

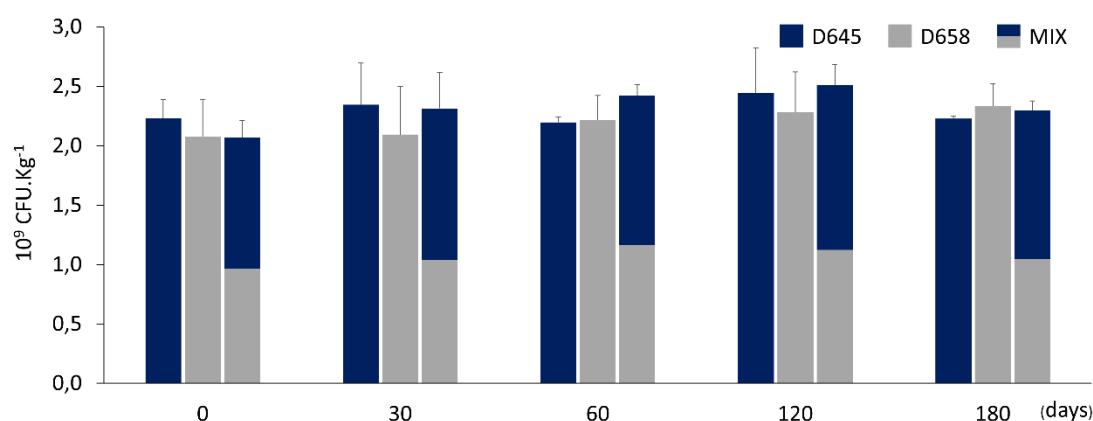


Figure 4.1 Spores' viability in the experimental diet during storage time at room temperature for 180 days.

Table 4.3 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
FE ^d	0.836 ± 0.001	0.850 ± 0.008	0.825 ± 0.005	0.865 ± 0.006	<0.001	0.119	0.081
PER ^e	1.86 ± 0.00	1.92 ± 0.02**	1.85 ± 0.01	1.96 ± 0.01**	<0.001	0.145	0.040

^aFBW (Final body weight) (g)

^bDGI (Daily Growth Index) (%): ((final body weight^{1/3} – initial body weight^{1/3})/time in days) × 100.

^cFI (Feed intake) (g kg ABW⁻¹ day⁻¹) with ABW (Average body weight) = (initial body weight + final body weight)/2.

^dFE (Feed efficiency): wet weight gain/dry feed intake.

^ePER (Protein efficiency ratio): wet weight gain/crude protein intake.

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).

Table 4.4 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).

The apparent digestibility coefficients (ADC) of dry matter, protein, and energy were higher in the D645 diet than in the control (Table 4.5). 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 4.6). The mean score of all evaluated parameters also did not differ among groups.

Table 4.5 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 4.6 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).

Dietary supplementation with FI645 led to higher plasma N-acetylglucosamine than with the other diets (Table 4.7). 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 (Figure 3.2). No further differences were observed in the expression of the other genes measured.

Table 4.7 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
<i>Metabolites</i>							
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
<i>Innate immunity</i>							
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.

No mortality was observed in the negative control group (PBS-treated fish) during the bacterial challenge (Figure 4.3). 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%).

4.4 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). This genus sporeforming ability is also industrially appealing, as spores are extremely resistant structures capable of overcoming the harsh feed manufacturing process and long feed storage times (Hong et al., 2005; Niu et al., 2018). Accordingly, the results of the present study confirmed that after inclusion in pelletized diets, spores maintained their viability for up to 180 days of storage at room temperature. 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

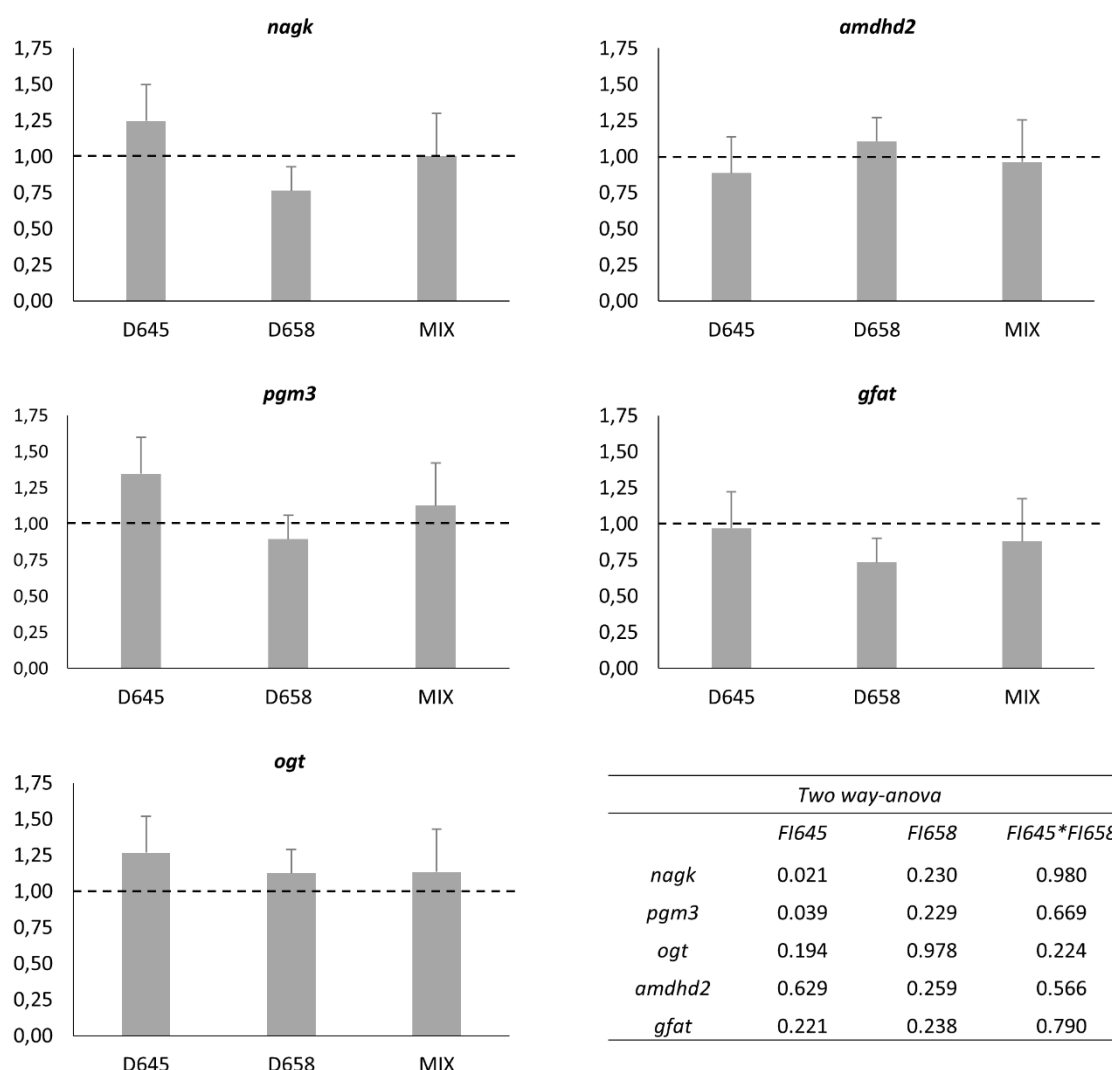


Figure 4.2 Gene expression of N-acetylglucosamine kinase (*nagk*), GlcNac phosphomutase (*pgm3*), O-GlcNac transferase (*ogt*), N-acetyl-D-glucosamine-6-phosphate deacetylase (*amdhd2*), and glutamine:fructose-6-phosphate amidotransferase (*gfat*) in fish fed the experimental diets. Values are expressed relative to the control group. Data are presented as mean (n = 8). The error bars represent the standard deviation.

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; Guerreiro et al., 2021; Karlsen et al., 2017; Mastoraki et al., 2020; Piccolo et al., 2017; Renna 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

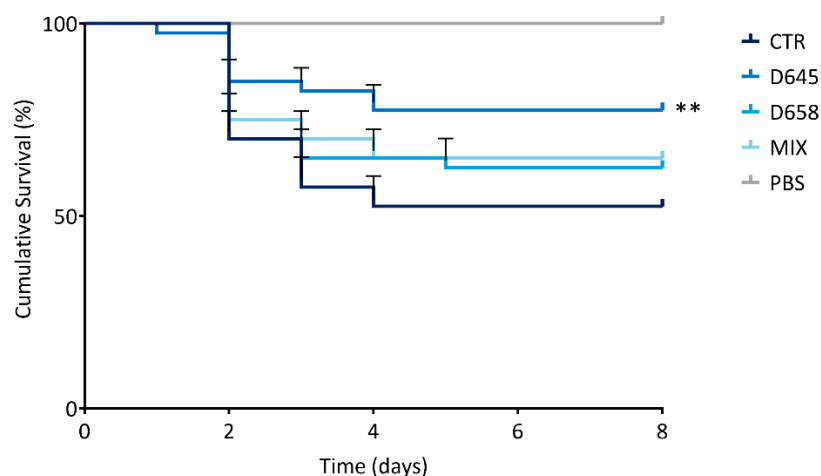


Figure 4.3 Cumulative survival of European sea bass (n=40) fed the experimental diets when challenged with *Vibrio anguillarum*. Fish treated with PBS was used as a negative control (PBS). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

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 the increase in total chitinolytic activity, as observed in diets supplemented with FI645. 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 increased observed in the MIX and D645 diets occurred in a FI645 concentration-dependent manner, opening promising prospects for further inclusion level optimization. As dietary FI645 supplementation 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). On the other hand, intestinal chitinolytic activity was also detected in fish fed the CTR diet. While this activity can be a result of HI-driven ESB intestinal microbiota modulation, as previously shown by (Rangel et al., 2022a), 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).

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). Present data showed that intestinal architecture was unchanged by dietary probiotic supplementation, indicating 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.

Arising from chitin digestion, metabolites such as COS or GlcNAc are now available to be used by the fish, with its metabolism, however, still being poorly understood. While GlcNAc uptake is hypothesized to occur by passive diffusion through the cell membrane (Krogdahl et al., 2005) (Figure 4.4), 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) (Figure 4.4). The HBP is considered a nutrient-sensing pathway as it integrates glucose, amino acids, fatty acids, nucleotides, and energy metabolism (Hardivillé and Gerald, 2014). Alterations in the HBP can modulate physiological cell response to nutrients and infection (Chang et al., 2020; Hardivillé and Gerald, 2014). Integration of GlcNAc in the HBP can be achieved by N-acetylglucosamine kinase (NAGK), which converts GlcNAc into N-acetylglucosamine-6-phosphate (GlcNAc-6P) (Figure 4.4) (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 (Figure 4.4). 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-

The diagram illustrates the metabolic and signaling pathways of GlcNAc. It is divided into three main regions: Gut lumen, Cytoplasm, and Nucleus.

Gut lumen: Chitin is broken down by the enzyme CHIT into (GlcNAc)_n, which is then converted to GlcNAc by the enzyme CTB.

Cytoplasm:

- Glucose and Glutamine transport:** Glucose and Glutamine enter the cell via transporters (purple and red ovals).
- Glucose metabolism:** Glucose is phosphorylated to G-6P by Hexokinase (HK). G-6P can be converted to F-6P by Glucose-6-phosphate isomerase (GPI) or enter Glycolysis.
- Glutamine metabolism:** Glutamine is converted to F-6P by Glutamine-dependent Nucleoside Phosphate-dependent Acetyltransferase (GNPDA). F-6P is then converted to GlcN-6p by Glutamine-dependent Fructose-6-phosphate acetyltransferase (GFAT).
- Acetyl-CoA metabolism:** Acetyl-CoA is converted to GlcN-6p by Acetyl-CoA-dependent Nucleoside Phosphate-dependent Acetyltransferase (AMDHD2). GlcN-6p is then converted to GlcNAc-6P by Glutamine-dependent Nucleoside Phosphate-dependent Acetyltransferase (GNPNAT).
- GlcNAc metabolism:** GlcNAc is converted to GlcNAc-6P by Nucleoside Phosphate-dependent Acetyltransferase (NAGK). GlcNAc-6P is then converted to GlcNAc-1P by Phosphate-dependent Glucosyltransferase (PGM3).
- UDP-GlcNAc synthesis:** GlcNAc-1P is converted to UDP-GlcNAc by UDP-GlcNAc 4-epimerase (UAP1).

Nucleus:

- Signaling pathways:** UDP-GlcNAc enters the nucleus and is converted to UDP-GlcNAc-6P by UDP-GlcNAc 4-epimerase (UAP1). UDP-GlcNAc-6P is then converted to UDP-GlcNAc-1P by Phosphate-dependent Glucosyltransferase (PGM3).
- O-GlcNAcylation:** UDP-GlcNAc-1P is used by O-GlcNAc transferase (OGT) to modify proteins (orange ovals) with O-GlcNAc. The modified proteins are then de-O-glcNAcylated by O-GlcNAcase (OGA) (green ovals).
- Gene regulation:** The O-GlcNAcylation cycle is involved in gene regulation, as indicated by the arrow pointing to the "Gene regulation" box.

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, cell growth and proliferation, and appetite regulation (Dai et al., 2018; Hardivillé and 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 mammal cells, it was shown that O-GlcNAcylation modulates oxidative stress (Groves et al., 2013; Issad et al., 2022), increases cellular neutrophils mobility (Kneass and Marchase, 2004), modulates macrophage phagocytic and proinflammatory responses (de Jesus et al., 2018; Newsholme and Parry-Billings, 1990), increases pre-B cells proliferation, maturation, homeostasis, and antibody response (Lee et al., 2020; Wu et al., 2017), and regulates natural killer cells cytotoxicity (Božič et al., 2018). 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 (Bjørngen and Koppang, 2022), namely in ESB (Chistiakov et al., 2007; Picchiatti 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., 2017; Kuebutornye et al., 2020; Monzón-Atienza et al., 2022; Santos et al., 2021) due to possess 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). Potential protection mechanisms against pathogens can, therefore, rely on other processes, such as anti-quorum sensing mechanisms (Santos et al., 2021; Vinoj et al., 2014), or immune modulation (Van Doan et al., 2020). The latter is a well described ability of *Bacillus* spp. (Nayak, 2021) that, in fish, can trigger, for example, the modulation of immune gene expression (Meloni et al., 2015; Monzón-Atienza et al., 2022), or the enhancement of innate immune parameters, such as lysozyme, nitric oxide, respiratory burst or total plasma immunoglobulins (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).

4.5 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.

Declarations

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.

Conflict of interests

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.

Authors' contributions

F.R., C.R.S., P.E., and A.O.T. designed and conceived the study. F.R., R.C., R.A.S., M.M., and P.E. performed the experiments. L.G. and F.G. produced and provided the *Hermetia illucens* meal. 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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Chapter 5.

General discussion, Conclusion and Future Perspectives

5.1 General discussion

Following the EU approval of IM as an ingredient for aquafeeds (European Union Directive; Regulation No 2017/893; July 1st, 2017), research on the effects of IM on fish feeds saw an exponential increase (Gasco et al., 2023). Overall, IM is regarded as a high-value protein source with an adequate amino acid profile for aquafeed applications (Tran et al., 2022a). IM is also regarded as a potential functional ingredient as it is often reported to possess immunostimulant (Gasco et al., 2018), and microbiota modulation abilities (Panteli et al., 2021; Rimoldi et al., 2021; Weththasinghe et al., 2022). However, IM is also frequently associated with decreased diets digestibility, mainly in carnivorous fish (Karlsen et al., 2017; Piccolo et al., 2017; Renna et al., 2017; Mastoraki et al., 2020; Guerreiro et al., 2021; Eggink et al., 2022). This outcome is the result of the carnivorous fish's inability to digest chitin (Olsen et al., 2006; Guerreiro et al., 2021; Eggink et al., 2022). In turn, chitin, by entrapping proteins and lipids within its matrix, limits proteases and lipases action, thus reducing their digestibility (De Marco et al., 2015; Marono et al., 2015).

Given carnivorous fish reliance on its gut microbiota for the digestion of non-starch polysaccharides such as chitin (Llewellyn et al., 2014; Egerton et al., 2018), potential readjustments of the host microbial communities in response to IM inclusion in the diets could contribute to increasing chitin digestion. As such, aiming to study the potential modulation of ESB gut microbiota communities to dietary IM inclusion, five isoproteic (45%) and isolipidic (18%) diets were fed to ESB over 8 weeks: a control FM-based diet (CTR), a chitin-enriched diet (CTR-like diet with 5% supplementation of commercial chitin from shrimp shells (CHIT5)), and three other diets containing 25% of IMs from TM or HI larvae meals, or HI exuviae meal (diets TM25, HM25 and HEM25, respectively). Microbial composition analysis of the allochthonous and autochthonous communities showed that mucosa-associated communities were less diverse and more resistant to dietary modulation than the digesta-associated microbiota (Chapter II). These results align with the observations made by Serra et al. (2021) in ESB fed with plant-based diets, and by Li et al. (2021) in Atlantic salmon fed with diets containing HI meal. This is, most likely, the result of the stable symbiotic relationships established between the fish host and mucosa-adhered microorganisms.

On the other hand, digesta-associated microbial communities presented marked shifts in fish fed with diets HM25 and HEM25, when compared to fish fed the CTR diet. Thus, diets HM25 and HEM25 increased ESB relative abundance of the phylum Firmicutes,

mostly resulting from an enrichment of the *Bacillaceae*, *Enterococcaceae*, and *Lachnospiraceae* families, known to have chitin-degrading (*Bacillaceae*) (Askarian et al., 2012; Ringø et al., 2020), or chitin-fermenting species (*Lachnospiraceae* and *Bacillaceae*) (Mao et al., 2013; Terova et al., 2016; Zhang and Davies, 2016; Bhat et al., 2019). Diet HEM25 further impacted the digesta-associated microbial communities, additionally increasing the abundance of other families from the Firmicutes phylum such as *Paenibacillaceae*, *Planococcaceae*, *Staphylococcaceae*, and *Brevibacteriaceae*. Compared to fish fed the CTR diet, the digesta-associated microbial communities of ESB fed with HM25 or HEM25 diets also presented an increased relative abundance of *Actinomycetaceae*, a family belonging to the Actinobacteria phylum, also known to encompass several highly proficient chitin-degrading bacteria (Callegari et al., 2020; Foysal and Gupta, 2022). These results are also in line with previous reports in several carnivorous fish fed with diets containing HI meal, such as rainbow trout (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2021), Atlantic salmon (Li et al., 2021; Weththasinghe et al., 2022), gilthead seabream (Panteli et al., 2021), Siberian sturgeon (Józefiak et al., 2019) and pearl gentian grouper (Huang et al., 2022). Additionally, the recurring emergence of carbohydrate-fermenting bacteria (e.g., LAB) but, particularly, the increase of bacteria from families containing chitin-degrading bacteria (e.g., *Bacillaceae* and *Actinomycetaceae*), points towards modulation of the gut microbiota that could result in higher chitinase activity (Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019; Li et al., 2021; Weththasinghe et al., 2022).

This agrees with results from the study by Huang et al. (2022), which observed in pearl gentian grouper fed HI meal an increase in chitinase activity in the distal intestine and the relative amount of Firmicutes in gut microbiota concomitant with the increase of dietary chitin levels. However, despite the increase in chitinase activity, in that study, both FBW and FCR were negatively affected by dietary HI meal inclusion (Huang et al., 2022). Eggink et al. (2022) further demonstrated that despite the increase in chitinase activity in the intestine of rainbow trout fed with diets containing HI meal (25%), dietary chitin digestibility remained low (11.5%). Additionally, both Guerreiro et al. (2021) and Coutinho et al. (2021) showed that in meagre fed diets containing 10%, 20%, or 30% of HI meal or TM meal, no intestinal chitinase activity could be measured and chitin remained undigestible. Together with the large decrease in protein digestibility concomitant with increasing levels of HI and TM meals in the diets (Piccolo et al., 2017; Chemello et al., 2020; Basto et al., 2021; Weththasinghe et al., 2021; Tran et al., 2022b; Stejskal et al., 2023), evidence suggests that an increase of the chitinase activity is

attainable by including HI meal in the fish diet, possibly due to diet-induced fish gut microbiota modulation, but is most likely insufficient to significantly impact on chitin digestibility.

With the diet-induced microbiota modulation being consistently insufficient to allow effective digestion of chitin, the present work had as its main aim to select one or multiple bacteria with a high chitinolytic ability that could be used as a probiotic able to increase chitin digestibility in IM-containing fish feeds. As previously mentioned, feeding ESB with either HM25 or HEM25 diets enriched the digesta with members of the other Bacillales (*Bacillaceae* and *Paenibacillaceae* families) (Chapter II). These families are particularly interesting for industrial applications as they can produce spores (Setlow and Johnson, 2019). These structures are highly resistant to external insults (e.g., pressure, temperature, dehydration, and radiation) such as those to which the probiotic is submitted during feed extrusion processes (Setlow, 2006; Huang et al., 2017; Niu et al., 2018). Moreover, spores are also able to maintain their viability throughout the harsh gastrointestinal tract of fishes, being able to germinate and colonize the host's gut (Tam et al., 2006; Nayak, 2021). Given these highly appealing characteristics for industrial application in fish feeds, the target bacteria in this study were sporeformers (Chapter III). To prompt the isolation of aerobic sporeforming bacteria, grounded on the previous work carried out by (Serra et al., 2019), digesta from the ESB fed with the diets CTR, HM25, TM25, HEM25, and CHIT5 was collected, serially diluted, and submitted to a heat treatment to eliminate non-sporulating or vegetative cells, before its inoculation on Luria Bertani medium agar plates.

Of the 366 aerobic sporeforming bacteria obtained from the digesta of ESB, most originated from HM-based diets (HM25 and HEM25). However, chitinolytic bacteria with the ability to form a hydrolysis halo in a chemically defined chitin-containing solid media were mostly present in the intestines of fish fed with diet HEM25. Among the sporeforming chitinolytic bacteria obtained, *Bacillus* was the predominant genus (85.2%). This is in line with the enrichment of the *Bacillaceae* family in the digesta-associated microbial communities of ESB fed with HM25 and HEM25 diets (Chapter II; (Rangel et al., 2022)). However, the underlying mechanisms triggering the increased emergence of chitinolytic microorganisms in ESB fed with the HEM25 diet were not yet fully elucidated. While several authors postulate that chitin-degrading bacteria increase with increased dietary chitin levels (Bruni et al., 2018; Antonopoulou et al., 2019; Huyben et al., 2019; Rimoldi et al., 2019; Rimoldi et al., 2021; Weththasinghe et al., 2022), in the present study both HM25 and HEM25 diets possessed similar dietary chitin levels (1.7%

vs. 1.8%, respectively) and resulted in different outcomes. As such, we hypothesized that these results were most likely triggered by differences in chitin's bioavailability resulting from the process of the insects' exoskeleton shedding. During this process, insects lose and split their old cuticle and simultaneously release chitinase and peptidase-rich molting fluids, which will further disrupt the chitin matrix (Belles, 2020). The disruption of the crystalline structure of chitin increases bacterial or enzyme access, thus facilitating bacterial colonization and chitin use (Belles, 2020; Purkayastha and Sarkar, 2020). Moreover, the proliferation of several families of the Firmicutes phylum with chitinolytic potential has been shown to increase in the presence of high amounts of GlcNac monomers and dimers (Hui et al., 2020; Shimoï et al., 2020). As such, the increased presence of these molecules resulting from the insect's exoskeleton shedding (Zhu et al., 2008; Muthukrishnan et al., 2019; Belles, 2020), can help to explain why families such as *Bacillaceae* and *Planococcaceae* were more prevalent in ESB fed with the HEM25 diet. This work is the first to establish HEM's potential to modulate fish gut microbiota toward the establishment of chitinolytic microorganisms.

Regardless of the dietary origin, all obtained aerobic sporeforming microorganisms capable of degrading chitin were subjected to a thorough evaluation concerning their potential hemolytic activity, antibiotic resistance, sporulation efficiency, spore survival and chitinolytic activity in gastrointestinal-like conditions. Our results identified two isolates closely related to *B. licheniformis*, FI645 and FI658, as the most promising chitinolytic probiotics, and their potential was further assessed *in vivo* (Chapter IV). To do so, the two chitinolytic sporeforming fish isolates (FI645 and FI658) were incorporated individually or as a mixture (1:1) at 2×10^9 CFU kg⁻¹ in ESB diets with high inclusion levels (30%) of defatted HI larvae meal and evaluated regarding their viability throughout diets storage and their effects on ESB growth performance, digestibility, and ability to increase ESB survival to *V. anguillarum* infection.

After inclusion in pelletized diets, FI645 and FI658 maintained their viability for up to 180 days of storage at room temperature, overall confirming these spores' resilience to long storage times (Christie and Setlow, 2020). Dietary supplementation with FI645 significantly increased chitin digestibility, along with dry matter, protein, and energy digestibility. Ultimately, these results culminated in increases in FE and PER, in fish fed with diets supplemented with FI645.

These results demonstrate the effectiveness of the probiotics selection methodology (previously described by Serra et al. 2019), allowing the selection and characterization of an aerobic sporeforming fish isolate, closely related to *B. licheniformis* able to increase

chitin digestibility and feed efficiency of ESB. Overall, the *Bacillus*-induced increase in FE could be associated with either an improvement of the fish gut intestinal morphology (Liu et al., 2020; Du et al., 2021) or an increase in the activity of digestive enzymes, such as proteases, lipases, and amylases (Adorian et al., 2019; Ghanei-Motlagh et al., 2021a; Ghanei-Motlagh et al., 2021b; Wang et al., 2021). In this study, given that no alterations were found in the ESB gut morphology upon dietary probiotic supplementation, the increased nutrient digestibility and FE are most likely due to increased chitin digestibility, prompted by FI645-induced increase in total chitinase activity (close to statistical significance, $p = 0.052$). As both total chitinolytic activity and chitin digestibility increased concomitantly with the dietary supplementation of FI645, these results provide encouraging possibilities for further optimization of dietary probiotic inclusion level.

Given that dietary supplementation with FI645 doubled chitin digestibility when compared to the CTR diet (47.8% vs. 23.8% with the CTR diet), these results also demonstrated the crucial role played by fish gut microbiota in the degradation of chitin, as previously suggested by several authors (Sugita et al., 1999; Itoi et al., 2006; Ray et al., 2012). On the other hand, the chitinolytic activity detected in fish fed the CTR diet cannot be disregarded. While the measured chitinase activity can be the result of HI-driven ESB intestinal microbiota modulation, ESB can also potentially possess some endogenous chitinase activity, a hypothesis further supported by ESB's ability to express an acidic mammalian chitinase like gene in its intestine (Calduch-Giner et al., 2016).

Dietary supplementation strategies that increase nutrient digestibility are not exclusively achieved using probiotics. For example, exogenous enzymes addition to diets were already demonstrated to increase PF digestibility in fish feeds (Dalsgaard et al., 2012; Castillo and Gatlin III, 2015; Liang et al., 2022). However, coupled with their ability to hydrolyze carbohydrates, probiotic *Bacillus* spp., often carry additional benefits for fish health, such as increased disease resistance, thus providing a multifunctional solution. Due to their well-documented antibiofilm, anti-adhesive, antimicrobial, and immunostimulant activities (Nithya and Pandian, 2010; Giri et al., 2019; Monzón-Atienza et al., 2021; Santos et al., 2021) *Bacillus* spp. were already demonstrated to be able to increase fish survivability to *Vibrio* spp. (Gao et al., 2017; Kuebutornye et al., 2020; Ghanei-Motlagh et al., 2021a; Ghanei-Motlagh et al., 2021b) namely to *V. anguillarum* (Chen et al., 2016; Monzón-Atienza et al., 2022), one of the most reported pathogens to infect ESB (Muniesa et al., 2020; FAO, 2022; Kapetanović et al., 2022). This ability was also assessed in this work, with diets supplemented with FI645 leading to an increase in ESB survival of 47.6% (from 52.5% to 77.5%) when challenged with *V. anguillarum*.

However, contrary to what was observed for *B. licheniformis* (Vinoj et al., 2014; Hamza et al., 2018), in the present study no antibacterial activity against *V. anguillarum* was detected in *in vitro* tests. As such, the increased disease resistance observed likely resulted from the general probiotic ability of *Bacillus* to enhance fish immune response (Newaj-Fyzul et al., 2007; Liu et al., 2012; Ghanei-Motlagh et al., 2021a; Ghanei-Motlagh et al., 2021b; Monzón-Atienza et al., 2022).

It should also be noted that the increased survivability to *V. anguillarum* was observed in ESB fed with the diet presenting the highest chitin digestibility (D645). During chitin degradation, *B. licheniformis* has been reported to express and secrete chitin bacterial lytic polysaccharide monooxygenase (Courtade et al., 2015). This enzyme cleaves large chitin molecules into smaller molecules designated COS. In turn, as the bioactivity of these molecules is related to their degree of polymerization, COS presents greater immunostimulant and antibacterial capacities when compared to longer molecules, such as chitin/chitosan (Kumar et al., 2020). Several studies reported that when included in carnivorous fish feeds, COS increased fish survivability to pathogen challenges (Luo et al., 2009; Lin et al., 2012; Lin et al., 2017; Zhang et al., 2021), and therefore could also explain the results observed herein.

As GlcNac monomers plasma concentrations increased in fish fed the diet with the highest chitin digestibility (D645), a potential immunostimulant effect triggered by this monomer should also be considered. In this work, dietary supplementation with FI645 increased the expression of genes coding for N-acetylglucosamine kinase (*nagk*) and GlcNac phosphomutase (*pgm3*). These enzymes are a key element in the cellular GlcNac salvage pathway, integrating this molecule into the hexosamine biosynthetic pathway (HBP) (Campbell et al., 2021). Nonetheless, this work provided the first evidence in fish of a potential metabolic pathway followed by GlcNac, hinting for its possible integration in the hexosamine biosynthetic pathway (HBP).

While in fish no information is available regarding the functions of HBP, in mammals it was shown that it modulates leucocyte-mediated responses to stress or disease by regulating, among others: neutrophils migration (Kneass and Marchase, 2004); macrophages phagocytic and proinflammatory responses (de Jesus et al., 2018); pre-B cells proliferation, maturation, homeostasis, and antibody response (Wu et al., 2017; Lee et al., 2020); NK cell cytotoxicity (Božič et al., 2018); and oxidative stress (Groves et al., 2013; Issad et al., 2022). While further elucidation on this matter is required, present data hints that increased chitin digestibility can also lead to enhanced disease resistance.

Altogether, the results obtained in the present thesis allowed the selection and characterization of a chitinolytic putative probiotic with digestive and immunomodulatory properties. Although further studies are needed to introduce FI645 as a commercially available probiotic, this work was able to demonstrate that probiotics can be used as an effective tool to increase chitin digestibility, thus allowing the inclusion of higher levels of IM in fish feeds. Moreover, FI645's ability to increase ESB survivability to *V. anguillarum* brings added value to this probiotic by providing a successful strategy in the mitigation of the mortalities caused by this pathogen in ESB aquaculture.

5.2 Conclusions

- The present study contributed to a better understanding of the gut microbiota dynamics of a carnivorous fish, ESB, in response to diets including different IM.
- The ESB digesta-associated communities were differentially impacted by the dietary inclusion of IM from different origins, with HM25 and HEM25 diets triggering modulations that favored the relative increase of families of the Firmicutes phylum (*Bacillaceae*, *Enterococcaceae*, and *Lachnospiraceae*), and of the Actinobacteria phylum (*Actinomycetaceae*), in detriment of the Proteobacteria phylum.
- Feeding ESB with the HEM25 diet contributed to a significantly higher copy number of genes encoding ChiA, a commonly used marker for the bacterial chitinolytic potential, resulting from the increase of the *Paenibacillaceae* family.
- Concomitantly with the reported increase of the sporeforming families *Bacillaceae* and *Paenibacillaceae* in the allochthonous communities, the digesta of fish fed with the diets HM25 and HEM25 contained the majority of the culturable aerobic sporeforming fish isolates, with the latter diet triggering the emergence of most chitinolytic microorganisms obtained.
- This study allowed the selection of 40 aerobic sporeforming chitinolytic microorganisms among which two (FI645 and FI658), closely related to *B. licheniformis*, demonstrated a high survivability and the best chitinolytic activity in ESB gut-like conditions.
- FI645 and FI658 spores included in pelletized diets were verified to withstand long storage times (up to 180 days) without losing their viability.
- Dietary inclusion of FI645 increased ESB total chitinolytic activity in the intestine, leading to significantly higher chitin, dry matter, protein, and energy digestibility, thus demonstrating that probiotic supplementation is a valid solution to increase IM digestibility in carnivorous fish feeds.
- Dietary inclusion of FI645 significantly increased ESB survivability to *V. anguillarum*, compared to the control diet, thus providing a novel tool to mitigate this pathogen impact on ESB aquaculture

5.3 Future perspectives

HI exuviae meal (HEM), a novel dietary component, was demonstrated to be significantly more impactful on the emergence of chitinolytic bacteria than *Hermetia illucens* and *Tenebrio molitor* larvae meals. As such, in the future, further attention should be given to this novel commodity. While the supplementation level used (25%) is not viable at an industrial level, smaller inclusion levels (1-5%) should be tested alone or in conjugation with either HI or TM meals to assess its ability to modulate gut microbiota, thus potentially increasing the host chitinolytic activity. Moreover, research to fully characterize HEM meal composition, regarding its antimicrobial peptide composition, chitin, and its derivatives (chitosan or COS) should be conducted. Finally, if the ingredient characterization shows promising levels of either antimicrobial peptides or COS, given these molecules' immunostimulant abilities, HEM meal should also be tested as a potential immunostimulant. The successful valorization of this by-product of insect production could help reduce insect production waste as well as potentially increase fish growth performance and health.

The performed work also allowed the development of a putative novel probiotic solution which, by increasing chitin digestibility, was shown to increase ESB feed efficiency and protein efficiency ratio. However, several questions remain to be answered. For example, in the future it is important to elucidate the effect of our chitinolytic probiotic supplementation in the fish gut microbiota modulation. Moreover, to better understand the probiotic modes of action that confer higher survivability to ESB fed probiotic-supplemented diets, subsequent *in vivo* pathogen challenges should contemplate the determination of humoral, cellular, and molecular immune parameters, before and after bacterial challenge. The determination of these parameters will be crucial to identify any probiotic-induced physiologic or genetic expression alterations during the infection period.

While promising, for a successful industrial application of FI645, several characterization and optimization steps must be performed. For example, sequencing both fish isolate genomes would allow the proper identification of the species, comprehensively assess their genomic potential regarding other carbohydrolitic enzymes or determine the origin of the detected antibiotic resistances. Moreover, industrial up-scaling viability tests, industrial processing viability tests (e.g., spray-drying and extrusion survival), and the establishment of optimal dietary supplementation levels are also required to understand the economic and practical viability of this solution in commercial aquafeeds. Co-

application with other chitinolytic bacterial species or with probiotics able to increase the fish feed utilization of plant-based ingredients (given plants are also a major component of fish feeds that contain other non-starch polysaccharides) should also be pondered as multi-strain probiotics are often more promising when compared to mono-strain ones.

Under the scope of the present work, several enzymes from the hexosamine biosynthetic (HBP) pathway that were modulated by dietary supplementation with FI645 were identified. Whether this outcome is the result of solely bacterial-induced cues or the rapid cell influx of GlcNac resulting from chitin digestion remains to be elucidated. As such, given the preponderance of the HBP pathway in animals' immune, appetite, and growth regulation processes, and the trend to increase of IM in fish diets, a full characterization of this metabolic pathway response to GlcNac should be performed, followed by an evaluation on how this pathway affects fish physiologic processes.

5.4 References

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