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Hermetia illucens larvae meal as an alternative protein source in practical diets for gilthead sea bream (*Sparus aurata*): A study on growth, plasma biochemistry and gut microbiota

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ABSTRACT

The effects of dietary *Hermetia illucens* (HI) larvae meal on growth, plasma biochemistry and gut microbiota was tested in gilthead sea bream. Four isonitrogenous and isolipidic extruded diets (50% protein; 14% lipid) with different levels of HI larva meal (0% CTRL, 5% HI5, 10% HI10, and 15% HI15) in partial substitution to fish meal (FM) were administered to triplicate fish groups over 113 days. Diets were designed to partially replace FM, using FM level for practical application. No significant differences ($p > 0.05$) were observed in terms of final body weight, specific growth rate (SGR), feed intake (FI), feed conversion rate (FCR) as well as feed efficiency parameters such as protein efficiency ratio (PER), gross protein efficiency (GPE), gross lipid efficiency (GLE). At the end of the trial there were not significant differences on growth, feed intake, feed conversion rate and protein efficiency. Among over 20 plasma parameters analyzed, HI inclusion level reduced iron (Fe), potassium (K), creatinine (CREA), aspartate aminotransferase (AST), alanine amino transferase (ALT), creatine kinase (CK), alkaline phosphatase (ALP), lactate dehydrogenase (LDH). The reduction of AST, ALT and ALP might suggest a potential beneficial role of HI for liver integrity and functionality. Concerning gut microbiome (GM) layout, HI was able to induce a shift in the GM structure at any inclusion level considered compared to the control diet increasing the abundance of *Bacillaceae* (mainly *Bacillus* and *Oceanobacillus*) and *Paenibacillaceae* (*Paenibacillus*). Taxa that can be involved in chitin degradation and has been recently recognized as novel probiotics for aquaculture. In conclusion, the results of feed intake, growth, feed utilization and plasma biochemistry indicate that HI larvae meal can be successfully incorporated up to 15% in practical aquafeed diets to partially replace FM without any negative effects on growth and feed efficiency. Beyond being a valid alternative protein source for fish meal replacement in this species, it displays gut health functional properties already at low inclusion level.

1. Introduction

Nowadays, access to high-quality protein is becoming a limitation due to several socio-economic factors such as the growing world population, urbanization, resource scarcity, climate change and, in this scenario, the aquaculture industry plays a central role all over the world (Alfiko et al., 2022). The State of World Fisheries and Aquaculture (FAO, 2020) estimated an increase in the growth of farmed fish from 114.5 million tonnes in 2018 to 201 million tons by 2030. Farmed fish species are mostly carnivorous, with nutritional requirements quite high in terms of quality and quantity of protein in the diet. Unfortunately, the European Union still has a deficit for high protein feed materials (30–50%), with severe concerns regarding feed security and the general competitiveness (FEFAC, 2018). For these reasons, the demand for a continuous and favourable protein supply is becoming a pressing issue also for fish feed.

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Fish meal (FM) has been traditionally considered the best protein source in aquafeed formulation. However, it is an animal-based protein product, and considering the depletion of natural resources in the last decades, it is destined to become increasingly scarce and expensive (Hidalgo et al., 2022). Consequently, the production of more economic and sustainable alternative proteins sufficient to satisfy demand is of vital importance (Nugroho and Nur, 2018).

In the last decades, several new alternative protein sources for aquafeed from various origins have been studied. Some of these are already adopted commercially, such as insect meal. Insects may represent an economically and ecologically valuable ingredient (Vargas-Abúndez et al., 2019) since efficiently convert food into proteins, have a limited need for arable land and water, have a low ecological cost (low emissions of greenhouse gases and CO_2), involve low investments in machinery, and have rapid reproduction cycles (Nugroho and Nur, 2018).

Insects are widely recognized as a source with a high nutritional value which makes them an excellent aquafeed ingredient. In fact, insects present a high protein content (50–82% dry matter), with an amino acidic profile (both essential and not essential), that satisfy the requirements of fish (Henry et al., 2015). Furthermore, they are rich in vitamins (e.g., pyridoxine, riboflavin, folic acid, and vitamin B12), minerals (potassium, calcium, iron, magnesium, zinc and selenium), and lipids (from 10 to 30%) (Henry et al., 2015).

Among the studied insect species, in recent years *Hermetia illucens* (HI) has received much attention as being of great interest as an alternative protein source to replace fish meal in fish feed. Only in the last years, HI larvae meal has been tested on several fish species, such as Atlantic salmon (*Salmo salar*) (Bruni et al., 2020a; Fisher et al., 2020; Li et al., 2020), Rainbow trout (*Oncorhynchus mykiss*), (Bruni et al., 2020b; Caimi et al., 2021; Hoc et al., 2021; Rimoldi et al., 2021), Mirror carp (*Cyprinus carpio*) (Xu et al., 2020), Hybrid tilapia (*Oreocromis niloticus x O. mozambique*) (Abu Bakar et al., 2021; Agbohessou et al., 2021; Tippayadara et al., 2021), African catfish (*Clarias gariepinus*) (Adeoye et al., 2020; Fawole et al., 2020; Ratika et al., 2020), European seabass (*Dicentrarcus labrax*) (Abdel-Latif et al., 2021; Abdel-Tawwab et al., 2020; Moutinho et al., 2021), Japanese seabass (*Lates calcarifer*) (Hender et al., 2021), Siberian sturgeon (*Acipenser baerii*) Meagre (*Argyrosomus regius*) (Guerreiro et al., 2021), Climbing fish (*Anabas testudineus*) (Vongvichith et al., 2020), Lemon fin Barb (*Hypsibarbus wetmorei*)

Table 1
An overview of the results where HI larvae meal was used to replace protein sources in diets for gilthead seabream.

Average fish weight		HI inclusion levels	Protein source replaced	Minimal FM inclusion	Length of the experiment	Observations	Reference
Initial	Final						
181.6 g	400 g	9.2, 18.4, 27.6%	FM	75 g/Kg	144 days	1. Fillet color (↔) 2. Saturated fatty acids (HI18, HI27) (↑) 3. n-3 PUFA (HI18, HI27) (↓)	Pulido-Rodriguez et al. (2021)
29.5 g	123 g	19.5%	FM	455 g/Kg	93 days	1.FBW (↔) 2.Protein retention (↔) 3.Fat retention (↓) 4.Plasma metabolites (↔) 5.Liver lipogenic enzymes (↔)	Mastoraki et al. (2022)
233 g	369 g	10%	FM	50 g/Kg	96 days	1. FBW (↔) 2. No anomalies in intestinal gross morphology and no signs of inflammation 3. Gastrointestinal transit (↓) 4. Excitatory cholinergic and serotoninergic transmission in the proximal and distal intestine (↓) 5. Inhibitory components of peristalsis in proximal intestine (↑), and distal intestine (↓)	Bosi et al. (2021)
6.77 g	25.4 g	5.6, 10.9%	FM	259 g/Kg	43 days	1. EPA and DHA (↓) (HI10.9)	Fabrikov et al. (2021)
48.8 g	186.2 g	16.2, 32.4%	VP		90 days	1. Incidence of intestine histological alterations and inflammatory response (↓) 2. Liver lipid deposition (↑) 3. FBW, SGR (↑) (16.2%), (↑↑) (32.4%) 4. RFI (↔) to VP, (↓) respect to FM 5. FCR (↓)	Randazzo et al. (2021)
29.5 g	123.6 g	19.5%	FM	455 g/kg	90 days	1. FBW, WG, DFI, SGR, FCR, Somatometric indices (↔) 2. In *Staphylococcus* sp., *Hafnia* sp., and *Aeromonas* sp. (↑)	Panteli et al. (2021)
48.8 g	345.5 g	8.10, 16.2, 32.4%	VP		147 days	1. FBW, FI, SGR, FCR (↔) 2. Fatty acids profile (↔)	Pulido-Rodriguez et al. (2021)
6.77 g	25.4 g	5.6, 10.9%	FM	259 g/Kg	43 days	1. FBW, FCR (↔) 2. WG (↓) (HI10.9) 3. (↔) Digestibility 4. (↑) Vmax of ALT and Km of AST (HI5.6)	Fabrikov et al. (2020)
49 g	188.7 g	8.1, 16.2, 32.4%	VP		84 days	1. FBW (↑) 2. Skin pigmentation (↔)	Pulcini et al. (2020)
2.4 g	10.8 g	5.8, 11.6, 17.4%	FM	290 g/kg	70-days	1.FBW, SGR, FI, PER, HSI, CF (↓), FCR (↑)	Karapanagiotidis et al. (2023)
65.3 g	163.5 g	5.0, 10%	FM	50 g/kg	112-days	1. FBW, LER (↓), SGR, FI, FCR, PER (↔), (10% inclusion) 2. Fillet n-3 PUFA, n-6 PUFA (↔) 3. Posterior gut *hsp90, hsp70, cox2, mhcii, tnfa, il-1b* gene expression (↔).	Carvalho et al. (2023)

(↔) equal; (↓) decrease; (↑) increase; (↑↑) higher increase; FM: Fish meal; FCR, Feed conversion rate; SGR, specific growth rate; PER, protein efficiency ratio; VP, vegetal proteins; FI, Feed intake; RFI, relative feed intake; DFI, daily feed intake; FBW, final body weight; WG, weight gain; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALT, Alanine aminotransferase; Vmax, the maximum rate of reaction of an enzyme; AST, Aspartate aminotransferase; Km, the affinity of an enzyme for its substrate.

(Kamarudin et al., 2021) with the purpose of observing its effects on growth performance, feed utilization, fish efficiency, fish welfare, gut health, digestive conditions, body composition, and fillet quality. Furthermore, several studies mostly on rainbow trout have investigated the potential of insect meal to modulate the gut microbiome in fish (Terova et al., 2019, 2021; Antonopoulou et al., 2019; Rimoldi et al., 2019). Although different effect on gut microbiome modulation were reported and results are still not conclusive (Hossain et al., 2023), recent outcomes are moving onward to confirm a positive modulation of the gut microbiota in terms of bacteria selection able to produce short chain fatty acids (mainly butyrate) induced by chitin fermentation (Biasato et al., 2022; Rimoldi et al., 2023).

Several studies have also evaluated the effect of HI dietary inclusion in gilthead sea bream as summarized in Table 1. Most of these publications are focused on performance, fillet quality (fatty acid composition) and potential intestinal morphology alterations. However, only one study evaluated growth and gut microbiota (Panteli et al., 2021). Here, the FM level adopted ranged between 45.5 and 65.0%, much higher than modern application for aquafeed (Carvalho et al., 2023).

This study aimed to evaluate the effect of increasing dietary level of HI larvae meal on growth, feed efficiency, plasma biochemistry, and gut microbiota during the on-growing stage of gilthead sea bream using diets with industrially-relevant FM level.

2. Materials and methods

2.1. Experimental diets

Four isoproteic (50%) and isolipidic (14%) experimental diets were formulated by Naturalleva (VRM s.r.l) and produced via extrusion technology by Sparos Lda (Olhão, Portugal) with a different inclusion level of *Hermetia illucens* (HI) larvae meal in substitution of FM: a control diet (CTRL) with a 0% of HI inclusion, a diet with the 5% of HI (HI5), a diet with the 10% of HI (HI10), and a diet with the 15% of HI (HI15) (ingredients and proximate composition in Table 2). The HI larvae meal inclusion level was chosen in order to partial replace FM up to 54%. Diets were formulated with FM and a mixture of vegetable ingredients as previously reported for this species (Busti et al., 2022; Marchi et al., 2023). The HI larvae meal used in this study was provided by MUTATEC (Caumont-sur-Durance, France).

Table 2
Ingredients and proximate composition of experimental diets.

Experimental diets				
Ingredients %	Control	HI5	HI10	HI15
Fish meal	22.0	18.1	14.1	10.1
Hermetia illucens larvae meal (Mutatec)[a]	0.00	5.01	10.0	15.0
Wheat flour	9.82	8.47	7.12	5.79
Wheat gluten meal	3.07	3.08	3.08	3.09
Soybean meal	11.4	11.5	11.5	11.5
Maize gluten meal	26.4	26.4	26.5	26.5
Soy protein Concentrate	13.2	13.2	13.2	13.3
Rapeseed oil	7.52	6.89	6.37	5.76
Fish oil	3.22	3.71	4.09	4.56
DL-Methionine	0.26	0.30	0.33	0.36
HCl Lysine	0.26	0.33	0.41	0.50
Taurine	0.22	0.24	0.26	0.27
Monammonium Phosphate	0.79	1.01	1.24	1.43
Vit. C	0.07	0.07	0.07	0.07
Premix Vitamins and Minerals	0.69	0.69	0.69	0.69
Hydrolized shrimp protein (liquid)	1.03	1.03	1.03	1.03
Proximate Composition, % wet weight				
Moisture	6.16	6.11	6.76	6.41
Crude protein	49.9	50.3	51.0	51.3
Crude fat	13.9	14.0	13.8	13.6
Ash	6.62	6.37	6.28	6.17

[a] *Hermetia illucens* defatted larvae meal, Proteins 55%, Lipids 10%, Fibres 10% Mutatec, France,

2.2. Fish and feeding trial

The experiment was conducted at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Fish were obtained from Panittica Pugliese (Torre Canne di Fasano, Brindisi, Italy). At the beginning of the trial, 50 specimens (initial weight: 98.6 ± 0.6 g) per tank were randomly distributed into 12 square tanks with 450 L of capacity. Experimental diets were assigned randomly and administered to triplicate groups to visual satiation twice a day, at h 8.30 and h 16.00, for 6 days a week, while on Sunday fish were fasted. The trial lasted 113 days, during which tanks were provided with natural seawater and connected to a closed recirculation system (RAS) (overall water volume: 7 $m^{-3;}$ Oxygen level 8.0 ± 1.0 mg L^{-1}; Temperature 24 ± 1.0 °C, Salinity 25 g L^{-1}). RAS utilized daily maintenance and measurements according to (Marchi et al., 2023).

2.3. Sampling

At the beginning, and at the end of the trial, all the fish in each tank were anesthetized by tricaine methanesulfonate (MS-222) at 100 mg L^{-1} and weighed. Specific growth rate (SGR), feed intake (FI) and feed conversion rate (FCR) were calculated. The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 10 fish and a pooled sample of 5 fish per tank at the end of the trial. Protein efficiency rate (PER), gross protein efficiency (GPE), and gross lipid efficiency (GLE) were calculated. At the end of the trial, 5 fish per tank (15 fish per treatment) were anesthetized and their blood was collected from the caudal vein. Blood samples were taken in a few minutes (<5) to avoid a probable increase in cortisol levels caused by manipulation (Molinero et al., 1997). Samples were then centrifuged (3000 xg, 10 min, 4 °C) and plasma aliquots were placed at −80 °C until analysis (Parma et al., 2023). At the same time, samples of distal intestine content from 5 fish per tank were individually collected and stored at —80C for gut bacterial community characterization through the 16S rRNA gene analysis using Illumina next-generation sequencing platform (Pelusio et al., 2021).

Overall experimental procedures were evaluated and approved by the Ethical-Scientific Committee for Animal Experimentation of the University of Bologna, under European directive 2010/63/UE relating to the protection of animals used for scientific purposes.

2.4. Gut bacterial community DNA extraction and sequencing

Total microbial DNA was extracted and analyzed from individual distal intestine content obtained from 5 fish per tank as previously reported in Parma et al. (2020). DNA was then quantified with NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) and stored at −20 °C until further processing. To perform the 16S rRNA gene analysis, the V3–V4 hypervariable regions were amplified using the 2 KAPA HiFi HotStart ReadyMix (KAPA Biosystems) using 341F and 785R primers with overhang Illumina sequencing adapters, as previously described (Pelusio et al., 2021). Briefly, the thermal cycle consisted of an initial denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 55C for the 30s and extension at 72 °C for 30 s, and a final extension step at 72 °C for 5 min. As recommended in the Illumina protocol "16S Metagenomic Sequencing Library Preparation" for the MiSeq system, PCR reactions were cleaned up by using Agencourt AMPure XP magnetic beads. A limited-cycle PCR was performed to obtain the indexed library using Nextera technology, followed by a second AMPure XP magnetic beads clean-up step. Sequencing was performed on the Illumina MiSeq platform using a 2 × 250 bp paired-end protocol according to the manufacturer's instructions (Illumina, San Diego, CA). At the end of the sequencing process, raw sequences were processed combining PANDAseq and QIIME2 pipelines (Bolyen et al., 2019; https://qiime2.org). High-quality reads, obtained after a filtering

step for length (min/max = 350/550 bp) and quality with default parameters, were cleaned using DADA2 (Callahan et al., 2016) and clustered into amplicon sequence variants (ASVs) using VSEARCH algorithm (Rognes et al., 2016). Taxonomy was assigned using RDP classifier against SILVA database (Quast et al., 2013). Three different metrics were used to evaluate internal ecosystem diversity (alpha-diversity) – Faith's Phylogenetic Diversity (faith_pd), Shannon_entropy index, and number of observed ASVs (observed features). Unweighted UniFrac distances were computed to estimate inter-sample ecosystem diversity (beta-diversity) and used as input for Principal Coordinates Analysis (PCoA).

2.5. Analytical methods

Diets and the whole body were analyzed for proximate composition. Moisture content was determined by weight loss after drying samples in an oven at 105 °C until a constant weight was achieved. Total lipids analysis was performed according to Bligh and Dyer's (1959) extraction method. Crude protein content was measured as total nitrogen (N) through the Kjeldahl method and multiplying N by 6.25. Ash content was evaluated by incineration to a constant weight in a muffle oven at 450 °C.

2.6. Metabolic parameters in plasma

Glucose (GLU), urea, creatinine (CREA), uric acid, total bilirubin (Tot bill), cholesterol (CHOL), high-density lipoprotein (HDL), triglycerides (TRIG), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatine kinase (CK), lactate dehydrogenase (LDH), calcium (Ca^{+2}), phosphorus (P), potassium (K^{+}), sodium (Na^{+}), iron (Fe), chloride (Cl), magnesium (Mg), and ALB/globulins (A/G) were measured in the plasma using samples of 500 μL on an automated analyzer (AU 480; Olympus/Beckman Coulter, Brea, CA, United State) according to the manufacturer's instructions (Parma et al., 2023). The A/G, ratio was calculated.

2.7. Statistical analysis

All data are presented in tables as mean ± standard deviation (SD). Results on growth, proximate composition, and nutritional indices were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Data were checked for normality (Anderson-Darling, D'Agostino-Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov tests) and homogeneity of variance (Bartlett and Brown–Forsythe tests). In case tests failed, data were analyzed by the non-parametric Kruskall-Wallis test. Plasma data showing high standard deviation were also checked for outliers by the Chauvenet's outliers test. Growth, nutritional indices and plasma parameters were also analyzed by linear regression. Statistical analyses were performed using GraphPad Prism 8.0 for Windows (Graph Pad Software, San Diego, CA, USA). Data were considered significant at $p < 0.05$.

Microbiota analysis and respective plots were produced using R software (https://www.r-project.org/) with "vegan" (http://www.cran.r-project.org/package-vegan/), "Made4"(Culhane et al., 2005) and "stats" packages (https://stat.ethz.ch/R-manual/R-devel/library/stats/html/00Index.html). Data separation was tested by a permutation test with pseudo-F ratios (function "Adonis" in "vegan" package). When required, Wilcoxon and Kruskal–Wallis test were used to assess significant differences in alpha diversity and taxon relative abundance between groups. *p*-values were corrected for multiple testing with the Benjamini–Hochberg method, with a false discovery rate (FDR) < 0.05 considered statistically significant (function p.adjust in the "stats" package).

2.8. Calculations

The calculations for the determination of performance parameters were the following: Specific growth rate (SGR) (% day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Feed intake (FI) (% ABW^{-1} day^{-1}) = ((100 * total ingestion)/(ABW))/days)) (where average body weight, ABW = (IBW + FBW)/2; Feed conversion ratio (FCR) = feed intake / weight gain. Protein efficiency rate (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish. Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish.

3. Results

3.1. Growth

Data on growth performances (FBW and SGR), FI, and FCR at the end of the trial, are summarized in Table 3 and Fig. 1. No significant differences were observed in FBW, SGR, FCR, and FI during the overall period. Data on nutritional indices (PER, GPE, GLE) showed no significant differences among treatments.

3.2. Plasma biochemistry

The results of plasma parameters are shown in Table 4. No significant differences ($p > 0.05$) were observed in GLU, urea, Tot Bil, CHOL, TRIG, HDL, TP, ALB, Ca^{2+}, Mg. Lactate, uric acid, Na and Cl showed a significant dose response at increasing HI dietary inclusion level while CREA, AST, ALT, ALP, CK, LDH, K, Fe displayed an inverse correlation (linear regression $p < 0.05$). Uric acid and lactate were lower in CTRL than HI15. CREA was affected by the HI inclusion level, with higher values in CTRL and HI5 diets compared to HI15 and higher values in HI5 compared to HI10. AST, ALP and CK were higher in CTRL compared to HI15. K^{+} was higher in HI5 compared to the other treatments and higher in CTRL than HI15. Na^{+} was higher in HI15 than CTRL and HI5, while Fe was lower in HI15 compared to the other three diets. A/G displayed significantly lower values in HI10 compared to CTRL. P displayed a tendency for higher values in HI5 and HI15 although no significant

Table 3
Growth performance and nutritional indices of sea bream fed experimental diets over 113 days.

Diet	Control	HI5	HI10	HI15	*P* value
IBW (g)	98.7 ± 0.38	98.3 ± 1.35	98.8 ± 0.22	98.7 ± 0.16	*0.886*
FBW (g)	273.9 ± 7.86	282.3 ± 5.12	271.6 ± 8.69	273.3 ± 9.14	*0.400*
SGR	0.90 ± 0.03	0.93 ± 0.00	0.89 ± 0.03	0.90 ± 0.03	*0.184*
FI	1.10 ± 0.03	1.10 ± 0.03	1.08 ± 0.02	1.09 ± 0.04	*0.595*
FCR	1.32 ± 0.02	1.29 ± 0.03	1.31 ± 0.03	1.31 ± 0.01	*0.492*
PER	1.52 ± 0.02	1.54 ± 0.04	1.51 ± 0.02	1.49 ± 0.02	*0.164*
GPE	27.2 ± 0.14	27.4 ± 0.98	27.2 ± 0.75	26.6 ± 1.21	*0.756*
GLE	90.3 ± 8.34	103.9 ± 4.23	95.4 ± 2.23	98.0 ± 13.49	*0.311*

Data are given as the mean ($n = 3$) ± SD. No significant differences among treatments (One-way Anova $p > 0.05$).
IBW = Initial body weight.
FBW = Final body weight.
SGR = Specific growth rate (% day^{-1}) = 100 × (ln FBW- ln IBW) / days.
FI = Feed intake (% ABW^{-1} day^{-1}) = ((100*total ingestion)/(ABW))/days)).
FCR = Feed conversion rate = feed intake / weight gain.
PER = Protein efficiency ratio = ((FBW-IBW)/protein intake).
GPE (%) Gross protein efficiency = 100 × [(% final body protein × FBW) − (% initial body protein × IBW)]/total protein intake fish.
GLE (%) Gross lipid efficiency = 100 × [(% final body lipid × FBW) − (% initial body lipid × IBW)]/total lipid intake fish.

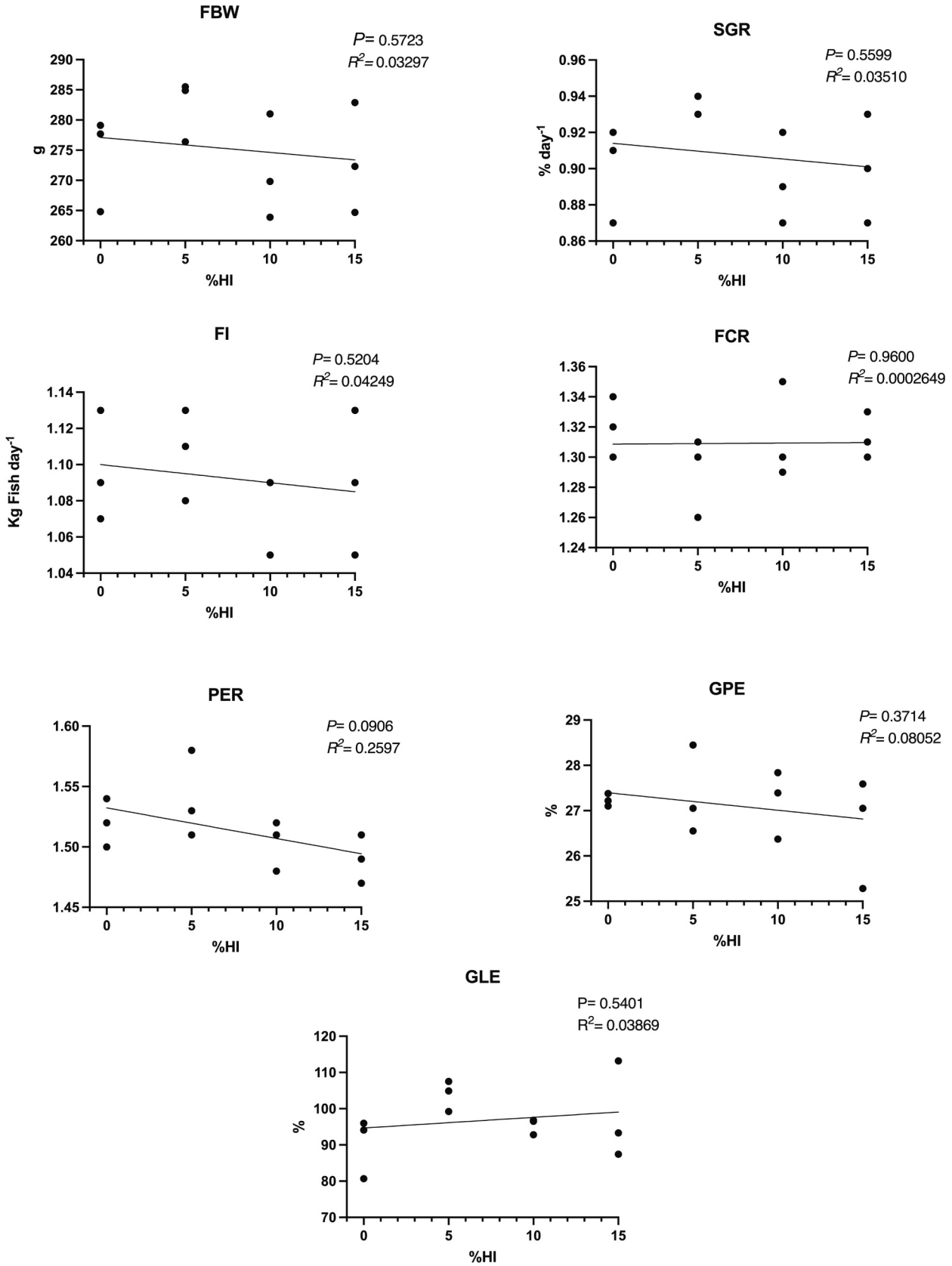


Fig. 1. Linear regression showing the effect of the increasing percentages of HI meal on final body weight (FBW,g), specific growth rate (SGR, % day^{-1}), feed intake (FI, Kg fish day^{-1}), feed conversion rate (FCR), protein efficiency ratio (PER), gross protein efficiency (GPE, %), gross lipid efficiency (GLE, %). No significant differences were detected ($P > 0.05$).

Table 4
Plasma biochemistry values for sea bream fed experimental diets over 113 days.

Parameters	Experimental diets						
	CTRL	HI5	HI10	HI15	P- value (One-way Anova)	P-value (Linear regression)	R^2
GLU	127.14 ± 18.0	131.75 ± 2.8	140.92 ± 25.2	135.73 ± 13.6	*0.3852*	*0.1786*	*0.0345*
Lactate (mg dL^{-1})	18.09 ± 4.98^{a}	20.72 ± 6.67^{ab}	22.69 ± 7.26^{ab}	23.92 ± 3.13^{b}	*0.0476*	*0.0228*	*0.0994*
Urea (mg dL^{-1})	14.58 ± 1.76	13.78 ± 2.67	13.15 ± 3.57	13.11 ± 2.36	*0.4247*	*0.1111*	*0.0480*
CREA	0.29 ± 0.08^{bc}	0.32 ± 0.07^{c}	0.23 ± 0.03^{ab}	0.21 ± 0.03^{a}	*<0.0001*	*0.0001*	*0.2628*
Uric acid (mg dL^{-1})	0.07 ± 0.08^{a}	0.23 ± 0.22^{ab}	0.22 ± 0.19^{ab}	0.23 ± 0.14^{b}	*0.0048*	*0.0176*	*0.1055*
Tot Bil (mg dL^{-1})	0.04 ± 0.03	0.03 ± 0.03	0.04 ± 0.03	0.05 ± 0.03	*0.1789*	*0.1271*	*0.0450*
CHOL	211.5 ± 34.3	224.0 ± 32.3	208.4 ± 49.5	219.79 ± 53.1	*0.8044*	*0.8138*	*0.0011*
HDL (mg dL^{-1})	77.6 ± 14.3	78.7 ± 16.2	75.6 ± 10.8	82.6 ± 21.5	*0.7286*	*0.5228*	*0.0082*
TRIG	677.2 ± 222.7	811.0 ± 267.8	650.5 ± 351.3	587.15 ± 152.8	*0.1407*	*0.1629*	*0.0385*
TP	4.01 ± 0.22	4.11 ± 0.36	4.21 ± 0.34	4.20 ± 0.48	*0.4218*	*0.1155*	*0.0488*
ALB (g dL^{-1})	1.17 ± 0.08	1.14 ± 0.14	1.12 ± 0.10	1.16 ± 0.11	*0.6386*	*0.7481*	*0.0020*
AST (U L^{-1})	97.14 ± 57.81^{b}	95.90 ± 88.34^{ab}	44.36 ± 20.74^{ab}	37.0 ± 6.36^{a}	*0.0067*	*0.0011*	*0.2081*
ALT (U L^{-1})	20.2 ± 13.8	16.7 ± 11.4	10.9 ± 5.4	8.5 ± 3.8	*0.0568*	*0.0011*	*0.2054*
ALP (U L^{-1})	273.8 ± 177.0^{b}	180.92 ± 68.61^{ab}	169.6 ± 59.9^{ab}	144.2 ± 48.0^{a}	*0.0135*	*0.0030*	*0.1688*
CK (U L^{-1})	1330.4 ± 1250.9^{b}	1200.7 ± 1092.2^{ab}	798.8 ± 478.9^{ab}	376.2 ± 345.4^{a}	*0.0347*	*0.0037*	*0.1592*
LDH (U L^{-1})	891.9 ± 899.1	1190.6 ± 1158.5	354.9 ± 232.1	281.3 ± 245.6	*0.0620*	*0.0097*	*0.1340*
Ca^{+2} (mg dL^{-1})	13.5 ± 0.49	14.2 ± 0.89	13.9 ± 0.91	14.0 ± 0.86	*0.1928*	*0.1715*	*0.0386*
P (mg dL^{-1})	11.1 ± 1.00	12 ± 1.23	11.1 ± 1.23	11.9 ± 1.02	*0.0492*	*0.2216*	*0.0297*
K^{+} (mEq L^{-1})	7.7 ± 2.2^{b}	10.1 ± 2.0^{c}	7.0 ± 1.8^{ab}	5.8 ± 0.6^{a}	*<0.0001*	*0.0009*	*0.1915*
Na^{+} (mEq L^{-1})	177.1 ± 3.37^{a}	177.9 ± 3.96^{a}	179.5 ± 3.15^{ab}	181.4 ± 3.04^{b}	*0.0075*	*0.0006*	*0.2052*
Fe (μg dL^{-1})	80.6 ± 26.3^{b}	92.0 ± 33.3^{b}	82.5 ± 29.3^{b}	51.3 ± 8.3^{a}	*0.0011*	*0.0046*	*0.1495*
Cl (mEq L^{-1})	149.4 ± 2.84	151.5 ± 2.16	151.5 ± 2.81	151.8 ± 2.26	*0.0642*	*0.0208*	*0.0984*
Mg (mg dL^{-1})	2.84 ± 0.24	3.13 ± 0.44	3.14 ± 0.45	3.09 ± 0.37	*0.1562*	*0.1095*	*0.050*
A/G	0.42 ± 0.04^{b}	0.38 ± 0.05^{ab}	0.37 ± 0.04^{a}	0.39 ± 0.04^{ab}	*0.0286*	*0.051*	*0.073*

Data are given as the mean (n = 3) ± SD. Different superscript letters indicate significant differences among treatments (One-way Anova $p \leq 0.05$). GLU, Glucose; Tot Bil, total bilirubin; CHOL, cholesterol; HDL, high density lipoprotein; TRIG, triglycerides; TP, total protein; ALB, albumin; AST, aspartate aminotransferase; ALT, alanine amino transferase; ALP, alkaline phosphatase; CK, creatine kinase; LDH, lactate dehydrogenase, Ca^{+2}, calcium; P, inorganic phosphorus; K+, potassium; Na+, sodium; Fe, iron; Cl, chloride; Mg, magnesium; A/G, albumin/globulins.

differences among treatments were detected in the post hoc test.

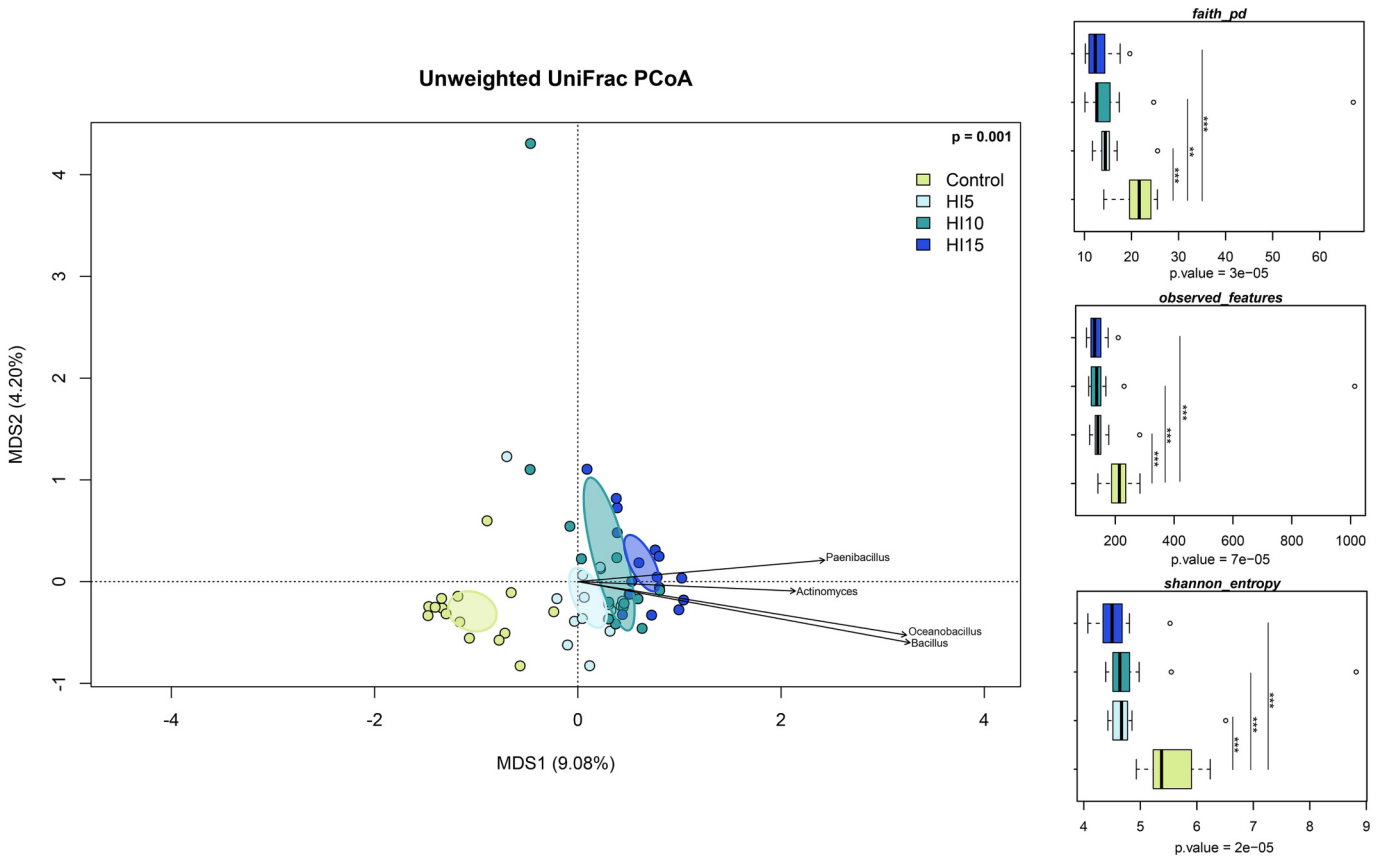
3.3. Gut bacterial community

In order to assess the possible effect that increasing dietary HI meal level could have on gut microbial community during the on growing stage of gilthead sea bream, at the end of the trial the 16S rRNA gene sequencing was performed on a total of 60 distal intestine content samples, 15 samples per each treatment, yielding 530′440 high-quality reads (mean ± SD, 8′840 ± 3′340) and clustered into a total of 9′830 ASVs. The gut microbiota (GM) variations between samples (beta-diversity) and the internal ecosystem diversity for each dietary group (alpha-diversity), were assessed respectively by the principal coordinates Analysis (PCoA) based on unweighted Unifrac distances (Fig. 2) and by the calculation of three different metrics: PD_whole_tree, obvserved_feature (number of ASV) and Shannon index (Fig. 2). Results showed that dietary HI levels significantly affected the GM in terms of overall GM composition, in fact HI5, HI10, and HI15 showed a significant variation compared the CTRL which resulted to be clearly separated from other diets (CTRL vs HI5/HI10/HI15; "pairwise Adonis permutation test", $p = 0.001$) (Fig. 2). For what concern internal ecosystems diversity, a significant reduction in all the 3 metrics calculated was observed according to the dose level, compared to the control group (Wilcoxon rank-sum test controlled for multiple testing using FDR; HI0 vs HI5/HI15 $p < 0.001$; HI0 vs HI10, $p < 0.01$) (Fig. 2). To highlight the GM composition of gilthead sea bream in the different dietary groups, the overall composition at various phylogenetic levels was investigated, at phylum (Fig. 3a) and family level (Fig. 3b). Specifically, at phylum level all treatments presented Firmicutes as dominant phyla (range mean relative abundance from 87 to 97%). In Diet CTRL, HI5 and HI10 groups, the other two dominant phyla were Actinobacteriota and Proteobacteria. In HI15 diet group was also detected the presence of Bacteroidota as the second dominant phyla (mean relative abundance ± SD; 3.1 ± 11.5%). At a family level *Lactobacillaceae* was the dominant family in the control group, followed by *Bacillaceae* and *Streptococcaceae* families (respectively with a mean relative abundance ± SD; 37.4 ± 7.0, 19.1 ± 14.7 and 9.8 ± 4.8). In the HI groups instead, the dominant bacterial families, all belonging to Firmicutes phylum, were *Bacillaceae*, followed by *Paenibacillaceae* and *Lactobacillaceae* (respectively with a range of mean relative abundance from 60 to 71%, 11 to 18% and 3.5 to 11%) (Fig. 3b). Considering a lower taxonomic level, several compositional differences in terms of bacterial genera relative abundance among dietary groups were observed, taking into account only bacterial genera with a mean relative abundance higher than 1% in at least one group (Wilcoxon rank-sum test controlled for multiple testing using FDR; $p < 0.05$) (Fig. 4). According to our data, the relative abundance of *Oceanobacillus*, *Bacillus* and *Paenibacillus* genera was significantly higher in all HI groups compared to the control group (Wilcoxon $p < 0.001$). On the other hand, the relative abundance of *Leuconostoc*, *Ligilactobacillus*, *Lactobacillus*, *Weissela*, *Limisolactobacillus*, *Streptococcus*, *Staphylococcus*, *Lactococcus* and *Peptostreptococcus* genera was significantly lower in the HI groups compared to the control group (Wilcoxon $p < 0.05$). Concerning the differences among the HI groups only *Weissella* and *Peptostreptococcus* genera showed a significant lower relative abundance in H15 group compared to HI5 group (Wilcoxon <0.05).

4. Discussion

Several studies were carried out to test the application of insect meal as alternative FM in gilthead sea bream at juveniles and on-growing stage (Table 1). However, few studies have tested the HI larvae meal in the context of modern feed with low FM dietary level (Carvalho et al., 2023). Data concerning the plasma and gut microbiota were also poorly investigated and more informations are needed to properly define optimal HI larvae meal inclusion level for this species.

In the present study, feeding gilthead sea bream with diets containing different inclusion levels of HI did not show significant differences in FBW. These results are in line with Bosi et al. (2021) where 10% of HI inclusion in replacement of FM did not affect growth in gilthead sea bream. Furthermore, in the same species, it has been observed that the inclusion of HI meal up to 32% as the sole animal protein source could lead to an increase in FBW (Pulcini et al., 2020; Randazzo et al., 2021).



Figs. 2. Beta diversity and alpha diversity of gut microbiota of gilthead seabream fed increasing HI meal dietary levels. On the left panel, Principal Coordinates Analysis (PCoA) based on unweighted UniFrac distances between gut microbiota structure of animals fed diets HI0, HI5, HI10, HI15. Samples are significantly separated (permutation test with pseudo-F ratios Adonis; $p = 0.001$). Black arrows are obtained by fitting the genus family relative abundance values for each sample within the ordination space (function envfit of the "vegan" R package, with a p-value <0.001), only genera with a higher load into the treated groups are plotted. On the right panel, boxplots show alpha-diversity values measured by PD_whole_tree, Shannon index and observed_features (number of ASVs). All metrics highlighted a significant reduction of internal ecosystem diversity in treated groups compared to control group (Wilcoxon rank-sum test, $p \leq 0.001$).

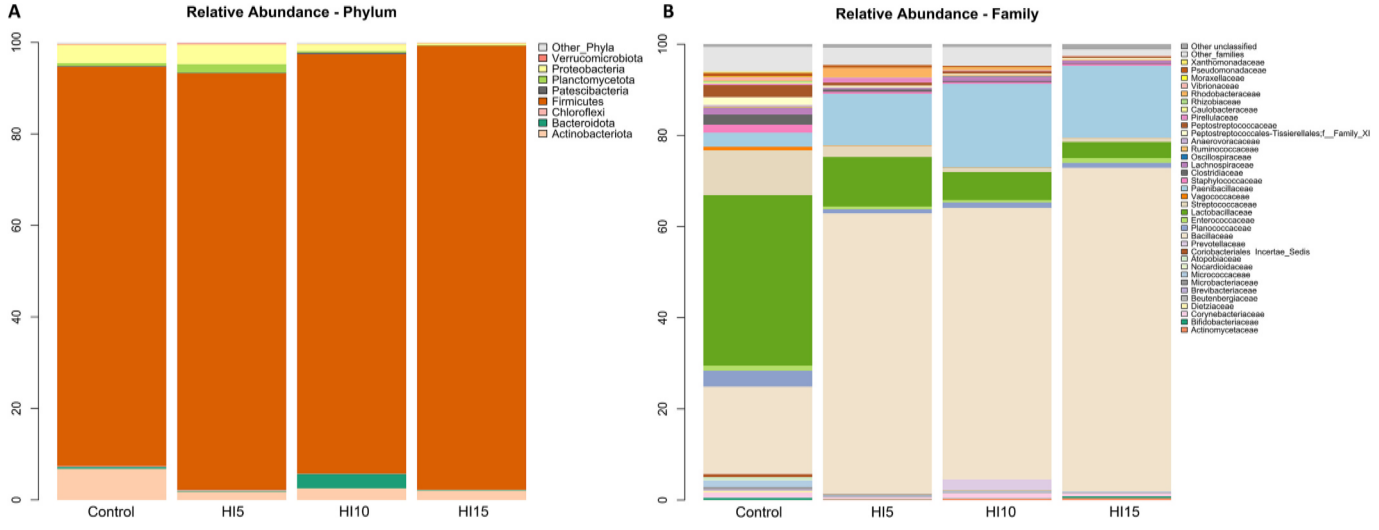


Fig. 3. Microbiota composition (%) of distal gut content of gilthead sea bream fed increasing HI meal dietary levels. Bar plot summarizing the microbiota composition at phylum (a) and family (b) level of fish feces. Only phyla and families with a relative abundance >0.5% in at least 1 sample are represented.

In general, previous studies on gilthead sea bream showed contradictory results in terms of optimal HI dietary inclusion level to replace FM. In juveniles, Mastoraki et al. (2022) and Fabrikov et al. (2020) reported no significant differences in growth when HI was added up to 19.5% and 10.9%, respectively, while 10% was an optimal level tested at on-growing stage (Bosi et al., 2021). On the contrary, according to Karapanagiotidis et al. (2023), the HI dietary inclusion of 17.4% induces a decrease in growth performances, while Carvalho et al. (2023), reported

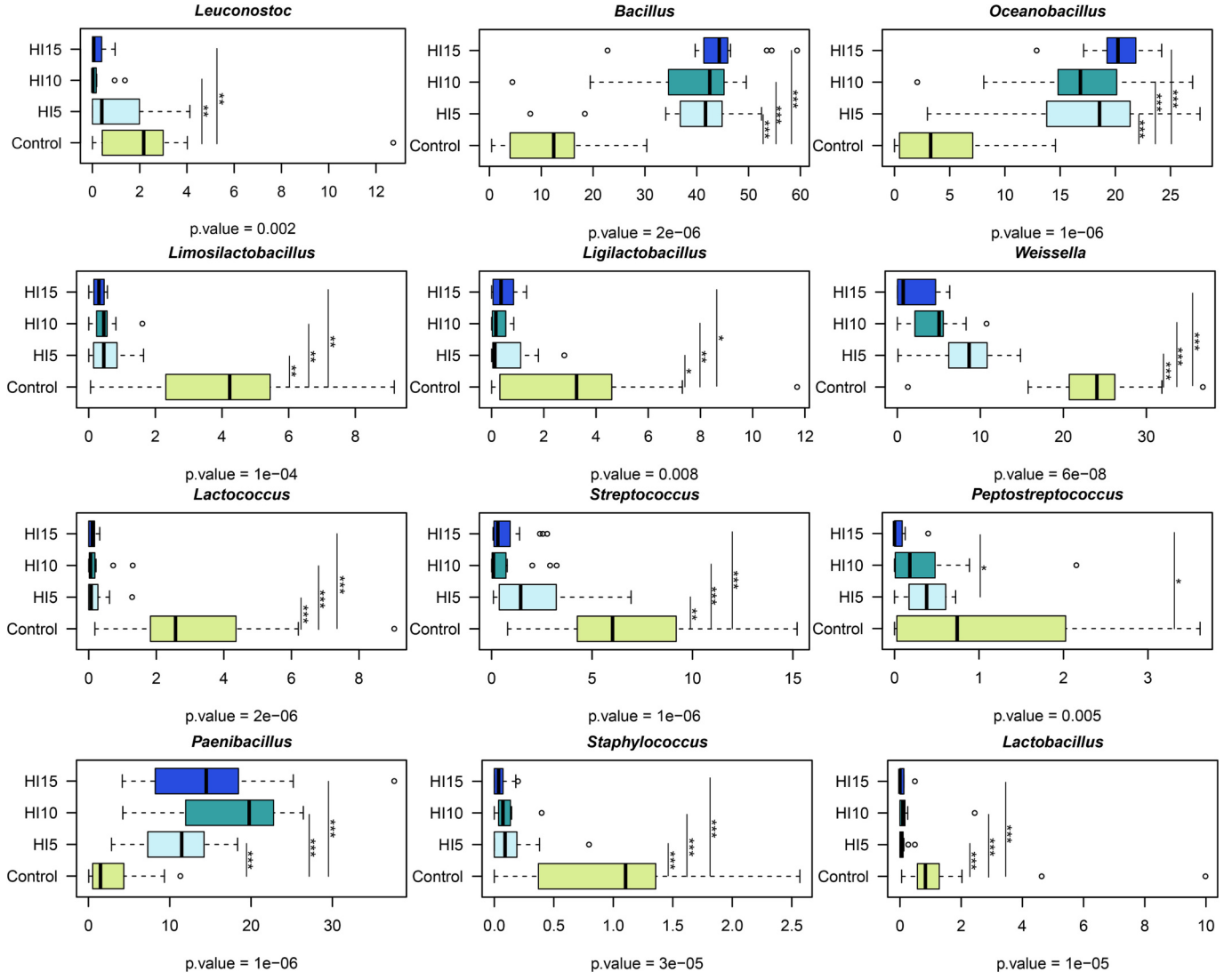


Fig. 4. Taxonomic composition of bacterial communities of distal gut content of gilthead sea bream fed increasing HI meal dietary levels. Distributions of relative abundance of genera that showed a significant variation between groups fed with different diets (Wilcoxon rank-sum test, *** $p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$). Only genera with a mean relative abundance ≥1.0% in at least one group were represented. The central box of each dataset represents the distance between the 25th and the 75th percentiles. The median between them is marked with a black line.

a reduction in final weight with 10% HI dietary inclusion (Table 1). These differences may be explained by the different FM level employed as reported in Table 1. Nevertheless, another possibility could be related to the different level of chitin present in the insect meal adopted. Carvalho et al. (2023) suggested that gilthead sea bream might be a highly-sensitive species to chitin, which may decrease feed digestibility and palatability. Even if in the present study chitin was not measured, this hypothesis was not confirmed according to the nutrient efficiency data here reported. Here, the absence of significant differences in FI, SGR, and FCR states that HI meal can be included up to 15% without affecting growth, feed consumption, and feed utilization when FM is maintained at a minimum of 10%. In addition, the absence of differences in the nutritional indices PER, GPE, and GLE indicates that the inclusion of HI meal did not affect the lipid and protein efficiency. No data were found in the literature regarding GPE and GLE, while PER results are consistent with those reported on other fish species fed with similar HI meal inclusion levels (Guerreiro et al., 2020; Caimi et al., 2021; Carvalho et al., 2023).

Plasma biochemistry analyses have been extensively used to monitor the effects of nutritional status, stress conditions, metabolic disorders, feed ingredients, and rearing conditions (Peres et al., 2013; Guardiola et al., 2018; Bonvini et al., 2018a, 2018b; Parma et al., 2020). In this study, most of the plasma values were similar to that observed in sea bream under optimal nutritional conditions (Peres et al., 2013; Parma et al., 2020; Busti et al., 2020). The results are in line with previous studies' showing that HI dietary inclusion up to 30% did not lead to a significant difference in GLU, TRIG, TP, ALB, Cl, Ca^{+2}, Tot Bil (Dumas et al., 2018; Guerreiro et al., 2020; Mastoraki et al., 2022). Moreover, no reduction in plasma cholesterol attributed to chitosan lowering properties as previously reported in fish (Magalhães et al., 2017; Wang et al., 2019; Khieokhajonkhet et al., 2022), or other animals fed insect meal (Pezzali and Shoveller, 2021; Marschall et al., 2023) was detected.

Interestingly, HI dietary level was associated with a significant linear decrease in the level of plasma AST, ALT, ALP, CK, and LDH. The plasma levels of these non-specific enzymes are generally considered to be indicators of fish health. An increased level outside the normal range for the species under study may indicate tissue damage in organs such as liver, muscles, spleen, and kidney (Peres et al., 2013; Guardiola et al., 2018). Few data are available on these parameters in fish in relation to insect meal dietary inclusion. According to Dumas et al. (2018), HI dietary inclusion did not lead to a significant difference in CK values in rainbow trout, while Khieokhajonkhet et al. (2022) associated a

reduction of AST and ALT when goldfish were fed increasing HI larvae meal. In the present study the concomitant reduction of AST, ALT, ALP and LDH might suggest a potential beneficial role of HI for liver integrity and functionality although further morphological analysis of the liver are needed to corroborate this hypothesis. Infact, recent studies have shown that the chitin or other bioactive molecules present in HI larvae meal were able to attenuates liver steatosis and dyslipidemia in rats (Marschall et al., 2023). In addition, chitosan oligosaccharides showed a significant antioxidant and anti-inflammatory effects able to reduce the levels of serum ALT and AST as well as ameliorate drug-induced liver oxidative damage (Xiang et al., 2021). Also, in poultry fed HI live larvae, the reduction of gamma-glutamyl transferase (GGT) concentration in plasma suggested a beneficial effect on liver health status (Bongiorno et al., 2022).

Recent studies have highlighted the ability of HI diet inclusion to modulate the gut microbiota of fish species of commercial interest. However, as reviewed by Foysal and Gupta (2022) most data are available in rainbow trout and to best of our knowledge no data in gilthead sea bream have been reported. In addition, according to the same authors, studies on fish gut microbiota in relation to HI have been performed only in the last three years and further efforts are necessary to reach conclusive results. In the present study, HI was able to induce a shift in the GM structure at any inclusion level considered compared to the control diet. In particular HI led to a significant enrichment of Bacillaceae (mainly *Bacillus* and *Oceanobacillus*) and Paenibacillaceae (*Paenibacillus*) with a concomitant general reduction of some lactic acid bacteria (LAB). Our data are consistent with a general GM reconfiguration induced by HI dietary inclusion using both FM or vegetable-based diet approach in rainbow trout, (Biasato et al., 2022; Rimoldi et al., 2021; Terova et al., 2019), pikeperch (Tran et al., 2021); and European sea bass (Rangel et al., 2022a). Interestingly, Rangel et al. (2022a) found a sharp increase of *Paenibacillus* abundance from the intestine of European sea bass fed practical diet with 25% HI dietary inclusion level. The authors, after having isolated and characterized this bacteria species, proposed this taxon as an interest novel candidate for probiotic application in aquaculture (Rangel et al., 2022b; Ringø et al., 2022). Infact, beyond its ability to degrade chitin and complex polysaccharides, *Paenibacillus* can produce antimicrobial compounds capable of disrupting bacterial and fungal pathogens (Grady et al., 2016) as well as enhancing the immune status of aquatic animals (Gupta et al., 2016; Amoah et al., 2020). Similarly, the dominance of genus *Bacillus* and *Oceanobacillus* further support the potential beneficial effect of HI in promoting the gut health status of gilthead sea bream. The positive effect of *Bacillus* in the GM of fish species are largely documented and includes positive contribution to nutrition, to the immune system and to disease resistance (Soltani et al., 2019; Marchi et al., 2023). In particular, the presence of *Bacillus* in relation to dietary HI has been related to its ability to ferment chitin leading to short-chain fatty acids (SCFAs) production in rainbow trout and other animals (Biasato et al., 2022; Rimoldi et al., 2021, Borrelli et al., 2021). A general decreasing trend observed in the present study in LAB agreed recent observation found in rainbow trout where a decreased in *Lactobacillus, Lactococcus, Leuconostoc* and *Weissella* were observed. Among LAB, *Weissella*, even with a significant reduction, was able to maintain relevant high abundance especially at 5% HI inclusion. This bacteria species is of relevant interest since is recognized as key GM taxon in healthy gilthead sea bream (Pelusio et al., 2021). Even if further studies on metagenomics and metabolomics are needed to clarify specific GM function, the enrichment of the aforementioned taxa induced by HI are likely to positively contribute to the gut health and fish welfare of gilthead sea bream.

5. Conclusion

In conclusion, the results of feed intake, growth, feed utilization and plasma biochemistry indicate that HI larvae meal can be successfully incorporated up to 15% in practical aquafeed diets to partially replace fish meal (54% of fish meal replacement) without any negative effects on growth and feed efficiency. The reduction of plasma AST, ALT and ALP might indicate a potential beneficial role of HI on liver integrity and functionality. Furthermore, the inclusion of HI at each level tested, determined a positive GM reconfiguration promoting beneficial taxa such as *Paenibacillus* and *Bacillus* potentially able to support chitin digestion and local immunity.

CRediT authorship contribution statement

Serena Busti: Methodology, Investigation, Writing – original draft, Writing – review & editing. **Alessio Bonaldo:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Marco Candela:** Conceptualization, Methodology. **Daniel Scicchitano:** Methodology, Investigation, Writing – original draft, Writing – review & editing. **Giulia Trapella:** Methodology, Investigation, Writing – review & editing. **Fabio Brambilla:** Conceptualization, Investigation, Writing – review & editing. **Côme Guidou:** Conceptualization, Writing – review & editing. **Christophe Trespeuch:** Conceptualization, Writing – review & editing. **Federico Sirri:** Conceptualization, Writing – review & editing. **Francesco Dondi:** Methodology, Investigation, Writing – review & editing. **Pier Paolo Gatta:** Conceptualization, Writing – review & editing. **Luca Parma:** Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

"*Hermetia illucens* larvae meal as an alternative protein source in practical diets for gilthead sea bream (*Sparus aurata*): A study on growth, plasma biochemistry and gut microbiota".

Data availability

Data will be made available on request.

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