Hermetia illucens meal inclusion in low-fishmeal diets for rainbow trout (Oncorhynchus mykiss): Effects on the growth performance, nutrient digestibility coefficients, selected gut health traits, and health status indices

Animal Feed Science and Technology

Dietary Hermetia illucens meal inclusion in commercial diets for rainbow trout (Oncorhynchus mykiss): effects on nutrient digestibility, growth performance, and fish health

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Abstract:	The effects of including Hermetia illucens (HI) meal in diets for rainbow trout have already been widely characterized, but data related to its utilization in commercial diets (especially when gut microbiota is considered) are quite scarce. This study aimed to investigate the impact of HI meal inclusion in commercial diets for rainbow trout by assessing fish growth performance, nutrient digestibility, histomorphological traits of intestine and main organs, and intestinal microbiota. In the 133-days growth trial, 600 rainbow trout were randomly distributed to 4 dietary treatments (3 replicate tanks/diet, 50 fish/tank): a low fishmeal-based diet as control (HI0), and three experimental diets including 80, 160 e 320 g/kg of HI meal as fed as replacement of 25, 50 and 100% of fishmeal (HI25, HI50 and HI100, respectively). At the end of the trial, growth parameters, condition factor and somatic indices were assessed, and gut, stomach, liver and spleen samples (12 fish/diet) were collected for histomorphological analyses. Feed and posterior intestine content were also sampled to characterize the feed and gut microbiota respectively. In the digestibility trial, 216 trout (3 tanks/treatment, 18 fish/tank) were used to evaluate the in vivo apparent digestibility coefficients (ADC) of the same diets. Unaffected growth performance, condition factor, somatic indices, nutrient digestibility, and histomorphological features were observed in the HI-fed rainbow trout ($P > 0.05$). Increasing levels of HI meal inclusion in the feeds determined a progressive increase in the relative abundance of Firmicutes and Actinobacteria

	phyla, and Staphylococcus , Enterococcus , Oceanobacillus and Actinomyces genera, whereas Proteobacteria – as well as Lactobacillus and Listeria – displayed a gradual reduction. Dietary HI meal inclusion increased the Chao1 index of the fish gut microbiota, but, at the same time, reduced the Shannon index ($P < 0.05$). The HI25 and HI50 fish also displayed higher relative abundance of Actinobacteria when compared to the other dietary treatments, as well as decreased Bacteroidetes (False Discovery Rate [FDR] < 0.05). Furthermore, Actinomyces , Bacillus , Enterococcus , Staphylococcus , and Oceanobacillus resulted to be enriched in the posterior gut microbiota of the HI-fed fish (FDR < 0.05). Differently, dietary HI meal inclusion determined a reduction of Campylobacter and Listeria , as well as Clostridium , Lactobacillus , Leuconostoc , Pediococcus , unclassified members (U.m.) of Peptostreptococceae, Weissella , Vagococcus , and Lactococcus . In conclusion, HI meal can be used in commercial diets for rainbow trout up to high inclusion levels (32%) without negatively affecting the growth performance, nutrient digestibility, somatic indices and histomorphological features of the animals. Furthermore, a positive modulation of the gut microbiota in terms of selection of short chain fatty acids (SCFAs)-producing bacteria and reduction of foodborne disease-causing pathogens was herein observed.
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To: Dr. Kumar
Co-Editor
Animal Feed Science and Technology

January 08th, 2022

Dear Dr. Kumar,

I am pleased to submit an original research article entitled "Dietary *Hermetia illucens* meal inclusion in commercial diets for rainbow trout (*Oncorhynchus mykiss*): effects on nutrient digestibility, growth performance, and fish health" for consideration for publication in *Animal Feed Science and Technology*.

In this manuscript, we investigated the effects of insect meal inclusion in commercial diets for rainbow trout. Despite several studies about the impact of insect meal utilization having already been performed in different fish species (rainbow trout included), this manuscript represents the first scientific evidence not only about the insect-related gut microbiota modulation when a commercial diet is administered, but also the first scientific evidence ever related to the characterization of the microbiota of an insect-based feed.

We believe that this manuscript is appropriate for publication by *Animal Feed Science and Technology* because it provides novel and useful information about the role of taurine in rainbow trout.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Thank you for your consideration.

Best regards,

Dr. Francesco Gai

Highlights

- *Hermetia illucens* meal does not alter the growth performance of rainbow trout
- *Hermetia illucens* meal does not affect nutrient digestibility of rainbow trout
- *Hermetia illucens* meal does not impair the health status of rainbow trout
- *Hermetia illucens* meal does positively modulate the gut microbiota of rainbow trout

Author statement

Ilaria Biasato: conduct the experiment, sampling, statistical analysis, and writing the initial draft,

Giulia Chemello: conduct the experiment, sampling, statistical analysis, and writing the initial draft,

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Francesco Gai: planning the research activity and reviewing the final draft,

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Laura Gasco: coordination, funding acquisition, planning the research activity, and reviewing the final draft.

Declaration of Competing Interest

There are no competing financial, professional, or personal interests that might have influenced the presentation of the work described in this manuscript.

Dietary *Hermetia illucens* meal inclusion in commercial diets for rainbow trout (*Oncorhynchus mykiss*): effects on nutrient digestibility, growth performance, and fish health

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Abstract

The effects of including *Hermetia illucens* (HI) meal in diets for rainbow trout have already been widely characterized, but data related to its utilization in commercial diets (especially when gut microbiota is considered) are quite scarce. This study aimed to investigate the impact of HI meal inclusion in commercial diets for rainbow trout by assessing fish growth performance, nutrient digestibility, histomorphological traits of intestine and main organs, and intestinal microbiota. In the 133-days growth trial, 600 rainbow trout were randomly distributed to 4 dietary treatments (3 replicate tanks/diet, 50 fish/tank): a low fishmeal-based diet as control (HI0), and three experimental diets including 80, 160 e 320 g/kg of HI meal as fed as replacement of 25, 50 and 100% of fishmeal (HI25, HI50 and HI100, respectively). At the end of the trial, growth parameters, condition factor and somatic indices were assessed, and gut, stomach, liver and spleen samples (12 fish/diet) were collected for histomorphological analyses. Feed and posterior intestine content were also sampled to characterize the feed and gut microbiota respectively. In the digestibility trial, 216 trout (3 tanks/treatment, 18 fish/tank) were used to evaluate the *in vivo* apparent digestibility coefficients (ADC) of the same diets. Unaffected growth performance, condition factor, somatic indices, nutrient digestibility, and histomorphological features were observed in the HI-fed rainbow trout ($P > 0.05$). Increasing levels of HI meal inclusion in the feeds determined a progressive increase in the relative abundance of Firmicutes and Actinobacteria phyla, and *Staphylococcus*, *Enterococcus*, *Oceanobacillus* and *Actinomyces* genera, whereas Proteobacteria – as well as *Lactobacillus* and *Listeria* – displayed a gradual reduction. Dietary HI meal inclusion increased the Chao1 index of the fish gut microbiota, but, at the same time, reduced the Shannon index ($P < 0.05$). The HI25 and HI50 fish also displayed higher relative abundance of Actinobacteria when compared to the other dietary treatments, as well as decreased Bacteroidetes (False Discovery Rate [FDR] < 0.05). Furthermore, *Actinomyces*, *Bacillus*, *Enterococcus*, *Staphylococcus*, and *Oceanobacillus* resulted to be enriched in the posterior gut microbiota of the HI-fed fish (FDR < 0.05). Differently, dietary HI meal inclusion determined a reduction of *Campylobacter* and *Listeria*, as well as *Clostridium*, *Lactobacillus*,

Leuconostoc, *Pediococcus*, unclassified members (U.m.) of Peptostreptococceae, *Weissella*, *Vagococcus*, and *Lactococcus*. In conclusion, HI meal can be used in commercial diets for rainbow trout up to high inclusion levels (32%) without negatively affecting the growth performance, nutrient digestibility, somatic indices and histomorphological features of the animals. Furthermore, a positive modulation of the gut microbiota in terms of selection of short chain fatty acids (SCFAs)-producing bacteria and reduction of foodborne disease-causing pathogens was herein observed.

Keywords

Black soldier fly, commercial feed, fish, growth performance, gut microbiota, insect meal, nutrient digestibility.

Abbreviations

AA, amino acid; ADF, acid detergent fiber; ADC, apparent digestibility coefficient; CF, coefficient of fatness; CY, carcass yield; CP, crude protein; DM, dry matter; EE, ether extract; FA, fatty acid; FAME, fatty acid methyl esters; FCR, feed conversion ratio; FDR, false discovery rate; FM, fishmeal; HE, Haematoxylin & Eosin; HI0, control diet; HI25, *Hermetia illucens* meal as replacement of 25% of fishmeal; HI50, *Hermetia illucens* meal as replacement of 50% of fishmeal; HI100, *Hermetia illucens* meal as replacement of 100% of fishmeal; HSI, hepatosomatic index; iFBW, individual final body weight; iIBW, individual initial body weight; iWG, individual weight gain; NDF, neutral detergent fiber; OTUs, Operational Taxonomic Units; PER, protein efficiency ratio; SCFAs, short chain fatty acids; SGR, specific growth rate; TFA, total fatty acids; Vh, villus height; VSI, viscerosomatic index.

Introduction

The commercial rearing of insects for feed production represents a market that has grown rapidly in the recent years, being also ready to scale up production (All About Feed, 2020). Recent economic

projections highlighted that the global edible insects market is expected to reach around USD 8 billion and a volume of 730,000 tonnes by 2030, with a CAGR of 24.4% and 27.8%, respectively, during the forecast period from 2019 to 2030 (Meticulous Research®, 2020). Since the use of insect proteins was firstly authorized in aquafeed by the EU (Annexe II of Regulation 2017/893 of the 24th of May, 2017), the aquaculture segment dominated the insect market, with a consumption of more than 50% (around 5,000 tonnes) of the European animal feed produced from insects (IPIFF, 2019). The rapid development of the insect sector is related to the strong ability of insects to transform the nutrients losses (food waste) back into the food chain in forms of protein-rich animal feed, thus allowing them to fully embrace the concept of "circular economy" (Ojha et al., 2020). Among the farmed insect species, the black soldier fly (*Hermetia illucens*, HI) represents the most popular choice for mass production, because of its short life cycle, better feed conversion ratio (FCR), and the efficiency in bioconversion (50–60%) and recovery of nutrients from a wide spectrum of organic materials (Sheppard et al., 1994). This scenario has stimulated the insect producers in the EU to invest more than € 600 million in scaling up their production in 2019, with more than € 2.5 billion being even invested in 2020 (All About Feed, 2020). However, this growth is strictly connected with two important challenges, such as the meet of consumer's expectations (in terms of consumption of safe, nutritious, and high-quality products) and the update of the regulatory framework (as no animal-based foodstuff can be used to feed insects, with the exception of the ones listed in the Reg. (EU) 2021/1372). In order to overcome these barriers (and, accelerate the scale up process), the insect producers need to currently test their products in the experimental setup.

In order to assess if a novel feed ingredient (such as insect-based products) can be suitable for fish feeding, a two-way approach is commonly adopted. First, the nutritional profile of the feed source needs to be fully characterized, as well as the feed acceptance, the growth performance and the nutrient digestibility by the fish (Rawski et al., 2020). Secondly, the implications for animal health must be investigated, with the attention being mainly directed towards the role of the gut. Indeed, the health status of the intestine (in terms of morphological development, mucin production, and

microbiota/microbiome) is fundamental to guarantee a proper health and growth of the fish (Józefiak et al., 2019; Caimi et al., 2020). So far, the administration of fishmeal (FM)-based diets containing high levels of HI meal up to 40% has been reported to not influence (Renna et al., 2017; Cappellozza et al., 2019; Cardinaletti et al., 2019) or worsen (St-Hilarie et al., 2007; Sealey et al., 2011; Dumas et al., 2018) the growth performance of rainbow trout (*Oncorhynchus mykiss*), with some authors also reporting unaffected (Renna et al., 2017) or reduced (Dumas et al., 2018; Cardinaletti et al., 2019) length of the intestinal villi. In parallel, the gut mucin production has been described as unaltered (Elia et al., 2018), while a positive modulation of the intestinal microbiota in terms of increased microbial diversity, selection of potentially beneficial bacteria, and reduction of potential pathogens has been identified in HI-fed fish (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019). However, the potential of using HI-based products in commercial diets – which are low-FM feeds with plant-derived proteins as additional protein sources – has recently started being explored at low inclusion levels only (3-15%; Caimi et al., 2021). Furthermore, no data about gut microbiota modulation in rainbow trout fed HI-based commercial diets are available yet.

Therefore, the present study aims to investigate the effects of including increasing levels of a partially defatted HI meal in commercial diets for rainbow trout as partial or total replacement of FM. In particular, the attention was herein focused on the fish growth performance, nutrient digestibility, and gut health parameters.

Materials and Methods

Two experimental trials (a digestibility and a growth trial, respectively) were conducted at the Experimental Facility of the Department of Agricultural, Forest, and Food Sciences (DISAFA) of the University of Turin (Italy). The experimental protocol was designed according to the guidelines of the European and Italian regulations on the care and use of experimental animals (European directive 86 609/EEC, put into law in Italy with D.L. 116/92). The experimental protocol was approved by the Ethical Committee of the University of Turin (protocol n° 143811).

Experimental diets

Two diets containing FM (206 g/kg as fed; HI0) or a partially defatted HI meal produced in the experimental facility of a Dutch insect producer (Protix BV, Dongen, The Netherlands – 320 g/kg as fed; HI100) in substitution of 100% of FM were formulated by Research Diet Services BV (Utrecht, The Netherlands) and DISAFA. For nutrient digestibility evaluation, 10 g/kg as fed of Diamol (an acid insoluble ash) was added as inert marker. The two diets were formulated to be isonitrogenous, isolipidic, and isoenergetic. After that, two additional experimental diets were prepared by mixing: 1) 750 g/kg as fed of HI0 and 250 g/kg as fed of HI100 (HI25), and 2) 500 g/kg as fed of HI0 and 500 g/kg as fed of HI100 (HI50). The control diet (HI0) was formulated to mimic a commercial diet for rainbow trout, while the four experimental diets included increasing levels of HI meal in substitution of 0% (HI0), 25% (HI25), 50% (HI50) and 100% (HI100) of FM (corresponding to dietary HI meal inclusion levels of 0, 80, 160 and 320 g/kg as fed, respectively). The four diets (shown in Table 1) were prepared as extruded feed by Research Diet Services BV and shipped to the Experimental Facility of DISAFA. The diets were stored at 0-4°C and 85-90% RH in dark room before feeding to the fish.

Chemical analyses of feed

Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and analysed for dry matter (DM; AOAC #934.01), crude protein (CP; AOAC #984.13), acid detergent fiber (ADF; AOAC# 973.18) and ash (AOAC #942.05) contents according to AOAC International (2000). Feed samples were also analysed for ether extract (EE; AOAC #2003.05) content according to AOAC International (2003), while the neutral detergent fiber (NDF) was analysed according to Van Soest et al. (1991); α-amylase (Sigma Aldrich, Saint Louis, MO, USA) was added, but no sodium sulphite, and the results were corrected for the residual ash content. The GE content was determined using an adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany). The fatty acid (FA)

composition of the experimental diets was assessed using the method described by Schmid et al. (2009). Fatty acid methyl esters (FAME) were separated, identified and quantified on the basis of the chromatographic conditions reported by Renna et al. (2014). The results were expressed as g/100 g of DM of total detected fatty acids (TFA). All the chemical analyses of the feeds were performed in duplicate (proximate composition) and triplicate (FA composition). The proximate composition and the FA profile of the experimental diets are shown in Table 1 and 2, respectively. Feed were also sampled for the microbiota assessment (please, see "*DNA extraction and 16S rRNA amplicon target sequencing*" subsection).

Digestibility trial

An *in vivo* digestibility trial was performed in order to determine the apparent digestibility coefficients (ADC) of the diets. A total of two hundred and sixteen trout (purchased from a private fish hatchery ["Troticoltura Bassignana", Cuneo, Italy], with a weight of 160.25 ± 8.24 g) were divided into twelve 250-L cylindroconical tanks (3 replicate tanks/diet, 18 fish/tank) connected to a flow-through open system where artesian well water (constant T of 13 ± 1 °C) was supplied (tank water inflow: 8 L/min), and the dissolved oxygen levels were measured every two weeks (range: 7.6-8.7 mg/L). After 14 days of acclimatization with the experimental diets, the fish were fed by hand to visual satiety twice a day (8:00 am and 3:00 pm). The ADC were measured using the indirect acid-insoluble ash method, with 1% Diamol being used as inert marker. The faeces were collected daily from each tank for four consecutive week, using a continuous automatic device, as described by Chemello et al. (2020). The faeces were frozen (−20 °C) and successively freeze-dried and stored until chemical analyses. The ADC of DM (ADCDM), crude protein (ADCCP), ether extract (ADCEE) and gross energy (ADCGE) were calculated according to Chemello et al. (2020).

Growth trial

A total of six hundred rainbow trout were purchased from a private fish hatchery ("Troticoltura Bassignana", Cuneo, Italy). After a four-week period of acclimation (during which the fish were fed a commercial diet [42% CP and 22% EE; Skretting Italia Spa, Mozzecane, Verona, Italy]), the rainbow trout were submitted to a light anaesthesia (MS-222; PHARMAQ Ltd., Sandleheath, UK; 60 mg/L), individually weighed (112.86 ± 8.41 g) using electronic scales (KERN PLE-N v. 2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany; d: 0.1), and randomly allotted to twelve 300-L, rectangular-shaped tanks (three replicate tanks per diet, fifty fish per tank) connected to the same flow-through open water system of the digestibility trial. The fish were fed 1.4% of the tank biomass for the first 123 days of trial, while the feeding rate was reduced to 1.1% for the remaining 20 days. In particular, the fish were fed by hand, twice a day (08:00 and 15:00) and six days per week. Feed intake was checked at each administration, and feed distribution was immediately interrupted if fish stopped eating. In order to update the daily feeding rate, the biomass tanks were weighed in bulk every 14 days. Mortality was daily checked. The experimental trial lasted 133 days.

Growth performance

At the end of the growth trial, the fish were left unfed for one day, submitted to a light anesthesia (MS-222; PHARMAQ Ltd., Sandleheath, UK; 60 mg/L) and individually weighed (KERN PLE-N v.2.2; KERN and SOHN GmbH, Balingen-Frommern, Germany; d: 0.1). The following performance indices were calculated per each tank:

1. Survival (%) = 100 – [(number of dead fish / number of fish at start) × 100]
2. Individual weight gain (iWG, g) = average individual final body weight (iFBW, g) – average individual initial body weight (iIBW, g)
3. Feed conversion ratio (FCR) = total feed supplied (g, DM) / WG (g)
4. Protein efficiency ratio (PER) = WG (g) / total protein fed (g, DM)
5. Specific growth rate (SGR, % day^{-1}) = [(lnFBW – lnIBW) / number of feeding days] × 100

Condition factor and somatic indices

At the end of the growth trial, twenty-one fish per dietary treatment (seven fish/tank) were killed by over anaesthesia (MS-222; PHARMAQ Ltd., Sandleheath, UK; 500 mg/L). The fish were individually weighted (KERN PLE-N v.2.2; KERN and SOHN GmbH, Balingen-Frommern, Germany; d: 0.1), and fish total length was manually measured to determine the Fulton's condition factor (K). The fish were then dissected in order to calculate the carcass yield (CY). Liver, gut and perivisceral fat were successively weighted (KERN PLE-N 420-3N; KERN and SOHN GmbH, Balingen-Frommern, Germany; d: 0.001) to calculate the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF) as follows:

- $K = [\text{fish weight (g)} / (\text{body length})^3 \text{ (cm)}] \times 100$;
- CY (%) = [total weight without gut and gonad (g) / fish weight (g)] × 100;
- HSI (%) = [liver weight (g) / fish weight (g)] × 100;
- VSI (%) = [gut weight (g) / fish weight (g)] × 100;
- CF (%) = [perivisceral fat weight (g) / fish weight (g)] × 100.

Sampling and processing

At the end of the growth trial, twelve fish per dietary treatment (four fish per tank) were also killed by over anaesthesia (MS-222; PHARMAQ Ltd., Sandleheath, UK; 500 mg/L) and submitted to morphometric and histopathological investigations. Anterior (the tract immediately after the pyloric caeca) and posterior (the tract 1 cm before the anus) gut segment samples (approximately 2 cm in length) were excised, flushed with 0.9% saline to remove all the content, and fixed in 10% buffered formalin solution for histomorphological investigations, as well as liver, spleen and stomach. All the tissues were routinely embedded in paraffin wax blocks, sectioned at 5 μm thickness, mounted on glass slides, and stained with Haematoxylin & Eosin (HE) for morphometric (gut) and histopathological (gut, liver, spleen and stomach) investigations. The posterior intestine content was

also collected into sterile plastic tubes with appropriate squeezing, cooled at 4 °C (for a maximum of 2 hours) and frozen at -80°C until DNA extraction.

Histomorphological investigations

One slide per each intestinal segment was examined by means of light microscopy, and one randomly selected high power field per each slide was captured with a Nikon DS-Fi1 digital camera (Nikon Corporation, Minato, Tokyo, Japan) coupled to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germania) using a 2.5× objective lens. The NIS-Elements F software (Nikon Corporation, Minato, Tokyo, Japan) was then used for image capturing. All the morphometric measurements were performed by Image–Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, Rockville, MD, USA) on 10 well-oriented and intact villi in order to evaluate the villus height (Vh, from the villus tip to submucosa) (Renna et al., 2017). The observed histopathological findings were evaluated in all the organs using the semi-quantitative scoring system previously established (Elia et al., 2018): absent (score = 0), focal to multifocal, mild (score = 1), multifocal, moderate (score = 2), and multifocal to diffuse, severe (score = 3). Gut histopathological findings were separately assessed for mucosa (inflammatory infiltrates) and submucosa (inflammatory infiltrates and Gut-Associated Lymphoid Tissue [GALT] activation) for each segment, according to Biasato et al. (2019). The total score of each gut segment was obtained by adding up the mucosa and submucosa scores. All the slides were blind assessed by two independent observers and the discordant cases were reviewed, using a multi-head microscope, until unanimous consensus was reached.

DNA extraction and 16S rRNA amplicon target sequencing

The total genomic DNA (gDNA) was extracted using the RNeasy Power Microbiome KIT (Qiagen, Milan, Italy), according to the manufacturer's instructions. One µL of RNase (Illumina Inc,, San Diego, CA, USA) was added to digest RNA in the DNA samples with an incubation of 1 h at 37°C. The DNA was then quantified using the NanoDrop and standardized at 5 ng/µL. The gDNA was used

to assess the microbiota composition by the amplification of the V3-V4 region of the 16S rRNA gene (Klindworth et al., 2013). The PCR products were purified according to the Illumina metagenomic standard procedure (Illumina Inc., San Diego, CA, USA). The sequencing was performed by a MiSeq Illumina instrument with V3 chemistry and generated 250 bp paired-end reads, according to the manufacturer's instructions.

Bioinformatics and statistical analysis

The experimental unit was the tank for growth performance and nutrient digestibility, and the fish for somatic indices, histomorphological findings and 16S rRNA sequences.

Paired-end reads were first merged using FLASH software with default parameters (Magoc and Salzberg, 2011). Joint reads were further quality filtered (at Phred < Q20) using QIIME 1.9.0 software (Caporaso et al., 2010) and the pipeline recently described (Biasato et al., 2018). The Operational Taxonomic Units (OTUs) clustering was obtained at 97% of similarity and taxonomy assignment was assessed by Greengenes 16S rRNA gene database v. 2013. The OTUs table was rarefied at the lowest number of sequence and displayed the highest taxonomy resolution. The vegan package of R (Dixon, 2003) was used to calculate the alpha diversity. The diversity indices were further analyzed by pairwise comparisons using Wilcoxon rank sum test to assess differences among the dietary treatments. The OTUs table filtered for relative abundance (>0.2% in at least five samples) was used to perform Anosim statistical test in R environment, and Pairwise Kruskal-Wallis tests allowed to find significant differences in microbial taxa abundance according to the dietary treatment. P-values were adjusted for multiple testing and a false discovery rate (FDR) < 0.05 considered as significant.

The statistical analysis of growth performance, nutrient digestibility, somatic indices and histomorphological findings was performed using IBM SPSS Statistics v, 26,0 (IBM, Armonk, NY, USA). Growth performance, nutrient digestibility and somatic indices data were analysed by one-way ANOVA. The Shapiro–Wilk test was used to check dependent variables for normality. The assumption of equal variances was assessed by Levene's homogeneity of variance test. If such an

assumption did not hold, the Brown-Forsythe statistic was performed to test for the equality of group means instead of the F one. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's test and Tamhane's T2 in the cases of equal variances assumed or not assumed, respectively). The morphometric indices were analysed by fitting a general linear model that allowed the morphometric indices (Vh) to depend on three fixed factors (diet, intestinal segment, and interaction between diet and intestinal segment). The interactions between the levels of the fixed factors were evaluated by pairwise contrasts. Histopathological scores were analysed by Chi-square test. The results obtained from normally distributed data were expressed as mean (growth performance, nutrient digestibility and somatic indices) or least square mean (morphometric indices) and pooled standard error of the mean (SEM), while those obtained from not normally distributed data (histopathological findings) as n (%). P values ≤ 0.05 were considered statistically significant.

Results

Chemical analyses of the feed

The experimental diets were not fully comparable in terms of macronutrients (Table 1). In particular, the HI100 diet showed numerically higher DM and CP, and lower EE when compared to the HI0 diet (+2.21%, +4.86%, and -8.13%, respectively). However, the proximate composition of the HI25 and HI50 diets was overall similar to that of the HI0 (Table 1). As far as the FA profile is concerned (Table 2), the lauric (C12:0), myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1 c9) and linoleic (C18:2 n6) acids were the most represented FA in all the experimental diets. In particular, the lauric, myristic, and palmitic acids increased with increasing HI meal inclusion levels, while the oleic and linoleic acids displayed the opposite trend (Table 2). Subsequently, the total saturated fatty acids (SFA) increased following the increased inclusion of the insect meal, whereas the total monounsaturated and polyunsaturated fatty acids (MUFA and PUFA, respectively) decreased (Table 2). The decrease in the PUFA was determined by the decrease in the arachidonic (C20:4 n6), eicosapentaenoic (EPA, C20:5 n3), docosapentaenoic (DPA, C22:5 n3) and docosahexaenoic (DHA,

C22:6 n3) acids, thus furtherly explaining the progressive reduction in the n3 and n6 FA (and the n6/n3 as well) identified in the experimental diets (Table 2).

Digestibility trial

Dietary HI meal inclusion did not influence the nutrient digestibility of the rainbow trout ($P > 0.05$, Table 3).

Growth trial

Growth performance

Growth performance of the rainbow trout are summarized in Table 4. The fish readily accepted all the experimental diets, with all the supplied feed being consumed and no feed refusals being recorded during the experimental trial. Survival was high for all the dietary treatments (range: 95.33-97.33), being also unaffected by dietary HI meal inclusion ($P > 0.05$, Table 4). Similarly, all the other growth parameters were not affected by insect meal utilization ($P > 0.05$, Table 4).

Condition factor and somatic indices

Dietary HI meal inclusion did not influence either the condition factor or the somatic indices of the rainbow trout ($P > 0.05$, Table 5).

Histomorphological investigations

Data regarding the morphometric measurements of the Vh in the anterior and posterior gut are reported in Table 6. The Vh was not influenced by the diet and the interaction between the diet and the intestinal segment ($P > 0.05$, Table 6), but it only depended on the intestinal segment ($P < 0.001$, Table 6). Independently of the dietary HI meal inclusion, the Vh showed a proximo-distal increasing gradient from the anterior to the posterior gut ($P < 0.001$, Table 6).

The histopathological alterations observed in liver, spleen, stomach, anterior and posterior gut are summarized in Table 7. In liver, absent to mild, focal to multifocal lymphoplasmacytic infiltrates, as well as absent to moderate, multifocal to diffuse fatty changes of the hepatocytes were observed in all the dietary treatments. Mild, focal to multifocal hemosiderosis, along with moderate, focal to multifocal white pulp hyperplasia were also recorded in all the experimental groups. No signs of immune cell infiltration were observed in the stomach, except for the HI100 group (8.3%). All the fish displayed mild, focal to multifocal lymphoplasmacytic infiltrates in both the anterior and the posterior intestine. However, dietary HI meal inclusion did not influence either the severity or the distribution of the observed histopathological alterations ($P > 0.05$, Table 7).

Feed 16S rRNA amplicon target sequencing

The feed samples were overall characterized by a simple microbiota, with Firmicutes, Cyanobacteria and Proteobacteria representing the main bacterial phyla, and *Lactobacillus*, *Listeria*, *Leuconostoc*, *Streptococcus* and *Photobacterium* the most abundant genera (Figure 1). Increasing levels of HI meal inclusion in the feeds determined a progressive increase in the relative abundance of Firmicutes and Actinobacteria phyla, whereas Proteobacteria displayed a gradual reduction (Figure 1A). Furthermore, the relative abundance of *Lactobacillus* and *Listeria* decreased with increasing levels of dietary HI meal inclusion, while *Staphylococcus*, *Enterococcus*, *Oceanobacillus* and *Actinomyces* showed the opposite trend (Figure 1B).

Posterior gut 16S rRNA amplicon target sequencing

After sequencing and quality filtering, 895,348 reads were used for the downstream analysis (with a median value of 18,371 ± 10,395 reads/sample). The rarefaction analysis and the estimated sample coverage indicated that there was a satisfactory coverage of all the samples (ESC median value of 96%). The alpha diversity analysis also revealed a significant increase in the Chao1 index of the posterior gut microbiota from the HI-fed rainbow trout, whereas the Shannon index displayed the

opposite trend ($P < 0.05$, Figure 2). By plotting the Principal Component Analysis (PCA), a clear separation between the fish fed the control and the HI-based diets was also observed, with a higher beta diversity being furtherly identified in the posterior gut microbiota from the HI25 rainbow trout when compared to the HI50 and HI100 groups ($P < 0.001$, Figure 3).

The characterization of the posterior gut microbiota of the rainbow trout overall revealed Firmicutes, Actinobacteria and Proteobacteria as predominant phyla (Figure 4A), while *Staphylococcus*, *Lactobacillus*, *Enterococcus*, *Oceanobacillus*, *Actinomyces*, *Streptococcus* and *Weissella* resulted to be the most abundant genera (Figure 4B). At phylum level (Figure 5), the HI25 and the HI50 fish showed a significant increase in the relative abundance of Actinobacteria in comparison with the HI0 group (FDR < 0.05). On the contrary, Bacteroidetes phylum was significantly less abundant in the rainbow trout fed the HI25 and the HI50 diets when compared to the HI0 one (FDR < 0.05). As far as genus level is concerned (Figure 6), the HI-fed fish showed a significant increase in the relative abundance of *Actinomyces*, *Bacillus*, *Enterococcus*, *Oceanobacillus*, and *Staphylococcus* (FDR < 0.05). Differently, the relative abundance of *Clostridium*, *Campylobacter*, *Listeria*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, unclassified members (U.m.) of Peptostreptococceae, *Vagococcus*, and *Weissella* genera was significantly decreased in the rainbow trout fed the HI-based diets in comparison with the HI0 group. No changes related to the different HI meal inclusion levels were, however, identified for both the phyla and the genera (FDR > 0.05).

Discussion

Digestibility trial

The apparent digestibility of the nutrients and the energy of the HI-based diets was analogous to that recorded for the C diet, as already underlined by previous research (Renna et al., 2017; Caimi et al., 2021). This is indicative of a good, proper nutrient availability, which reasonably explains the unaffected growth performance highlighted in the HI-fed rainbow trout.

Growth trial

Growth performance

The growth performance of the rainbow trout of the present study were not affected by dietary HI meal inclusion, as already underlined by previous research (Renna et al., 2017; Cappellozza et al., 2019; Cardinaletti et al., 2019; Caimi et al., 2021). This represents a positive finding, since increasing levels of HI larva (26.4% [Dumas et al., 2018]) and prepupa (29.8% [St-Hilarie et al., 2007] or 32.80% [Sealey et al., 2011]) meals in the diets for rainbow trout have also been reported to worsen either the weight gain (St-Hilarie et al., 2007; Sealey et al., 2011) or the feed efficiency (St-Hilarie et al., 2007; Dumas et al., 2018) of the fish. Those different outcomes could be attributed to a potential, reduced nutrient availability of the HI diets (Sealey et al., 2011), which, in turn, is partially related to the use of full-fat HI meals (St-Hilarie et al., 2007; Sealey et al., 2011). Indeed, lower lipid (St-Hilarie et al., 2007; Sealey et al., 2011), GE (St-Hilarie et al., 2007), and CP (Dumas et al., 2018) contents were identified in the whole body (St-Hilarie et al., 2007; Dumas et al., 2018) and the muscle (Sealey et al., 2011) of rainbow trout fed the HI-based diets than the control. Despite no whole-body composition analysis having been performed in the current research, the unaffected nutrient digestibility herein observed in the HI-fed fish reasonably suggests no alterations in the nutrient availability as well. Apart from the nutritional composition, the quality of the HI meal in terms of rearing substrates on which the HI larvae were reared may exert a significant influence as well. Indeed, the use of manure from swine (St-Hilarie et al., 2007) and dairy cows (Sealey et al., 2011) may not represent an optimal rearing substrate when compared to the vegetable waste (Cappellozza et al., 2019; Cardinaletti et al., 2019; Caimi et al., 2021).

Condition factor and somatic indices

Both the condition factor and the somatic indices of the rainbow trout of the present study were not significantly influenced by dietary HI meal inclusion. This is in agreement with previous research studies assessing the effects of HI meal utilization in rainbow trout, which also reported analogous K

(Renna et al., 2017; Cardinaletti et al., 2019), HSI (Sealey et al., 2011) and VSI (Bruni et al., 2018) values. All the dietary treatments showed K values higher than 1, thus implying that fish are in good physiological state of well-being and, in turn, that dietary HI meal inclusion does not alter the condition, fatness, or wellbeing of fish (Muddasir and Imtiaz, 2016; Renna et al., 2017). The unaffected HSI and VSI are indicative of the absence of significant diseases in either the liver or the gastrointestinal tract of the HI-fed rainbow trout, as altered values of HSI have previously been ascribed to metabolic problems or liver deficiencies (Dernekbaşi, 2012), and no HI-related hepatic or gastrointestinal histopathological alterations were herein identified. The unaltered values of CF in the fish fed the HI-based diets is also indicative of a proper nutrient availability (Sealey et al., 2011).

Histomorphological features

Dietary HI meal inclusion did not significantly affect the gut morphology of the rainbow trout of the current research, as already reported by Renna et al. (2017). This is in agreement with the unaffected growth performance herein observed in the HI-fed fish, thus suggesting no negative repercussions on either the digestion or the absorption of the nutrients by the intestine. A shortening of the gut villi (Dumas et al., 2018) and fold (Cardinaletti et al., 2019) has also previously been reported in rainbow trout fed diets containing HI meal, with the growth performance of the fish being, however, impaired with the highest inclusion level only (26.4% [Dumas et al., 2018]). Independently of HI utilization, the posterior intestine of the rainbow trout of the present study showed longer villi than the anterior segment. This appears to be in contrast with Khojasteh et al. (2009), which reported progressively shorter villi toward the posterior intestine. However, the simultaneous presence of short and long villi in both the gut segments – as well as the villi length changes throughout the fish cycle – has recently been reported in rainbow trout (Verdile et al., 2020), thus making further investigations needed.

The histopathological alterations observed in the fish of the current research were also not significantly influenced by HI meal utilization, thus suggesting no negative effects of HI on fish health. Elia et al. (2018) previously described similar findings in the liver, spleen and anterior

intestine of rainbow trout, with no HI-related alterations being analogously identified. The fatty and inflammatory changes in liver and gastrointestinal tract, respectively, are the common result of the high-energy diet administered to salmonids, while the spleen reactivity appears to be aspecific. Furthermore, the histopathological alterations were identified in both the control- and the HI-fed fish, also resulting to be predominantly mild to moderate.

Feed and gut microbiota

Firmicutes, Cyanobacteria and Proteobacteria phyla dominated the microbiota of the feed used in the present study. This is partially in agreement with Terova et al. (2019), which identified a predominance of Firmicutes, Proteobacteria and Actinobacteria in FM-based diets for rainbow trout. However, the detection of high percentages of Cyanobacteria represents an unexpected finding. Cyanobacteria has recently been found in the gut microbiota of marine (Salas-Leiva et al., 2020) and freshwater (Jiang et al., 2020; Zeng et al., 2020) species, being also one of the most abundant prokaryotes in sea (Korlević et al., 2016; Quéméneur et al., 2020) and anthropogenic-induced eutrophied freshwaters (Zhang et al., 2021). Considering that the biomass which supplies the FM industry is mainly composed of small pelagic species (Péron et al., 2010), it seems reasonable that the feed microbiota herein characterized reflect the gut microbiota of the fish species (and their rearing environment as well) used to produce the FM. The identification of high relative abundances of Firmicutes and Proteobacteria – which are two of the dominant bacterial phyla of the fish gut microbiota (Butt and Volkoff, 2019) – further supports such hypothesis. A similar consideration can also be made for the most represented bacterial genera detected in feed microbiota, as *Lactobacillus* (Tarnecki et al., 2017; Huyben et al., 2020; Yu et al., 2021), *Leuconostoc*, *Streptococcus* (Tarnecki et al., 2017) and *Photobacterium* (Huyben et al., 2020) constitute the core microbiota of several marine species, with the latter OTU being particularly characteristic of piscivores such the pelagic species (Huang et al., 2020). Differently, the detection of high percentages of *Listeria* may rise worrying concerns in terms of food safety, as some species (especially *L. monocytogenes*) are

involved in foodborne outbreaks of listeriosis (Buchanan et al., 2017). Since the consumption of raw and smoked seafood is one of the most common predisposing factor to develop such disease and *Listeria* has frequently been isolated in marine finfish (Basha et al., 2019), the fish species herein used to produce the FM could have potentially carried *Listeria* to the feeds.

The HI-based diets used in the current research were characterized by a progressive increase in the relative abundance of Firmicutes and Actinobacteria phyla, whereas Proteobacteria displayed a gradual reduction. This is in agreement with Terova et al. (2019), which described the same scenario in feeds containing increasing levels of HI prepupa meal as FM replacement. This represents the logical consequence of substituting the FM (which is obtained by carnivorous fish) with the insect meal (which is obtained by larvae reared on vegetable substrates). Indeed, plant ingredients in the diet are commonly associated with a higher Firmicutes:Proteobacteria ratio when compared to animal protein-based diet, which, on the contrary, stimulates the proliferation of Proteobacteria (Rimoldi et al., 2018). A clear increase in the relative abundance of *Staphylococcus*, *Enterococcus*, *Oceanobacillus* and *Actinomyces* was also identified in the HI-based diets, thus partially agreeing with the findings reported by Terova et al. (2019). The detection of increasing percentages of *Oceanobacillus* represents, however, a novel, difficult-to-explain result, as this taxon has been reported to dominate the gut microbiota of healthy shrimp, crab and clam (Sun et al., 2019). Furthermore, despite Rimoldi et al. (2021) having recently discovered *Oceanobacillus* in HI-based feed only, its relationship with insects remains to be fully elucidated. High amounts of *Lactobacillus* in diets containing HI meal are also common (Terova et al., 2019; Rimoldi et al., 2021), while the HI-based feeds used in the present study displayed a progressive reduction of this genus. This finding – as well as the decrease of *Listeria* – is reasonably related to the FM replacement by insect meal, as these OTUs are herein hypothesized to depend on the fish species used to produce the FM.

Dietary HI meal inclusion increased the gut microbial richness in the fish of the current research, but, at the same time, reduced its diversity. This partially contrasts with the majority of the previous findings in rainbow trout, which identified unaffected or higher Chao1 and Shannon indices in the

HI-fed fish when compared to those fed the control diet (Bruni et al., 2018; Huyben et al., 2019; Rimoldi et al., 2019; Terova et al., 2019; Rimoldi et al., 2021). This represents a challenging scenario, as reduced bacterial diversity may determine less competition for incoming pathogens, thus favouring their colonization of the gastrointestinal tract of fish and the development of several diseases frequently related to several diseases (Terova et al., 2019). However, the rainbow trout fed the HI-based diets of the present study remained healthy throughout the experimental trial, also showing no significant histopathological alterations.

Firmicutes, Actinobacteria and Proteobacteria represented the dominant bacterial phyla in both the control- and HI-fed fish of the current research. These findings overall agree with the previous research carried out in rainbow trout (Desai et al., 2012; Wong et al., 2013; Ingerslev et al., 2014; Bruni et al., 2018; Rimoldi et al., 2018; Huyben et al., 2019; Terova et al., 2019; Pelusio et al., 2020). In relation to the genera composition, *Staphylococcus*, *Lactobacillus* and *Streptococcus* mainly colonized the posterior gut microbiota of the fish fed either the control or the HI-based diets in the present study. These findings are also in agreement with the previous studies, which observed *Lactobacillus* (Wong et al., 2013; Ingerslev et al., 2014; Rimoldi et al., 2018; Huyben et al., 2019; Terova et al., 2019; Pelusio et al., 2020), *Streptococcus* (Ingerslev et al., 2014; Rimoldi et al., 2018; Pelusio et al., 2020) and *Staphylococcus* (Bruni et al., 2018; Terova et al., 2019) as main bacterial genera in the cecal microbiota of rainbow trout.

In the current research, the utilization of HI meal at 25% and 50% inclusion levels determined higher relative abundance of Actinobacteria phylum in the fish posterior gut microbiota when compared to the HI0 group. A significant increase in Actinobacteria has also previously been reported in HI-fed rainbow trout (Huyben et al., 2019; Terova et al., 2019), as well as the increment in Firmicutes (Bruni et al., 2018; Huyben et al., 2019; Terova et al., 2019) and the reduction of Proteobacteria (Huyben et al., 2019; Terova et al., 2019). On one hand, the increase in Actinobacteria herein observed partially reflects the high relative abundance of this bacterial phylum detected in the HI-based diets; on the other, some genera belonging to Actinobacteria (such as *Actinomyces*) are often identified as chitin

degraders (Beier and Bertilsson, 2013), thus partially explaining its high abundance in the HI-fed rainbow trout. Despite Firmicutes and Proteobacteria percentages being similar among the experimental treatments, the HI25 and the HI50 fish of the present study also displayed lower relative abundance of Bacteroidetes in their posterior gut microbiota in comparison with the HI0 group. Bacteroidetes members are well-known to be involved in the fermentation of dietary non-starch polysaccharides (NSP; den Besten et al., 2013). Since the HI-based diets were characterized by a progressive reduction of wheat meal content (which has considerable quantity of NSP), the decrease in Bacteroidetes may represent a reasonable consequence. Chitin is another NSP, but the chitinolytic bacteria mainly belong to Firmicutes (Cody, 1989) and Actinobacteria (Beier and Bertilsson, 2013) phyla, thus furtherly explaining the reduction of Bacteroidetes herein observed.

Actinomyces, *Bacillus*, *Enterococcus*, *Oceanobacillus*, and *Staphylococcus* resulted to be enriched in the posterior gut microbiota of the HI-fed rainbow trout of the current research. On the one hand, this partially reflects the microbiota of the HI-based feeds (characterized by high percentages of *Actinomyces*, *Enterococcus*, *Oceanobacillus*, and *Staphylococcus*); on the other, these changes can be attributable to chitin. Indeed, apart from the already mentioned chitin degrading activity of *Actinomyces* (Beier and Bertilsson, 2013), many *Bacillus* species are chitinolytic (Cody, 1989). As lactic acid bacteria (LAB), *Enterococcus* is also capable of using chitin as prebiotic (Terova et al., 2019), while novel chitinolytic *Staphylococcus* species have recently been characterized (Gürkök and Görmez, 2016). In agreement with the findings herein observed, a significant increase in *Actinomyces*, *Enterococcus* (Terova et al., 2019), *Staphylococcus* (Bruni et al., 2018) and *Bacillus* (Rimoldi et al., 2021) has also been reported in rainbow trout fed diets containing HI meal. These changes can be beneficial for the health status of the fish gut, as bacterial fermentation of chitin leads to short-chain fatty acids (SCFAs) production (Borrelli et al., 2017; Yu et al., 2019). Indeed, SCFAs (such as butyric, propionic and acetic acids) act as energy source, promote the proliferation of intestinal epithelial cells, exert the antimicrobial activity by lowering intestinal pH, modulate the composition of intestinal microbiota, and enhance the immune response of the fish (Li et al., 2019). In the present study, dietary

HI meal inclusion also determined a significant reduction of *Clostridium*, *Campylobacter*, *Listeria*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, unclassified members (U.m.) of Peptostreptococceae, *Vagococcus*, and *Weissella* in the fish gut microbiota. The decrease in LAB such as *Lactobacillus*, *Leuconostoc* and *Pediococcus* – which have been reported to proliferate in HI-fed rainbow trout (Huyben et al., 2019; Terova et al., 2019; Rimoldi et al., 2021) – seems difficult to explain, especially because *Enterococcus* (previously described as LAB) was, however, significantly enriched. This discrepancy may be caused by the different HI meal adopted (prepupae [Terova et al., 2019] vs larvae), but the capability of insects to stimulate the growth of some LAB at the expense of others deserves future investigations. The reduction of *Clostridium* could not represent a relevant finding, since this taxon is characteristic of the intestinal microbiota from endotherms (Eckburg et al., 2005) and is involved in the degradation of the cellulolytic fibers (which are not predominant in diets for carnivorous fish) (Chapagain et al., 2019). A similar consideration can also be made for Peptostreptococcaceae family, whose members exert the generic function of utilizing proteinaceous substrates and carbohydrates (Fu et al., 2019). On the contrary, the decrease in *Weissella* may represent a potential challenging outcome, as this genus includes probiotic bacteria (Kühlwein et al., 2013) and displays antimicrobial activity against a wide range of microorganisms (Patterson et al., 2010). However, such reduction could have successfully been compensated by the chitin and the lauric acid contained in the HI meal, which have been reported to exert antimicrobial activity against both the Gram-negative (Marono et al., 2017) and the Gram-positive (Skrivanova et al., 2006) bacteria. As a reasonable consequence, the HI antimicrobial properties may have determined the decrease in *Lactococcus*, *Vagococcus*, *Campylobacter* and *Listeria*. Indeed, the reduction of *Lactococcus* and *Vagococcus* – whose distinct species have been related to the development of a growing number of diseases (Ringø and Gatesoupe, 1998) – can be considered a positive finding, but the most remarkable HI-related outcome is represented by the decreased proliferation of *Campylobacter* and *Listeria*. Similarly, to what was already pointed out for *Listeria*, *Campylobacter* is one of the most common agents of food-borne diseases (Kreling et al., 2020), thus making their

reduction particularly interesting within a food safety scenario. The reduced percentage of *Listeria* identified in the HI-based diets could also partially explain its reduction in the gut, but the difference in the corresponding relative abundances (about 7% vs 0.4%) reasonably suggests an active role of HI meal as well.

Conclusions

In conclusion, HI meal can be used in commercial diets for rainbow trout up to high inclusion levels (320 g/kg as fed) without negatively affecting the growth performance, nutrient digestibility, somatic indices and histomorphological features of the animals. Therefore, considering that the low FM-diets are nowadays the most adopted fish feeds from a sustainability perspective, the possibility of including either low or high inclusion levels of HI meal without incurring in adverse outcomes represents a promising scenario. Furthermore, a positive modulation of the gut microbiota in terms of selection of SCFAs-producing bacteria and reduction of foodborne disease-causing pathogens was herein observed for the first time when rainbow trout were administered with low FM-diets containing HI meal. In the light of such positive findings, future investigations also assessing the gut metagenome and metabolome are mandatory in order to fully characterize the HI way of action in the fish gut.

Author statement

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Declaration of Competing Interest

There are no competing financial, professional, or personal interests that might have influenced the presentation of the work described in this manuscript.

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Table 1. Feed ingredients and proximate composition of the experimental diets.

	HI meal	HI0	HI25	HI50	HI100
Ingredients, g/kg as fed					
Fish meal		206	154.50	103	0
Soybean protein concentrate		150	150	150	150
Wheat gluten meal		100	100	100	100
Corn gluten meal		70	70	70	70
Soybean meal		40	40	40	40
Wheat meal		240.50	218.23	195.95	151.40
HI meal		0	80	160	320
Fish oil		50	50	50	50
Soybean oil		123.50	111.38	99.25	75
Vit. min. premix (1%)		10	10	10	10
DL methionine		0	0.28	0.55	1.10
L-lysine HCL		0	0.60	1.20	2.40
Diamol		10	10	10	10
Lime fine		0	1.62	3.25	6.50
Monocalcium phosphate		0	2	4	8
Salt		0	1.63	3.25	5
Magnesium oxide		0	0.15	0.30	0.60
Proximate composition[a]					
DM, g/100g	96.24	93.93	94.81	94.41	96.01
CP, g/100g DM[b]	51.71	43.75	44.01	44.84	45.88
EE, g/100g DM	20.43	19.43	19.23	18.04	17.85
Ash, g/100g DM	5.65	6.85	6.65	6.47	6.71
NDF, g/100g DM	N.A.	20.89	25.08	8.26	9.91
ADF, g/100g DM	N.A.	1.63	2.21	3.08	3.88
NFE, g/100g DM[c]	N.A.	23.90	24.92	25.06	25.58
GE, MJ/Kg[c]	22.04	22.23	22.33	22.08	22.47

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50% of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal; DM, dry matter; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; ADFn, acid detergent fiber nitrogen; NDF, neutral detergent fiber; NFE, nitrogen-free extract. [a]Values are reported as mean of duplicate analyses; [b]Conversion factors of 5.62 for the HI meal (Janssen et al., 2017) and 6.25 for the experimental diets; [c]Calculated as 100 – [(100 – DM) + CP + EE + Ash); [d]Determined by calorimetric bomb.

Table 2. Fatty acid (FA) composition of the experimental diets.

	HI0	HI25	HI50	HI100
Fatty acids, g/100 g DM of TFA				
C10:0	0.00	5.90	12.32	14.87
C12:0	7.54	363.27	756.46	1298.35
C14:0	198.88	273.55	352.25	468.21
C15:0 iso	7.37	6.87	6.75	5.61
C15:0 anteiso	9.15	8.47	10.01	8.36
C14:1 c + C15:0	24.06	25.71	26.49	27.19
C16:0 iso	4.41	4.66	4.21	4.07
C16:0	1480.80	1570.42	1627.03	1663.50
C17:0 iso	20.61	19.82	19.09	15.73
C17:0 anteiso	19.35	17.37	20.76	18.41
C16:1 c	226.49	244.14	269.18	289.74
C17:1 c9	19.94	20.02	20.43	19.80
C18:0	486.70	491.09	476.63	443.73
C18:1 t	35.64	34.58	30.05	27.40
C18:1 c9	5186.36	5028.82	4851.09	4163.97
C18:1 c11	248.71	238.81	231.19	194.62
C18:1 c12	5.71	4.15	5.62	2.26
C18:1 c14 + t 16	18.77	13.95	15.35	9.80
C18:2 n6	6284.27	6029.43	5726.58	4698.82
C20:0	37.68	41.45	38.80	37.03
C18:3 n6	9.76	8.59	7.73	8.39
C20:1 c9	41.31	40.71	35.08	28.43
C20:1 c11	308.66	303.57	282.93	245.98
C18:3 n3	245.42	259.18	271.83	280.86
C20:2 n6	72.29	69.68	66.22	56.73
C18:4 n3	75.04	75.04	66.59	56.24
C22:0	9.60	10.09	10.44	9.19
C22:1 n9	322.80	290.28	270.81	227.25
C20:3 n6	41.46	39.34	35.67	30.64
C20:4 n6	24.29	22.58	19.59	12.26
C20:5 n3	295.22	279.60	251.54	193.51
C22:5 n3	62.39	60.51	59.32	52.80
C22:6 n3	235.30	192.50	175.99	132.08
Σ SFA	2371.59	2903.64	3417.38	4061.31
Σ MUFA	6414.39	6219.03	6011.73	5209.25
Σ PUFA	7279.99	6971.50	6624.90	5475.28
Σ n3	879.80	831.12	794.35	689.89
Σ n6	6405.91	6144.52	5836.18	4787.65
Σ n6/ Σ n3	7.28	7.39	7.35	6.94
TFA	16065.97	16094.16	16054.02	14745.84

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50% of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal; c, cis; t, trans; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids.

Table 3. Apparent digestibility coefficients of dry matter, protein, ether extract and gross energy of the rainbow trout (n=4)

	HI0	HI25	HI50	HI100	SEM	P-value
ADC DM (%)	84.54	87.50	84.71	84.67	0.67	0.346
ADC CP (%)	95.07	95.85	94.44	94.32	0.25	0.086
ADC EE (%)	98.43	98.73	98.37	98.33	0.08	0.298
ADC GE (%)	92.26	93.10	91.25	90.84	0.41	0.192

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50% of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal; SEM, standard error of the mean; P, probability; ADC, apparent digestibility coefficient; DM, dry matter; CP, crude protein; EE, ether extract; GE, gross energy.

Table 4. Survival and growth performance of the rainbow trout (n = 3).

	HI0	HI25	HI50	HI100	SEM	P-value
Survival (%)	96.00	95.33	97.33	96.67	0.48	0.557
IBW (g)	112.73	113.13	112.70	112.93	0.08	0.142
FBW (g)	467.53	463.60	469.37	474.70	3.33	0.756
iWG (g)	354.87	350.49	356.66	361.77	3.33	0.748
FCR	1.72	1.73	1.77	1.75	0.02	0.856
PER	1.33	1.32	1.26	1.25	0.02	0.299
SGR (% day^{-1})	0.90	0.90	0.89	0.91	0.01	0.762

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50% of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal; SEM, standard error of the mean; P, probability; iIBW, individual initial body weight; iFBW, individual final body weight; iWG, individual weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio.

Table 5. Condition factor and somatic indices of the rainbow trout (n = 21).

	HI0	HI25	HI50	HI100	SEM	p-value
K	1.19	1.12	1.16	1.16	0.02	0.548
CY	89.98	88.72	89.17	89.37	0.26	0.392
HSI	1.12	1.08	1.07	1.08	0.03	0.938
VSI	8.42	8.47	8.16	7.88	0.15	0.488
CF	3.52	3.86	3.62	3.26	0.14	0.465

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50% of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal; SEM, standard error of the mean; P, probability; K, condition factor; CY, carcass yield; HIS, hepatosomatic index; VSI, viscerosomatic index; CF, coefficient of fatness.

Table 6. Intestinal morphometric indices of the rainbow trout (n = 12).

	Diet (D)				Intestinal segment (IS)		SEM		P-value		
	HI0	**HI25**	**HI50**	**HI100**	**Anterior**	**Posterior**	**D**	**IS**	**D**	**IS**	**D x IS**
Vh (mm)	0.87	0.83	0.80	0.79	0.68^{a}	0.96^{b}	0.33	0.02	0.392	<0.001	0.982

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50% of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal; SEM, standard error of the mean; P, probability; Vh, villus height.
Means with different superscript letters (a, b) indicate significant differences.

Table 7. Histopathological alterations of the rainbow trout (n = 12).

	Dietary treatments				
Variables	**HI0**	**HI25**	**HI50**	**HI100**	**P-value**
Liver *n (%)*					
Inflammation					0.110
Absent	12 (100)	12 (100)	9 (75)	10 (83.3)	
Mild	0 (0)	0 (0)	3 (25)	2 (16.7)	
Degeneration					0.088
Absent	0 (0)	1 (8.3)	5 (42)	3 (25)	
Mild	5 (41.7)	7 (58.3)	5 (42)	8 (66.7)	
Moderate	6 (50)	4 (33.4)	2 (16.7)	0 (0)	
Severe	1 (8.3)	0 (0)	0 (0)	1 (8.3)	
Spleen *n (%)*					
White pulp hyperplasia					0.495
Absent	9 (81.8)	10 (90.9)	11 (91.7)	12 (100)	
Mild	2 (18.2)	1 (9.1)	1 (8.3)	0 (0)	
Hemosiderosis					0.347
Absent	3 (27.3)	6 (54.5)	4 (33.3)	7 (58.3)	
Mild	8 (72.7)	5 (45.5)	8 (66.7)	5 (41.7)	
Stomach inflammation *n (%)*					0.395
Absent	12 (100)	12(100)	12 (100)	10 (83.4)	
Mild	0 (0)	0 (0)	0 (0)	1(8.3)	
Anterior gut inflammation *n (%)*					1.00
Absent	9 (75)	9 (75)	9 (75)	9 (75)	
Mild	3 (25)	3 (25)	3 (25)	3 (25)	
Posterior gut inflammation *n (%)*					0.681
Absent	11 (91.7)	9 (75)	9 (75)	10 (83.3)	
Mild	1(8.3)	3 (25)	3 (25)	2 (16.7)	

Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50% of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal; SEM, standard error of the mean; P, probability.

Figure captions

Figure 1. Relative abundance of the main bacterial phyla (**A**) and genera (**B**) in samples of commercial feeds containing low content of fishmeal (HI0), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100).

Figure 2. Bacterial community alpha diversity in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets.

Figure 3. Bacterial community composition (weighted UniFrac beta diversity, PCA plots) in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets.

Figure 4. Relative abundance of the main bacterial phyla (**A**) and genera (**B**) in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets.

Figure 5. Relative abundance at phylum level of differentially abundant OTUs in in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia*

illucens meal as replacement of 100% of fishmeal (HI100) diets. Pairwise Kruskal-Wallis test, FDR < 0.05.

Figure 6. Relative abundance at genus level of differentially abundant OTUs in in posterior gut samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia illucens* meal as replacement of 100% of fishmeal (HI100) diets. Pairwise Kruskal-Wallis test, FDR < 0.05.

Figure 1A

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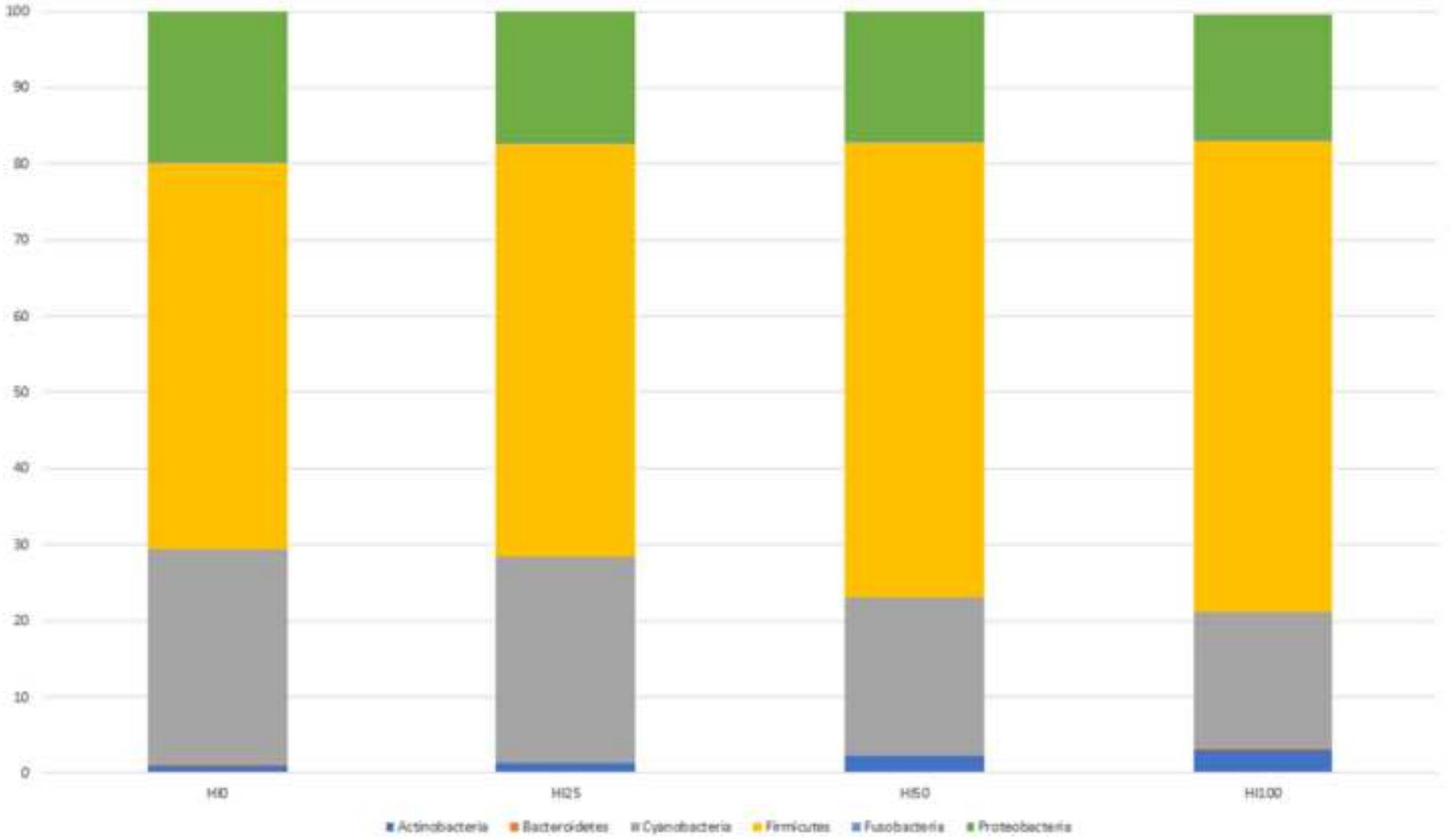

Figure 1B

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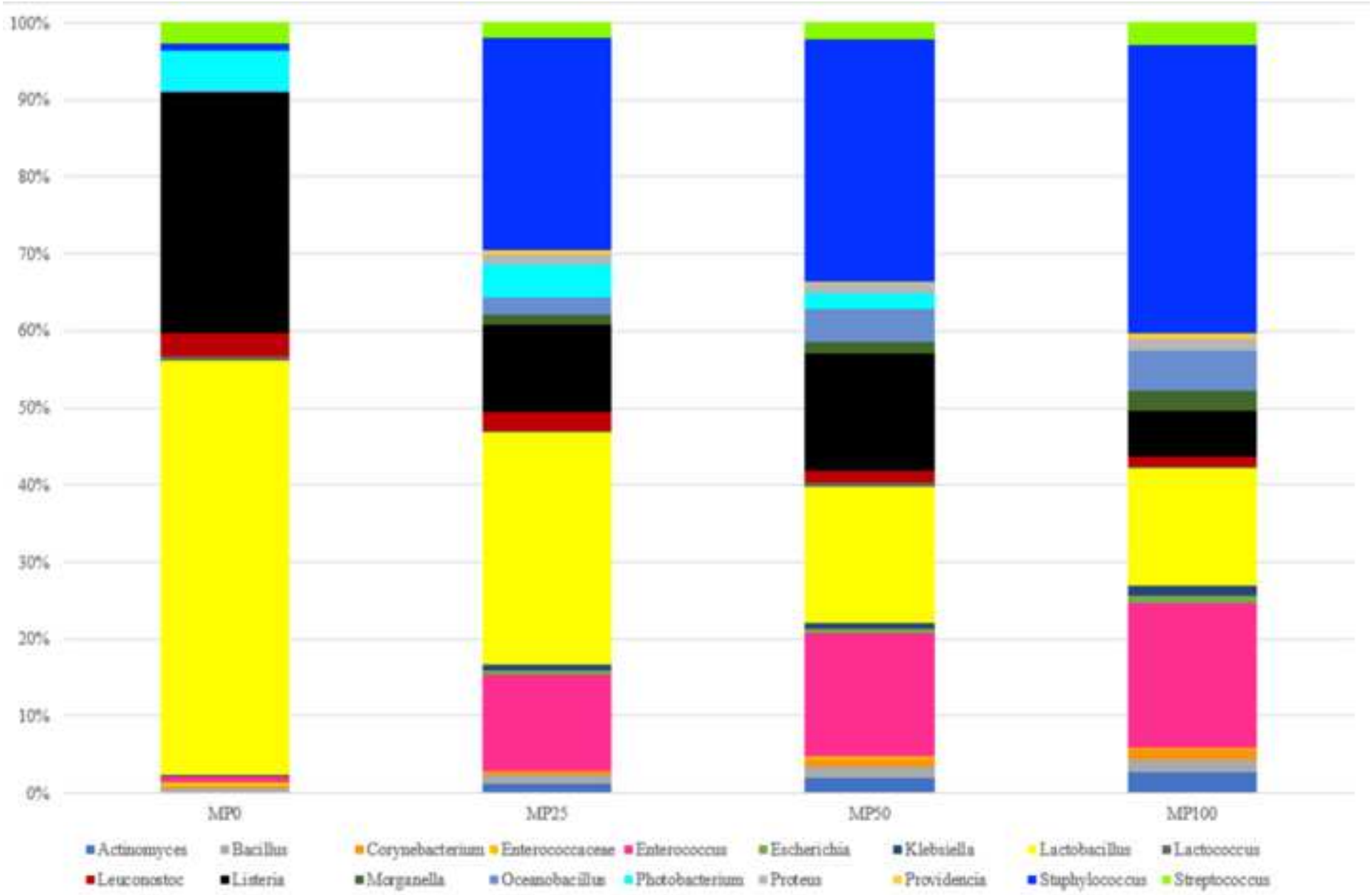

Figure 2

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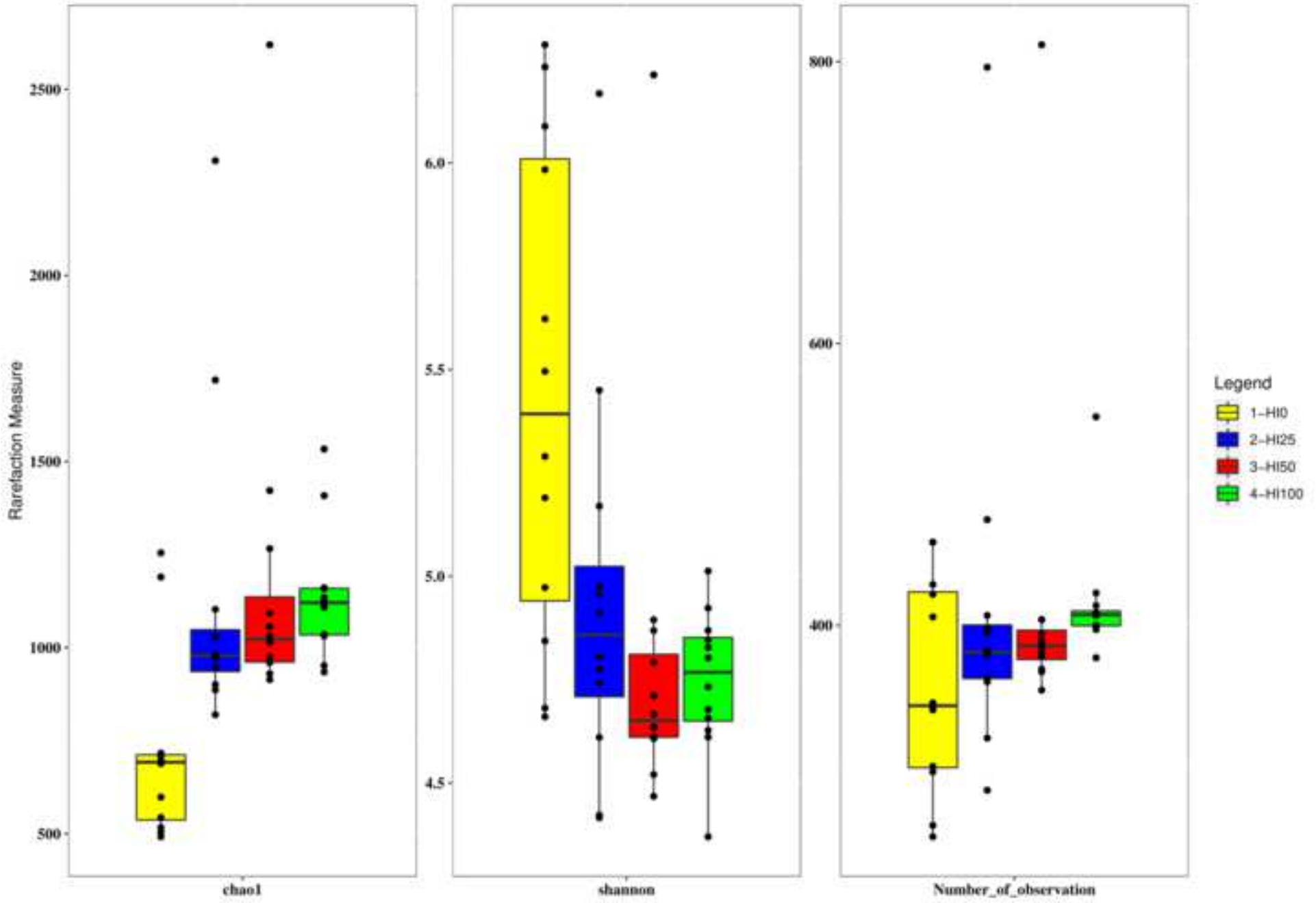

Figure 3

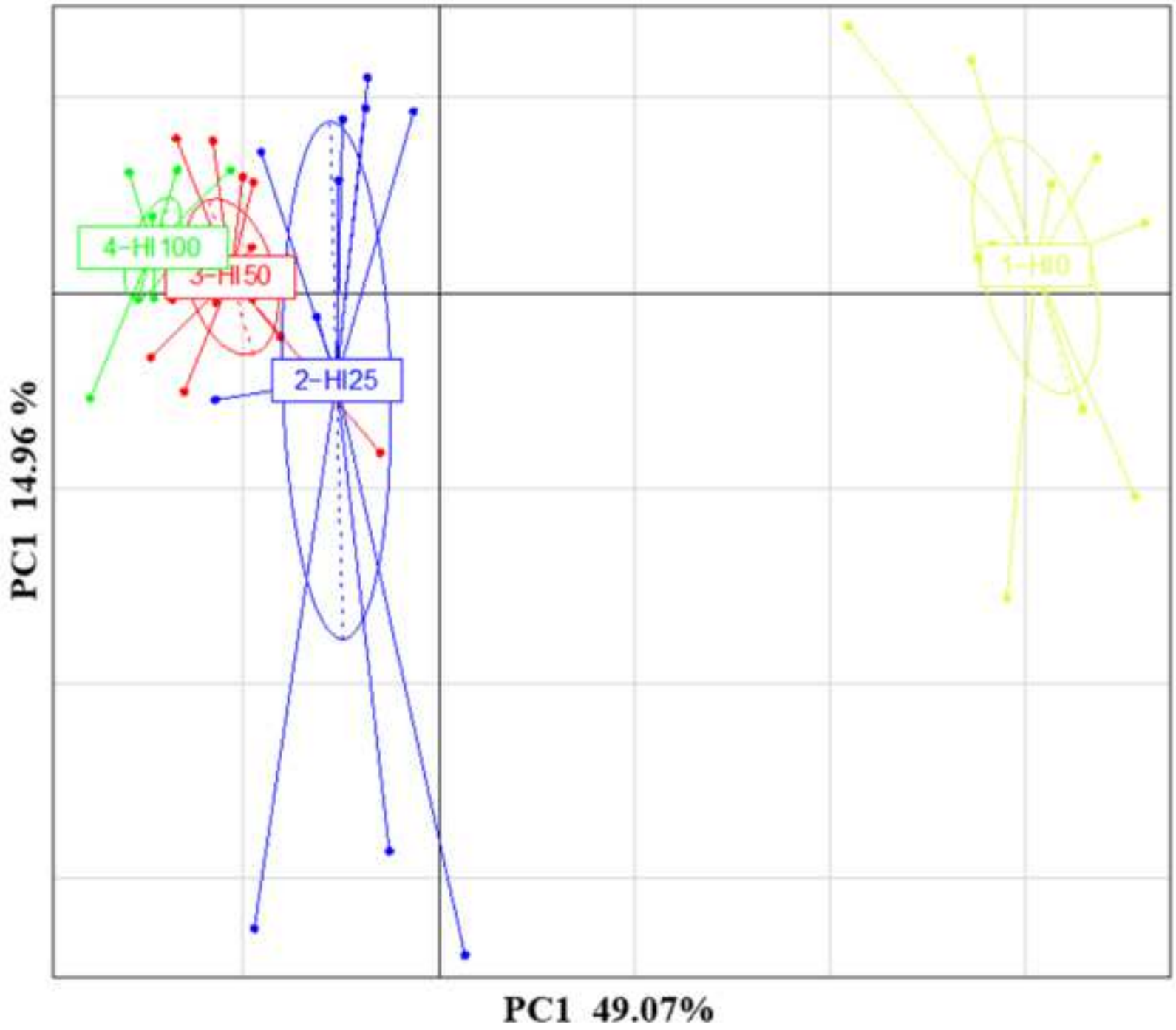

Figure 4A

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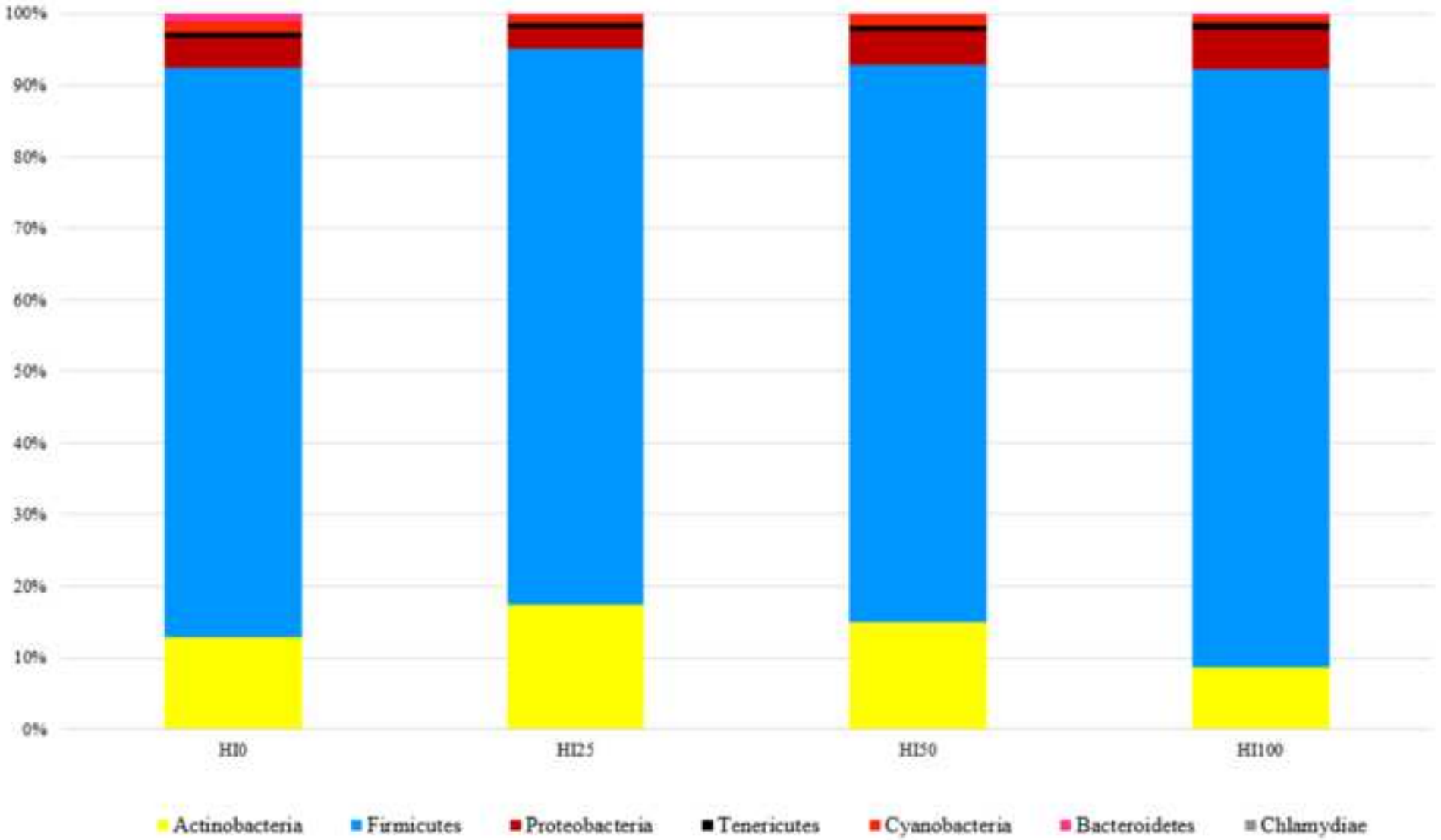

Figure 4B

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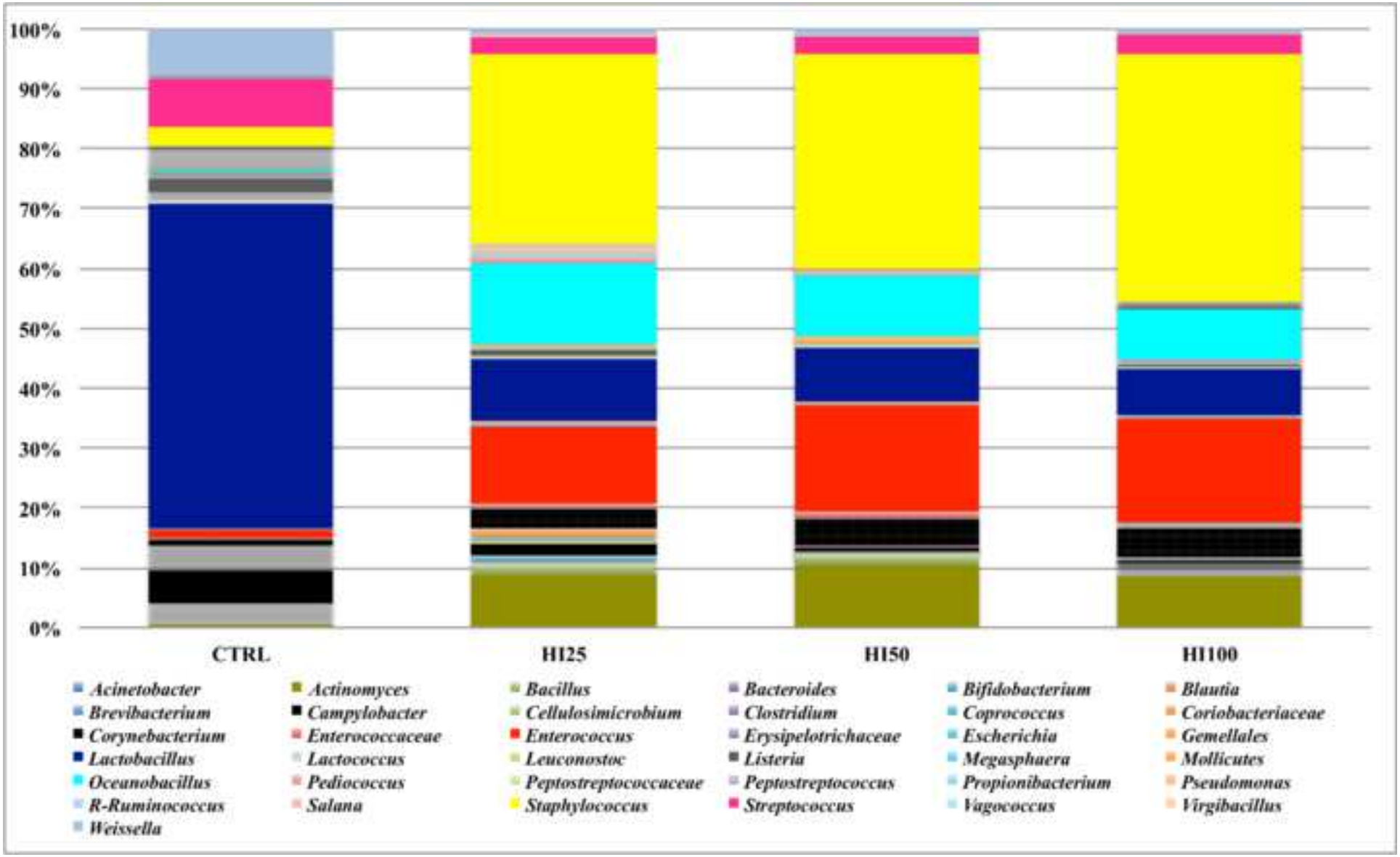

Figure 5

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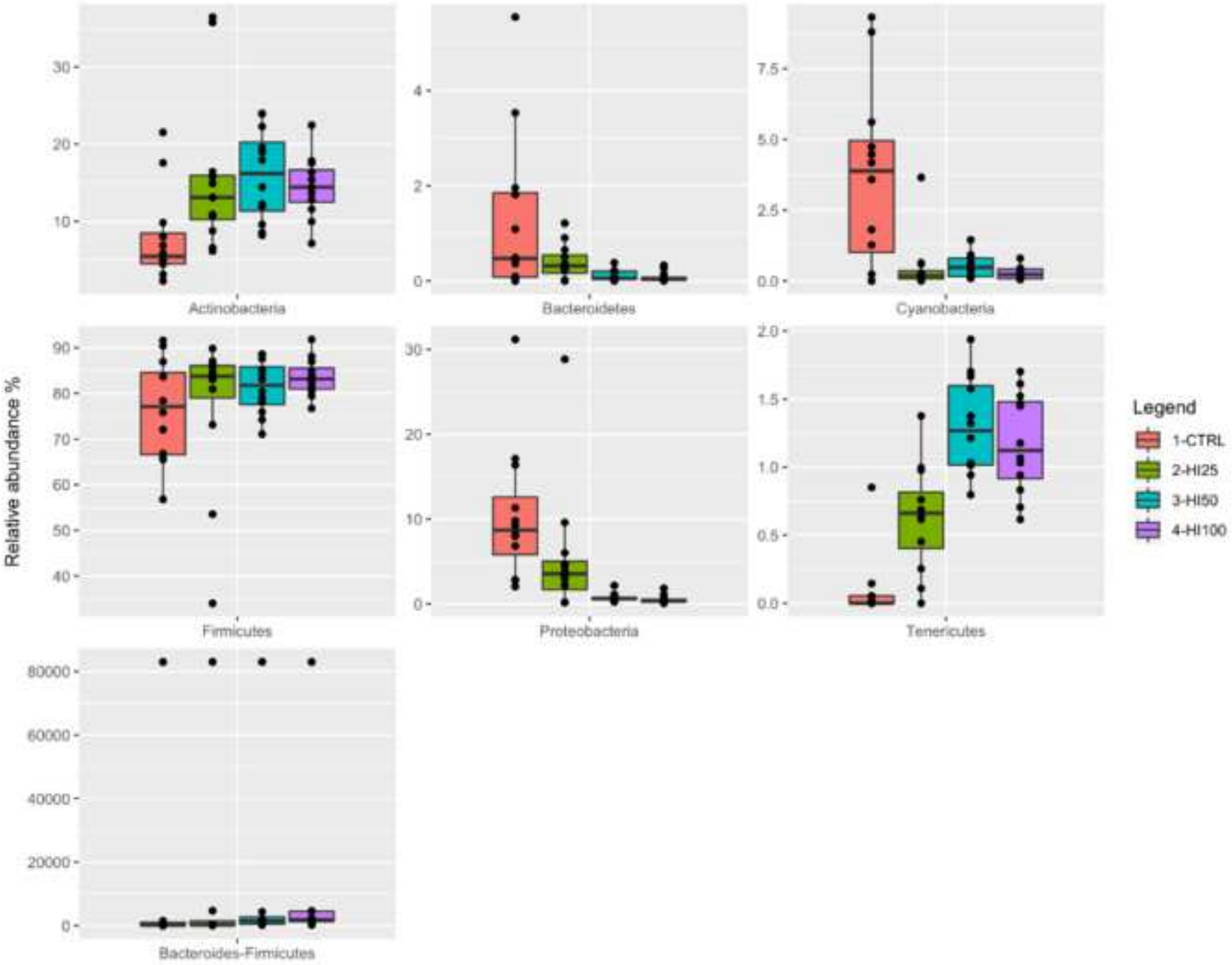

Figure 6

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