Animal Feed Science and Technology

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

--Manuscript Draft--

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Abstract:	The effects of including Hermetia illucens (HI) meal in rainbow trout diets have already been widely characterized, but data related to its utilization in commercial diets (especially when gut microbiota is considered) are quite scarce. The current research 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 fish (3 tanks/diet, 18 fish/tank) allowed evaluating the apparent digestibility coefficients (ADC) of the dietary nutrients. Unaffected growth performance, condition factor, somatic indices, nutrient digestibility, and histomorphological features were observed in the HI-fed rainbow trout ($P > 0.05$). Increasing percentages of HI meal 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. The Chao1 index of the fish gut microbiota increased when including HI meal, while the Shannon index displayed the opposite trend ($P < 0.05$). The HI25 and HI50 fish showed enrichment of Actinobacteria, but, at the same time, a reduction in Bacteroidetes (False Discovery Rate [FDR] < 0.05). Furthermore, Bacillus , Actinomyces , Staphylococcus Enterococcus , and Oceanobacillus displayed higher relative abundance in the HI-fed fish than the other groups (FDR < 0.05). On the contrary, HI meal utilization was accompanied by a decrease in Campylobacter and Listeria , as well as Lactobacillus , Clostridium , Pediococcus , Leuconostoc , unclassified members (U.m.) of Peptostreptococceae, Vagococcus , Lactococcus , and Weissella . In conclusion, HI meal can be used in commercial diets for rainbow trout up to high inclusion levels (32%) without negatively affecting the nutrient digestibility, growth performance, somatic indices and histomorphological features of the animals. Furthermore, a positive shift of the gut microbiota towards the selection of short chain fatty acids (SCFAs)-producing bacteria and reduction of foodborne disease-causing pathogens was herein observed.
Suggested Reviewers:	Ines Guerreiro imsguerreiro@gmail.com She is an expert in fish nutrition.
	Giuliana Parisi giuliana.parisi@unifi.it She is an expert in fish nutrition and the use of insect-based products in fish.
Opposed Reviewers:	
Response to Reviewers:	

From: Dr. Francesco Gai
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To: Dr. Kumar
Co-Editor
Animal Feed Science and Technology

January 14th, 2022

Dear Dr. Kumar,

I am pleased to submit an original research article entitled "Dietary *Hermetia illucens* meal inclusion in commercial feeds 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.

This is a resubmission, as the original version displayed a similarity percentage with prior publications non acceptable. We checked the paper with our plagiarism check system (i.e., Turnitin) and we found out that the huge majority of the overlapping was related to the Materials and Methods (as our previous studies shared similar design and methodologies with the current one). We rewrote all the sentences (simply citing our previous studies, without reporting in details all the information) and we reached a similarity percentage lower than 30%. In the new version, the actual overlapping is related to conjunctions, adjectives or specific names that it is not possible to replace, or to the cited references in the manuscript text. We hope that this version will allow the Editor properly assessing the originality of the research.

We believe that this manuscript is appropriate for publication by *Animal Feed Science and Technology* because it provides novel and useful information about the impact of insect-based products 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

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To Dr. Vikas Kumar

Editor-in-Chief

Animal Feed Science and Technology

Re: Decision on submission to Animal Feed Science and Technology – Manuscript Number: ANIFEE-D-22-00039

Dear Dr. Vikas Kumar,

I have read carefully your letter dated February 15, 2022 and the comments from the reviewer regarding the above manuscript. I think the comments of the referee are interesting, and generally contribute to the improvement of the manuscript. I rewrote the paper according to these comments. As you will see, I did my best efforts to meet the criticism received, and to improve the quality of the article. I hope this modified version will fully satisfy the reviewers and match the standards of the *Animal Feed Science and Technology.*

The comments of the referee have been addressed one by one, and are listed in the following pages.

Yours sincerely

Dr. Francesco Gai

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Università degli studi di Torino
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ANSWER TO EDITOR'S COMMENTS

Please check the proximate composition and gross energy content of diets.

The proximate analysis of all the experimental diets was repeated and performed in triplicate, after a proper homogenization of all the samples. The new results are reported in the revised version of the manuscript.

ANSWER TO REVIEWER'S COMMENTS

Reviewer 1

Thank you for the valuable comments that stimulated us on to improve our manuscript.

Dear Corresponding Author,
Please, find comments and suggestions listed line-by-line below. Next, clearly and in detail respond to all reviewer's queries and doubts.

Title - is it necessary to mentioned that the diet used in the study was commercial feed? The reviewer suggest to change as follow,
„Dietary *Hermetia illucens* meal inclusion rainbow trout (Oncorhynchus mykiss) diet: effects on the growth performance, nutrient digestibility coefficients, selected gut histomorphometric traits, and health status indices".

Following the Reviewer's suggestion, we modified the title as follows: "*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". We decided to replace "commercial feeds" with "low-fishmeal diets", as the majority of the studies evaluating the impact of insect meals in rainbow trout nutrition has mainly been focused on the use of high-fishmeal diets, and we would like to emphasize the novelty of our research study in this sense. We also opted for replacing "gut histomorphometric" with "gut health", as the gut microbiota was also herein evaluated.

Comment 1 - it is quite hard to revise the manuscript without the line numbering.

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We added the line numbering as requested.

Comment 2 - in terms of above-mentioned „commercial diet" please, correct and unify whole manuscript in this case.

We replace “commercial diet” with ”low-fishmeal diet” throughout the whole manuscript.

Abstract

- replicate tanks/diet, - per treatment

Corrected as requested.

- 80, 160 e 320 g/kg - „e" what does it mean?

It was a typo error. We replaced “e” with “and”.

- 3 tanks/diet 18 fish/diet - please do not use "…/diet" but „per group" or „per treatment"

Corrected as requested.

Comment 3 - If the Authors emphasized significant results please use p-value.

P-values were always indicated, with the only exception of feed microbiota (where a descriptive analysis only has been performed).

-Staphylococcus, Enterococcus

Corrected as requested.

- „short-chain fatty acids"

Corrected as requested.

Keywords - „commercial feed" - Reviewer do not understand this phenomenon, why the Authors always emphasized this term…

We replace "commercial diet" with "low-fishmeal diet" (please, see our previous comments).

Abbreviation

- „short-chain fatty acids"

Corrected as requested.

Introduction

Comment 4 - please, do not use AllAbout Feed, Meticulous Research®… only scientific sources should be used in the manuscript

Considering that we removed all the information related to insect branch and investments, this correction is no longer needed.

- please, change the ancient citation, i.e., Sheppard 1994, in the 2022 please, used the most latest papers since 2015…

We removed this reference.

Comment 5 - the first paragraph of the introduction section should be shorten significantly. There is no need a story about the development of the insect producing branch, investment, etc.

Following the Reviewer's suggestions, we significantly shortened the Introduction by removing the unnecessary parts of the section.

Comment 6- the aim of the study should contains all measurements, according to the proposed title.

We modified the aim as follows: "Therefore, the present study aims to evaluate the effects of including increasing levels of a partially defatted HI meal in low-FM 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 coefficients, selected gut health traits, and health status indices".

Material and methods

Comment 6 - „Two experimental trials" - from the reviewer point of view one experiment was conducted which was divided into two main parts, i.e., growth measurements and nutrient digestibility coefficients.

Corrected as requested.

Comment 7 - It is crucial to add information about the insect meal preparation as an experimental factor, before the subheadings „Experimental diets". Please, answer in the text the following questions, what kind of diet did insects eat? How were the invertebrates slaughtered (boiling, freezing, etc.), stored (temp. How long), and processed (drying, temp, how long)? It is absolutely important to emphasize the conditions of each processing. Thus, the quality of used HI meals will be known by future readers.

These information have been added in the manuscript (please, see lines 124-130).

Comment 8 - the information about the extrusion process will be very welcome, with the highlight of the technical device name, company name, country, and city.

This information has been added as follows (please, see lines 145-146): "Feed was extruded using a Clextral BC45 twin-screw extruder with electrical barrel heating (Clextral, Firminy, France)".

Chemical analyses of feed

- crude ash instead of ash

Corrected as requested.

Sampling and processing

Comment 9 - why the authors did not use dry ice to freeze the sample for DNA extraction as fast as possible… Based on the Reviewer's experience, the digesta samples changes in terms of microbial quality very quickly… during storage in the 4C the sample freeze slowly and irreversible changes could appear resulting in different/incorrect microbial population levels...

As stated in the manuscript, samples were immediately cooled at 4 °C and keep for a maximum of 2 hours to avoid changes in microbiota. We do not believe that in such short time at this temperature microbiota can be modified. This issue could have been occurred if RNA was used as a target molecule.

DNA extraction

Comment 10 - this section is poorly written and should be significantly expanded, in terms of, full DNA extraction process, kits, conditions, etc. The same for sequencing, and metagenomics analysis.

Details on DNA extraction and bioinformatics were added in the manuscript.

Bioinformatics and statistical analysis

Comment 10 - why the Authors used the Wilcoxon rank sum test for diversity indives, when 4 treatments were used. Thus, if the results were not clasified as a normal distributed and the homogeneity of variance were significant, than the post-hoc Dunn's test after Kruskal-Wallis test should be used…

Data were analysed following Reviewer's comments using the function dunn.test of R (Kruskal-Wallis test p-value adjustment methods Benjamini-Hochberg). However, results did not change, therefore no correction was needed in both the Results and Discussion sections.

Comment 11 - there is lack of information about the bioinformatics activity…

Details on DNA extraction and bioinformatics were added in the manuscript.

Results

Comment 12 - the Authors mentioned in the material and methods section that „The two diets were formulated to be isonutrigenous, isolipidic, and isoenergetic", howerer in the results section shows that the experimental diet varied in terms of crude protein content and ether extract, thus the diet does not meet of those rules… crude protein differs nearly 5% (437,5 vs 458,8) as well as 8% for ether extract…
From the reviewer point of view it is a huge error in the experimentation preparation…
Other thing is to calculate the nitrogen to protein conversion factor which was probably used as usual, i.e., 6,25. However, it is mistake as well. Thus the protein in insects was overestimated.

The proximate analysis of all the experimental diets was repeated and performed in triplicate, after a proper homogenization of all the samples. The new results are reported in the revised version of the manuscript. The differences between CP and EE are still present, but they fall below the acceptance limit (5%). Furthermore, as reported in the Table 1 footnote, we used the correct conversion factor for calculating the protein content of the insect meal (5,62). The conversion factor of 6,25 was used to obtain the CP contents of the experimental diets only.

Feed 16S rRNA…

Comment 13 - Please, add the p-value for each part when the Authors mentioned terms, e.g., increase, reduce, etc.

Differently from what we did for the intestinal microbiota, we did not perform an analytical statistics for the feed microbiota (as we analysed it in duplicates per each feed type). Therefore, in order to make this aspect clear, we specified "numerical" or "numerically" along with such terms.

Discussion Comment 14 - please, do not discuss obtained non-significant results, i.e., digestibility, growth, somatic indices, histomorphometrics. Thus, remove those parts from the discussion section.

Following the Reviewer's suggestions, we shortened the Discussion of these parameters. We respectfully preferred not to remove all, as the non-significant results are still relevant results, and we think that this Discussion (even if short and concise) will be useful for those readers that may approach to the topic for the first time.

-the quality of the figures is very low and should be improved.

Quality of the figures was improved.

-if the authors mentioned the differences between treatments and the results are presented on the graphs also p-value should be appear.

Corrected as requested.

Reviewer 2

Thank you for the valuable comments that stimulated us on to improve our manuscript.

Excellent paper, but it has a lot of information, I recommend being objective or keeping the most important information. The work in the current format presents its readers with long and tiresome reading. There are parameters evaluated that have little representation for the work, especially those that did not show statistical differences.

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We thank the Reviewer for the positive feedback. Following the suggestions of both the Reviewers, we shortened the Discussion of these parameters. We respectfully preferred not to remove all, as the non-significant results are still relevant results, and we think that this Discussion (even if short and concise) will be useful for those readers that may approach to the topic for the first time.

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

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

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Abstract

The effects of including *Hermetia illucens* (HI) meal in rainbow trout diets have been widely characterized, but data related to its utilization in low-fishmeal (FM) diets are quite scarce. The current research investigated the impact of HI meal inclusion in low-FM diets for rainbow trout by assessing fish growth performance, nutrient digestibility, histomorphological traits of intestine and main organs, and gut microbiota. In the 133-days growth trial, 600 rainbow trout were randomly distributed to 4 dietary treatments (3 replicate tanks/treatment, 50 fish/tank): a low-FM diet as control (HI0), and three experimental diets including 80, 160 and 320 g/kg of HI meal as fed as replacement of 25, 50 and 100% of FM (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 their microbiota. In the digestibility trial, 216 fish (3 tanks/treatment, 18 fish/tank) allowed evaluating the apparent digestibility coefficients (ADC) of the dietary nutrients. Unaffected growth performance, condition factor, somatic indices, nutrient digestibility, and histomorphological features were observed in the HI-fed rainbow trout ($P > 0.05$). Increasing percentages of HI meal 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. The Chao1 index of the fish gut microbiota increased when including HI meal, while the Shannon index displayed the opposite trend ($P < 0.05$). The HI25 and HI50 fish showed enrichment of Actinobacteria, but a reduction in Bacteroidetes (False Discovery Rate [FDR] < 0.05). Furthermore, *Bacillus*, *Actinomyces*, *Staphylococcus*, *Enterococcus*, and *Oceanobacillus* displayed higher relative abundance in the HI-fed fish than the other groups (FDR < 0.05). On the contrary, HI meal utilization was accompanied by a decrease in *Campylobacter* and *Listeria*, as well as *Lactobacillus*, *Clostridium*, *Pediococcus*, *Leuconostoc*, unclassified members (U.m.) of Peptostreptococceae, *Vagococcus*, *Lactococcus*, and *Weissella* (FDR < 0.05). In

conclusion, HI meal can be used in low-FM diets for rainbow trout up to high inclusion levels (32%) without negatively affecting the fish nutrient digestibility, growth performance, somatic indices and histomorphological features. A positive shift of the gut microbiota towards the selection of short-chain fatty acids (SCFAs)-producing bacteria and reduction of foodborne disease-causing pathogens was also observed.

Keywords

Black soldier fly, low-fishmeal 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, especially in relation to the aquaculture segment (as a reasonable consequence of the early EU authorisation of the insect processed animal proteins [PAPs] in aquaculture feed in July

2017 [IPIFF, 2021]). The rapid development of the insect sector is related to the strong ability of insects to transform the food waste in forms of protein-rich animal feed, thus allowing them to fully embrace the concept of "circular economy". In particular, 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 (Ojha et al., 2020). However, the growth of the insect market 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 use of high-fishmeal (FM) 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 positive effects on the intestinal microbiota (i.e, 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 low-FM diets – which contain 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 low-FM diets are available yet.

Therefore, the present study aims to evaluate the effects of including increasing levels of a partially defatted HI meal in low-FM 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 coefficients, selected gut health traits, and health status indices.

Materials and Methods

The present experiment (consisting of both a digestibility and a growth trial) was 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 predisposed in respect of the guidelines of the European and Italian regulations on the care and use of experimental animals (European directive 86 609/EEC). The experimental protocol was approved by the Ethical Committee of the University of Turin (protocol n° 143811).

Insect meal preparation

The HI larvae were fed GMP+ certified feed of vegetal origin. Larvae were harvested, washed in cold water, slaughtered by direct mincing, pasteurized, separated by centrifugal force, and furtherly processed to obtain partially defatted meal and fat. Partially defatted HI meal used in the present study had a shelf life of 6 months and was stored according to supplier instructions (20 °C in a cool and dry place). Processing of partially defatted meal into feed was performed within one month since production.

131

132 *Experimental diets*

133 Two diets containing FM (206 g/kg as fed; HI0) or a partially defatted HI meal produced in the
134 experimental facility of a Dutch insect producer (Protix BV, Dongen, The Netherlands – 320 g/kg as
135 fed; HI100) in substitution of 100% of FM were formulated by Research Diet Services BV (Utrecht,
136 The Netherlands) and DISAFA. For nutrient digestibility evaluation, 10 g/kg as fed of Diamol (an
137 acid insoluble ash) was added as inert marker. The two diets were formulated to be isonitrogenous,
138 isolipidic, and isoenergetic. After that, two additional experimental diets were prepared by mixing:
139 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
140 500 g/kg as fed of HI100 (HI50). The control diet (HI0) was formulated to mimic a commercial (low-
141 FM) diet for rainbow trout, while the four experimental diets contained increasing levels of HI meal
142 in substitution of 0% (HI0), 25% (HI25), 50% (HI50) and 100% (HI100) of FM (corresponding to 0,
143 80, 160 and 320 g/kg as fed of HI meal, respectively). The four diets (shown in Table 1) were prepared
144 as extruded feed by Research Diet Services BV and shipped to the Experimental Facility of DISAFA.
145 Feed was extruded using a Clextral BC45 twin-screw extruder with electrical barrel heating (Clextral,
146 Firminy, France). The diets were stored at 0-4°C and 85-90% RH in dark room before feeding to the
147 fish.

148

149 *Chemical analyses of feed*

150 Feed samples were ground using a cutting mill (MLI 204; Bühler AG, Uzwil, Switzerland) and
151 analysed for dry matter (DM), crude protein (CP), acid detergent fiber (ADF) and crude ash contents
152 (AOAC International, 2000). Feed samples were also analysed for ether extract (EE; AOAC
153 International, 2003), neutral detergent fiber (NDF; Van Soest et al., 1991), and fatty acid (FA) profile
154 (Renna et al., 2017). An adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany) allowed
155 determining the GE content. All the chemical analyses of the feeds were performed in triplicate. The
156 proximate composition and the FA profile of the experimental diets are shown in Table 1 and 2,

157 respectively. Feed were also sampled for the microbiota assessment (please, see "*DNA extraction and*
158 *16S rRNA amplicon target sequencing*" subsection).
159
160 *Digestibility trial*
161 A total of 216 trout (purchased from a private fish hatchery ["Troticoltura Bassignana", Cuneo, Italy],
162 with a weight of 160.25 ± 8.24 g) were distributed into twelve 250-L cylindroconical tanks (3 replicate
163 tanks/diet, 18 fish/tank) connected to a flow-through open system where artesian well water was
164 supplied (tank water inflow: 8 L/min; T: 13 ± 1 °C; dissolved oxygen levels: 7.6-8.7 mg/L). After 14
165 days of acclimatization with a commercial diet (420 g/kg as fed CP and 220 g/kg as fed EE; Skretting
166 Italia Spa, Mozzecane, Verona, Italy), the fish were fed by hand to visual satiety twice a day (8:00
167 am and 3:00 pm). The ADC were measured using the indirect acid-insoluble ash method, with 1%
168 Diamol being used as inert marker. The faeces were collected daily from each tank for four
169 consecutive week as described by Chemello et al. (2020). The faeces were frozen (−20 °C),
170 successively freeze-dried, and stored until chemical analyses. The ADC of DM (ADCDM), crude
171 protein (ADCCP), ether extract (ADCEE) and gross energy (ADCGE) were calculated according to
172 Chemello et al. (2020).
173
174 *Growth trial*
175 A total of 600 rainbow trout were purchased from a private fish hatchery ("Troticoltura Bassignana",
176 Cuneo, Italy). After a four-week period of acclimation (during which the fish were fed the same
177 commercial diet used for the digestibility trial), the rainbow trout were submitted to a light anaesthesia
178 (MS-222; PHARMAQ Ltd., Sandleheath, UK; 60 mg/L), individually weighed (112.86 ± 8.41 g)
179 using electronic scales (KERN PLE-N v. 2.2; KERN & Sohn GmbH, Balingen-Frommern, Germany;
180 d: 0.1), and randomly allotted to twelve 300-L, rectangular-shaped tanks (three replicate tanks per
181 diet, fifty fish per tank) connected to the same flow-through open water system of the digestibility
182 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 choose the optimal daily feeding rate, all the biomass tanks were weighed every two weeks. 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 and individually weighed. The following performance parameters were calculated according to Renna et al. (2017): survival, individual weight gain (iWG), feed conversion ratio (FCR), protein efficiency ratio (PER), and specific growth rate (SGR).

Condition factor and somatic indices

At the end of the growth trial, 21 fish per diet (7 fish/tank) were killed by over anaesthesia (500 mg/L) after being individually weighted. The Fulton's condition factor (K), the carcass yield (CY), the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF) were calculated according to Renna et al. (2017).

Sampling and processing

At the end of the growth trial, 12 fish per dietary treatment (4 fish per tank) were also killed by over anaesthesia 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 morphometric investigations, while liver, spleen and stomach were sampled for histopathological examination. All the tissues were processed according to Chemello et al. (2021). The posterior gut content was also collected into sterile plastic

tubes after appropriate squeezing, cooled at 4 °C (for a period not longer than 2 hours), and frozen at -80°C until the extraction of the DNA.

Histomorphological investigations

Morphometric analysis of the gut was performed following the procedures described in details by Chemello et al. (2021). Briefly, the morphometric measurements of villus height (Vh, from the villus tip to submucosa) were performed by Image–Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, Rockville, MD, USA) on 10 well-oriented and not damaged villi (Renna et al., 2017). The observed histopathological findings were scored in all the organs according to Elia et al. (2018), while gut histopathological findings were characterized following the semi-quantitative scoring system proposed by Biasato et al. (2019).

DNA extraction and 16S rRNA amplicon target sequencing

The total genomic DNA (gDNA) was extracted from the posterior gut content (0.25 mg) by using the Qiagen power microbiome kit (Milan, Italy) following manufactured instructions. The RNA was digested by adding 0.1 U of RNAse (Promega, Milan, Italy), and thengDNA was quantified by using the Qubit assay and diluted to 5ng/μL. The V3-V4 region of the 16S rRNA gene was amplified by using the following primers (Klindworth et al., 2013): 16SF (5'-CCTACGGGNGGCWGCAG-3') and 16SR (5'-GACTACHVGGGTATCTAATCC-3'). The Illumina overhang adapter and PCR conditions were chosen according to the standard Illumina 16S Metagenomic Sequencing Library Preparation. Amplicons were then purified by using the Kapa pure beads (Roche, Milan, Italy) and tagged by using the Nextera XT dual index according to the manufactory instruction. Amplicons were then quantified by using the Qubit assay, normalized at the same concentration, and pooled. Sequencing was performed on a MiSeq platform (Illumina) using the MiSeq v2 reagent cartridge in paired end mode (2x250pb).

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.

After sequencing raw reads were imported in QIIME for denoising (>Q20) and chimera filtering step (Caporaso et al., 2010). The Operational Taxonomic Units (OTUs) were then picked at 97% of similarity and used for taxonomic assignment by using the RDP classifier against the greengenes database. The OTUs table was then build, singleton excluded and rarefied at the lowest number of sequencing per samples. The OTUs table was then filtered at >0.2% in at least five samples. The statistical analysis of 16S rRNA sequences was performed using R software. Alpha diversity was calculated using the vegan package of R (Dixon, 2003), and all the diversity indices were compared among the experimental diets by using the function *dunn.test* of R (Kruskal-Wallis test, p-value adjustment methods Benjamini-Hochberg). The OTUs table was then used to perform Anosim statistical test and analyze the beta diversity, with Pairwise Kruskal-Wallis tests allowing the identification of significant differences in OTUs abundance according to the dietary HI meal inclusion. 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. 27.0 (IBM, Armonk, NY, USA). One-way ANOVA was used to compare growth performance, nutrient digestibility and somatic indices data among the dietary treatments. The Shapiro–Wilk test assessed the normality or non-normality distribution of the dependent variables. The assumption of equal variances was assessed by Levene's homogeneity of variance test, and, if such an assumption did not hold, the Brown-Forsythe statistic was performed. Tukey's and Tamhane's T2 tests were chosen as post-hoc tests 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 the corresponding interaction). 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 all comparable in terms of macronutrients, as the differences between the lowest and the highest CP, EE and GE contents were lower than 5% (1.92%, 2.65%, and 1.33%, respectively; 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 on increasing 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

The ADCs of the nutrients observed in the rainbow trout were not significantly influenced by dietary HI meal inclusion ($P > 0.05$, Table 3).

287 *Growth trial*

288 *Growth performance*

289 Growth performance of the rainbow trout are summarized in Table 4. The fish did not refuse the
290 experimental diets, with all the supplied feed being daily consumed during the experimental trial.
291 Survival was high for all the dietary treatments (range: 95.33-97.33), being also not influenced by HI
292 meal utilization ($P > 0.05$, Table 4). Similarly, all the other growth parameters were not affected by
293 insect meal inclusion ($P > 0.05$, Table 4).

294

295 *Condition factor and somatic indices*

296 The inclusion of HI meal in rainbow trout diets did not influence either the condition factor or the
297 somatic indices of the fish ($P > 0.05$, Table 5).

298

299 *Histomorphological investigations*

300 Data regarding the morphometric measurements of the Vh in the anterior and posterior gut are
301 reported in Table 6. The Vh was not influenced by the diet and the interaction between the diet and
302 the intestinal segment ($P > 0.05$, Table 6), but it only depended on the intestinal segment ($P < 0.001$,
303 Table 6). Independently of the dietary HI meal inclusion, the Vh progressively increased from the
304 anterior to the posterior gut ($P < 0.001$, Table 6).

305 The histopathological alterations observed in liver, spleen, stomach, anterior and posterior gut are
306 summarized in Table 7. In liver, absent to mild, focal to multifocal lymphoplasmacytic infiltrates, as
307 well as absent to moderate, multifocal to diffuse fatty changes of the hepatocytes were observed in
308 all the dietary treatments. Mild, focal to multifocal hemosiderosis, along with moderate, focal to
309 multifocal white pulp hyperplasia were also recorded in all the experimental groups. No signs of
310 immune cell infiltration were observed in the stomach, except for the HI100 group (8.3%). All the
311 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 in the feeds determined a progressive, numerical increase in the relative abundance of Firmicutes and Actinobacteria phyla, whereas Proteobacteria displayed a gradual, numerical reduction (Figure 1A). Furthermore, the relative abundance of *Lactobacillus* and *Listeria* numerically decreased with increasing percentages of HI meal, 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 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 increased 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 fish.

Growth trial

Growth performance

The growth performance of the rainbow trout of the present study were not affected by dietary HI meal inclusion, thus reflecting the unaltered nutrient digestibility coefficients, and being already underlined by previous research (Renna et al., 2017; Cappellozza et al., 2019; Cardinaletti et al., 2019; Caimi et al., 2021).

364 *Condition factor and somatic indices*

365 Both the condition factor and the somatic indices of the rainbow trout of the present study were not
366 significantly influenced by dietary HI meal inclusion. This is in agreement with previous research
367 studies about HI meal utilization in rainbow trout, which also reported analogous K (Renna et al.,
368 2017; Cardinaletti et al., 2019), HSI (Sealey et al., 2011) and VSI (Bruni et al., 2018) values. The so-
369 obtained results confirm that HI meal utilization allows the preservation of a good physiological state
370 in fish, without determining metabolic problems or liver and gastrointestinal diseases (as analogously
371 confirmed by the histopathological examination).

372

373 *Histomorphological features*

374 Dietary HI meal inclusion did not significantly affect the gut morphology of the rainbow trout of the
375 current research, as already reported by Renna et al. (2017). This is in agreement with the unaffected
376 growth performance herein observed in the HI-fed fish, thus suggesting no negative repercussions on
377 either the digestion or the absorption of the nutrients by the intestine. Independently of HI utilization,
378 the posterior intestine of the rainbow trout of the present study showed higher villi than the anterior
379 gut. This scenario disagrees with that one underlined by Khojasteh et al. (2009), which reported a
380 progressive decrease in villi length from the anterior to the posterior intestine. However, the
381 concomitant identification of short and long villi in both the gut segments – as well as the villi length
382 changes throughout the fish cycle – has recently been reported in rainbow trout (Verdile et al., 2020),
383 thus making further investigations needed.

384 The histopathological findings observed in the fish of the current research were also not significantly
385 influenced by HI meal utilization, thus being in agreement with previous research studies (Elia et al.,
386 2018), and confirming no negative effects of HI on fish health. The fatty and inflammatory changes
387 in liver and gastrointestinal tract, respectively, are the common result of the high-energy diet
388 administered to salmonids, while the spleen reactivity appears to be aspecific. Furthermore, the

389 histopathological alterations were highlighted in both the control- and the HI-fed fish, also resulting
390 to be predominantly mild to moderate (and, in turn, of negligible relevance).
391
392 *Feed and gut microbiota*
393 Firmicutes, Cyanobacteria and Proteobacteria phyla dominated the microbiota of the feed used in the
394 present study. This is partially in agreement with Terova et al. (2019), which identified a
395 predominance of Firmicutes, Proteobacteria and Actinobacteria in FM-based diets for rainbow trout.
396 However, the detection of high percentages of Cyanobacteria represents an unexpected finding.
397 Cyanobacteria has recently been found in the gut microbiota of marine (Salas-Leiva et al., 2020) and
398 freshwater (Jiang et al., 2020; Zeng et al., 2020) species, being also one of the most abundant
399 prokaryotes in sea (Korlević et al., 2016; Quéméneur et al., 2020) and anthropogenic-induced
400 eutrophied freshwaters (Zhang et al., 2021). Considering that the biomass which supplies the FM
401 industry is mainly composed of small pelagic species (Péron et al., 2010), it seems reasonable that
402 the feed microbiota herein characterized reflect the gut microbiota of the fish species (and their
403 rearing environment as well) used to produce the FM. The identification of high relative abundances
404 of Firmicutes and Proteobacteria – which are two of the dominant bacterial phyla of the fish gut
405 microbiota (Butt and Volkoff, 2019) – further supports such hypothesis. A similar consideration can
406 also be made for the most represented bacterial genera detected in feed microbiota, as *Lactobacillus*
407 (Tarnecki et al., 2017; Huyben et al., 2020; Yu et al., 2021), *Leuconostoc*, *Streptococcus* (Tarnecki
408 et al., 2017) and *Photobacterium* (Huyben et al., 2020) constitute the core microbiota of several
409 marine species, with the latter OTU being particularly characteristic of piscivores such the pelagic
410 species (Huang et al., 2020). Differently, the detection of high percentages of *Listeria* may rise
411 worrying concerns in terms of food safety, as some species (especially *L. monocytogenes*) are
412 involved in foodborne outbreaks of listeriosis (Buchanan et al., 2017). Since the consumption of raw
413 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 during the feeding trial, also showing no remarkable histopathological lesions.

Firmicutes, Actinobacteria and Proteobacteria represented the major phyla in either the control- or the HI-fed fish of the current research. These findings are in overall agreement with the previous studies 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). As far as the genera are concerned, *Staphylococcus*, *Lactobacillus* and *Streptococcus* mainly colonized the posterior gut microbiota of the fish fed both the control and the HI-based diets in the present study. *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) have previously been reported as main bacterial genera in the cecal microbiota of rainbow trout, thus analogously confirming the identification of a physiological bacterial community.

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

518 in the corresponding relative abundances (about 7% vs 0.4%) reasonably suggests an active role of
519 HI meal as well.
520
521 **Conclusions**
522 In conclusion, HI meal can be used in low-FM diets for rainbow trout up to high inclusion levels (320
523 g/kg as fed) without negatively affecting the growth performance, nutrient digestibility, somatic
524 indices and histomorphological features of the animals. Therefore, considering that the low FM-diets
525 are nowadays the most adopted fish feeds from a sustainability perspective, the possibility of
526 including either low or high inclusion levels of HI meal without incurring in adverse outcomes
527 represents a promising scenario. Furthermore, a positive modulation of the gut microbiota in terms
528 of selection of SCFAs-producing bacteria and reduction of foodborne disease-causing pathogens was
529 herein observed for the first time when rainbow trout were administered with low FM-diets containing
530 HI meal. In the light of such positive findings, future investigations also assessing the gut
531 metagenome and metabolome are mandatory in order to fully characterize the HI way of action in the
532 fish gut.
533
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535 **Ilaria Biasato**: conduct the experiment, sampling, statistical analysis, and writing the initial draft,
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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.

Data availability

The metataxonomic sequences are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession numbers PRJNA783153.

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821 **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/kg	962.4	939.0	941.6	944.2	949.4
CP, g/kg DM[b]	517.1	443.5	445.6	447.8	452.0
EE, g/kg DM	204.3	189.0	187.8	186.5	184.0
Ash, g/kg DM	56.5	67.6	67.8	68.1	68.5
NDF, g/kg DM	N.A.	208.3	181.1	153.9	99.5
ADF, g/kg DM	N.A.	15.7	21.7	27.6	39.5
NFE, g/kg DM[c]	N.A.	238.9	240.4	241.9	244.9
GE, MJ/Kg[c]	22.04	21.74	21.85	21.57	21.56

822 Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50%
823 of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal;
824 DM, dry matter; CP, crude protein; EE, ether extract; ADF, acid detergent fiber; ADFn, acid detergent
825 fiber nitrogen; NDF, neutral detergent fiber; NFE, nitrogen-free extract. [a]Values are reported as mean
826 of duplicate analyses; [b]Conversion factors of 5.62 for the HI meal (Janssen et al., 2017) and 6.25 for
827 the experimental diets; [c]Calculated as 100 – [(100 – DM) + CP + EE + Ash); [d]Determined by
828 calorimetric bomb.
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837 **Table 2.** Fatty acid (FA) composition of the experimental diets.

	HI0	HI25	HI50	HI100
Fatty acids, g/100 g DM of TFA[a]				
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

838 Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50%
839 of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal;
840 c, cis; t, trans; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA,
841 polyunsaturated fatty acids; TFA, total fatty acids. [a]Renna et al. (2014).
842
843 **Table 3.** Apparent digestibility coefficients of dry matter, protein, ether extract and gross energy of
844 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

845 Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50%
846 of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal;
847 SEM, standard error of the mean; P, probability; ADC, apparent digestibility coefficient; DM, dry
848 matter; CP, crude protein; EE, ether extract; GE, gross energy.
849
850 **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

851 Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50%
852 of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal;
853 SEM, standard error of the mean; P, probability; iIBW, individual initial body weight; iFBW,
854 individual final body weight; iWG, individual weight gain; SGR, specific growth rate; FCR, feed
855 conversion ratio; PER, protein efficiency ratio.
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858
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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.

889 **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)	

890 Abbreviations: HI0, control diet; HI25, 25% of FM replaced by *Hermetia illucens* meal; HI50, 50%
891 of FM replaced by *Hermetia illucens* meal; HI100, 100% of FM replaced by *Hermetia illucens* meal;
892 SEM, standard error of the mean; P, probability.
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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. Box plots with different superscript letters (a, b) indicate significant differences among the treatments ($P < 0.05$).

Figure 3. Bacterial community composition (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*

931 *illucens* meal as replacement of 100% of fishmeal (HI100) diets. Box plots with different superscript
932 letters (a, b) indicate significant differences among the treatments (FDR < 0.05).
933
934 **Figure 6.** Relative abundance at genus level of differentially abundant OTUs in in posterior gut
935 samples of rainbow trout fed control (HI10), *Hermetia illucens* meal as replacement of 25% of
936 fishmeal (HI50), *Hermetia illucens* meal as replacement of 50% of fishmeal (HI50), and *Hermetia*
937 *illucens* meal as replacement of 100% of fishmeal (HI100) diets. Box plots with different superscript
938 letters (a, b) indicate significant differences among the treatments (FDR < 0.05).

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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.

Dietary *Hermetia illucens* meal inclusion in commercial feeds 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 rainbow trout diets have already been widely characterized, but data related to its utilization in commercial diets (especially when gut microbiota is considered) are quite scarce. The current research 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 fish (3 tanks/diet, 18 fish/tank) allowed evaluating the apparent digestibility coefficients (ADC) of the dietary nutrients. Unaffected growth performance, condition factor, somatic indices, nutrient digestibility, and histomorphological features were observed in the HI-fed rainbow trout ($P > 0.05$). Increasing percentages of HI meal 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. The Chao1 index of the fish gut microbiota increased when including HI meal, while the Shannon index displayed the opposite trend ($P < 0.05$). The HI25 and HI50 fish showed enrichment of Actinobacteria, but, at the same time, a reduction in Bacteroidetes (False Discovery Rate [FDR] < 0.05). Furthermore, *Bacillus*, *Actinomyces*, *Staphylococcus Enterococcus*, and *Oceanobacillus* displayed higher relative abundance in the HI-fed fish than the other groups (FDR < 0.05). On the contrary, HI meal utilization was accompanied by a decrease in *Campylobacter* and *Listeria*, as well as *Lactobacillus*, *Clostridium*, *Pediococcus*, *Leuconostoc*, unclassified members

(U.m.) of Peptostreptococceae, *Vagococcus*, *Lactococcus*, and *Weissella*. In conclusion, HI meal can be used in commercial diets for rainbow trout up to high inclusion levels (32%) without negatively affecting the nutrient digestibility, growth performance, somatic indices and histomorphological features of the animals. Furthermore, a positive shift of the gut microbiota towards the 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 food waste 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 use 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 positive effects on the intestinal microbiota (i.e, 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 evaluate 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 predisposed in respect of the guidelines of the European and Italian regulations on the care and use of experimental animals (European directive 86 609/EEC). 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 contained increasing levels of HI meal in substitution of 0% (HI0), 25% (HI25), 50% (HI50) and 100% (HI100) of FM (corresponding to 0, 80, 160 and 320 g/kg as fed of HI meal, 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), crude protein (CP), acid detergent fiber (ADF) and ash contents (AOAC International, 2000). Feed samples were also analysed for ether extract (EE; AOAC International, 2003), neutral detergent fiber (NDF; Van Soest et al., 1991), and fatty acid (FA) profile (Renna et al., 2017). An adiabatic calorimetric bomb (C7000; IKA, Staufen, Germany) allowed determining the GE content. 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

A total of 216 trout (purchased from a private fish hatchery ["Troticoltura Bassignana", Cuneo, Italy], with a weight of 160.25 ± 8.24 g) were distributed into twelve 250-L cylindroconical tanks (3 replicate tanks/diet, 18 fish/tank) connected to a flow-through open system where artesian well water was supplied (tank water inflow: 8 L/min; T: 13 ± 1 °C; dissolved oxygen levels: 7.6-8.7 mg/L). After 14 days of acclimatization with a commercial diet (42% CP and 22% EE; Skretting Italia Spa, Mozzecane, Verona, Italy), 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 as described by Chemello et al. (2020). The faeces were frozen (−20 °C), 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 600 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 the same commercial diet used for the digestibility trial), 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 choose the optimal daily feeding rate, all the biomass tanks were weighed every two weeks. 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 and individually weighed. The following performance parameters were calculated according to Renna et al. (2017): survival, individual weight gain (iWG), feed conversion ratio (FCR), protein efficiency ratio (PER), and specific growth rate (SGR).

Condition factor and somatic indices

At the end of the growth trial, 21 fish per diet (7 fish/tank) were killed by over anaesthesia (500 mg/L) after being individually weighted. The Fulton's condition factor (K), the carcass yield (CY), the hepatosomatic index (HSI), the viscerosomatic index (VSI), and the coefficient of fatness (CF) were calculated according to Renna et al. (2017).

Sampling and processing

At the end of the growth trial, 12 fish per dietary treatment (4 fish per tank) were also killed by over anaesthesia 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 morphometric investigations, while liver, spleen and stomach were sampled for histopathological examination. All the tissues were processed according to Chemello et al. (2021). The posterior gut content was also collected into sterile plastic

tubes after appropriate squeezing, cooled at 4 °C (for a period not longer than 2 hours), and frozen at -80°C until the extraction of the DNA.

Histomorphological investigations

Morphometric analysis of the gut was performed following the procedures described in details by Chemello et al. (2021). Briefly, the morphometric measurements of villus height (Vh, from the villus tip to submucosa) were performed by Image–Pro Plus 6.0 software (Media Cybernetics Inc., Bethesda, Rockville, MD, USA) on 10 well-oriented and not damaged villi (Renna et al., 2017). The observed histopathological findings were scored in all the organs according to Elia et al. (2018), while gut histopathological findings were characterized following the semi-quantitative scoring system proposed by Biasato et al. (2019).

DNA extraction and 16S rRNA amplicon target sequencing

The total genomic DNA (gDNA) was extracted by the posterior gut content, and, after being quantified and standardized, used to characterize the microbiota composition by sequencing the V3-V4 region of the 16S rRNA gene (Biasato et al., 2018).

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.

Bioinformatic data analysis was performed using the Greengenes as a database for the taxonomic assignment according to Biasato et al. (2018). The statistical analysis of 16S rRNA sequences was performed using R software. Alpha diversity was calculated using the vegan package of R (Dixon, 2003), and all the diversity indices were compared among the experimental diets by pairwise comparisons using Wilcoxon rank sum test. The OTUs table (relative abundance >0.2% in at least five samples) was then used to perform Anosim statistical test and analyze the beta diversity, with

Pairwise Kruskal-Wallis tests allowing the identification of significant differences in OTUs abundance according to the dietary HI meal inclusion. 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. 27.0 (IBM, Armonk, NY, USA). One-way ANOVA was used to compare growth performance, nutrient digestibility and somatic indices data among the dietary treatments. The Shapiro–Wilk test assessed the normality or non-normality distribution of the dependent variables. The assumption of equal variances was assessed by Levene's homogeneity of variance test, and, if such an assumption did not hold, the Brown-Forsythe statistic was performed. Tukey's and Tamhane's T2 tests were chosen as post-hoc tests 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 the corresponding interaction). 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 on increasing 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

The ADCs of the nutrients observed in the rainbow trout were not significantly influenced by dietary HI meal inclusion ($P > 0.05$, Table 3).

Growth trial

Growth performance

Growth performance of the rainbow trout are summarized in Table 4. The fish did not refuse the experimental diets, with all the supplied feed being daily consumed during the experimental trial. Survival was high for all the dietary treatments (range: 95.33-97.33), being also not influenced by HI meal utilization ($P > 0.05$, Table 4). Similarly, all the other growth parameters were not affected by insect meal inclusion ($P > 0.05$, Table 4).

Condition factor and somatic indices

The inclusion of HI meal in rainbow trout diets did not influence either the condition factor or the somatic indices of the fish ($P > 0.05$, 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 progressively increased 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 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 percentages of HI meal, 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 increased 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 fish.

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 about 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 were in a good physiological state and, in turn, that dietary HI meal inclusion did not undermine the fish wellbeing (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 higher villi than the anterior

gut. This scenario disagrees with that one underlined by Khojasteh et al. (2009), which reported a progressive decrease in villi length from the anterior to the posterior intestine. However, the concomitant identification 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 findings 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 highlighted in both the control- and the HI-fed fish, also resulting to be predominantly mild to moderate (and, in turn, of negligible relevance).

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 during the feeding trial, also showing no remarkable histopathological lesions.

Firmicutes, Actinobacteria and Proteobacteria represented the major phyla in either the control- or the HI-fed fish of the current research. These findings are in overall agreement with the previous studies 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). As far as the genera are concerned, *Staphylococcus*, *Lactobacillus* and *Streptococcus* mainly colonized the posterior gut microbiota of the fish fed both the control and the HI-based diets in the present study. *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) have previously been reported as main bacterial genera in the cecal microbiota of rainbow trout, thus analogously confirming the identification of a physiological bacterial community.

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.

Data availability

The metataxonomic sequences are available at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession numbers PRJNA783153.

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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/kg	962.4	939.3	948.1	944.1	960.1
CP, g/kg DM[b]	517.1	437.5	440.1	448.4	458.8
EE, g/kg DM	204.3	194.3	192.3	180.4	178.5
Ash, g/kg DM	56.5	68.5	66.5	64.7	67.1
NDF, g/kg DM	N.A.	208.9	250.8	82.6	99.1
ADF, g/kg DM	N.A.	16.3	22.1	30.8	38.8
NFE, g/kg DM[c]	N.A.	239.0	249.2	250.6	255.8
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[a]				
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. [a]Renna et al. (2014).

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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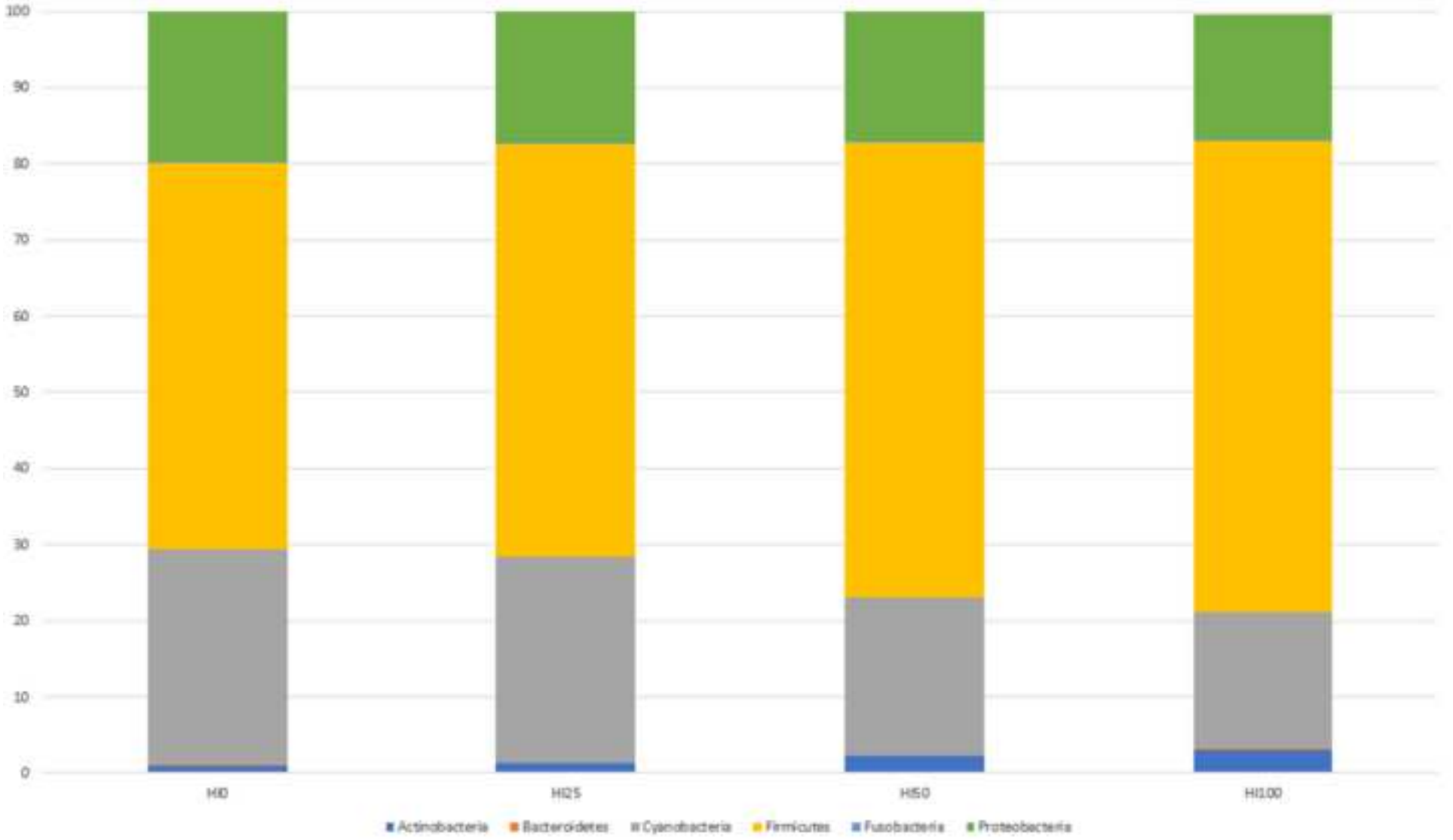

Figure 1B

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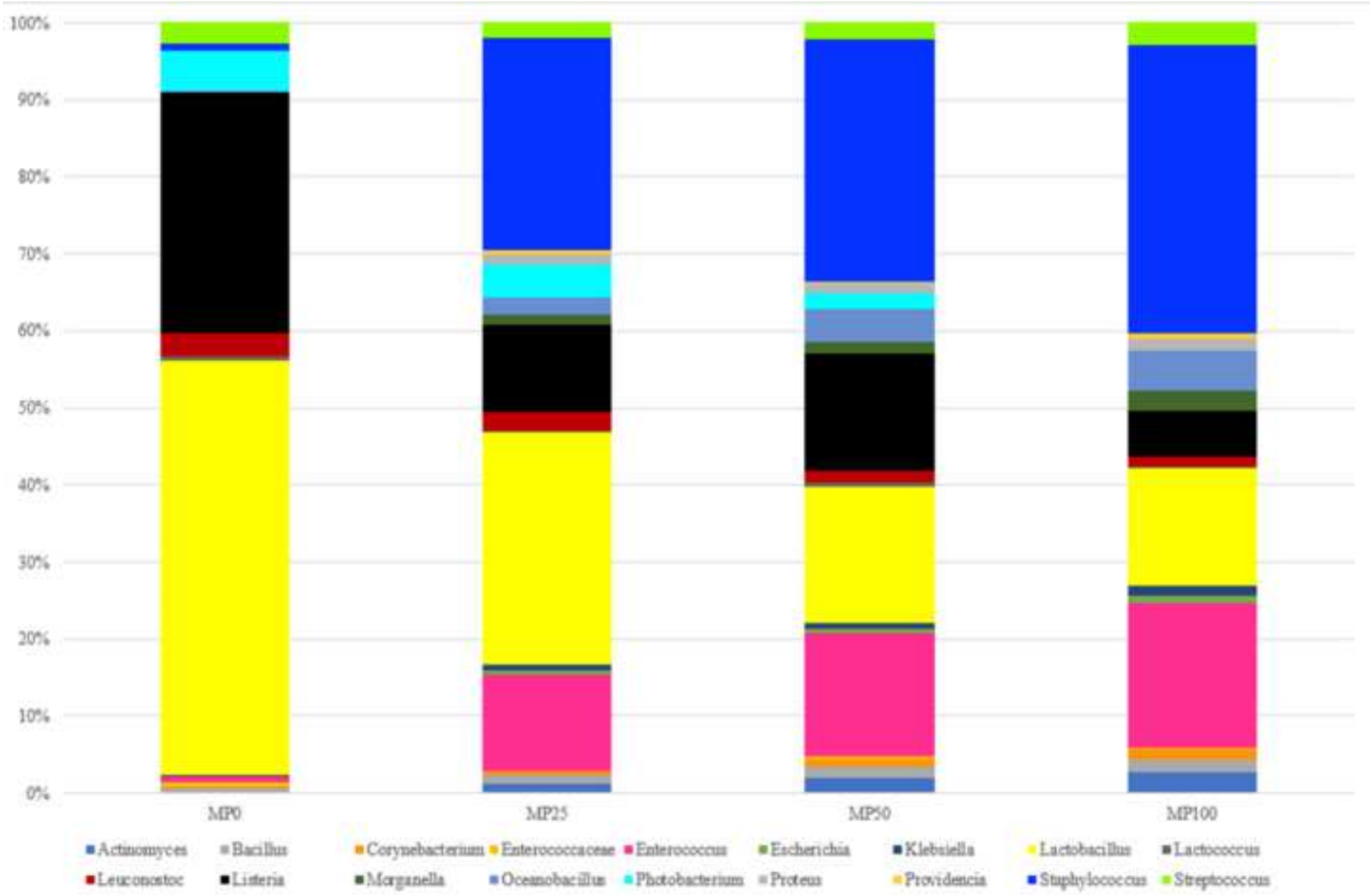

Figure 2

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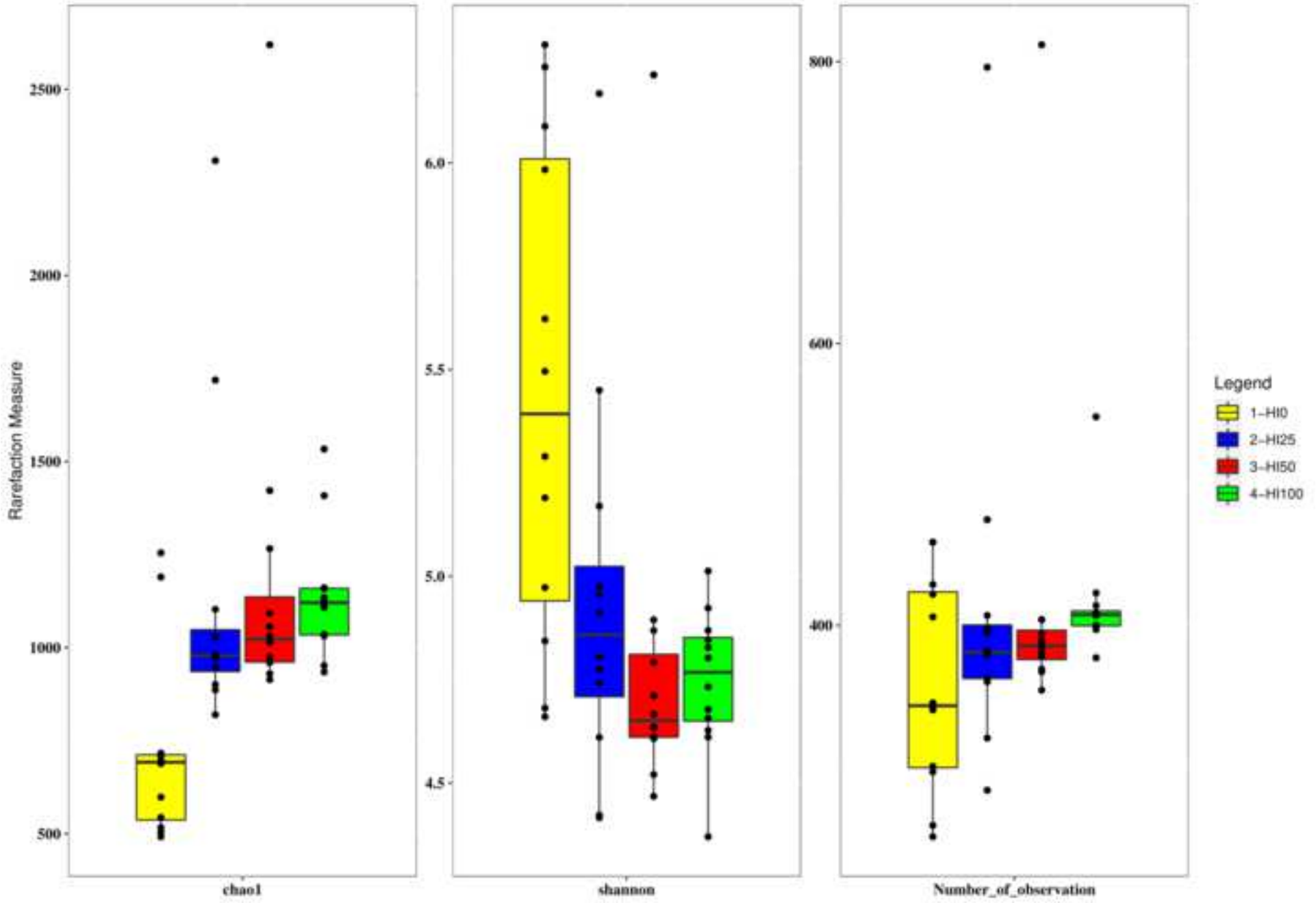

Figure 3

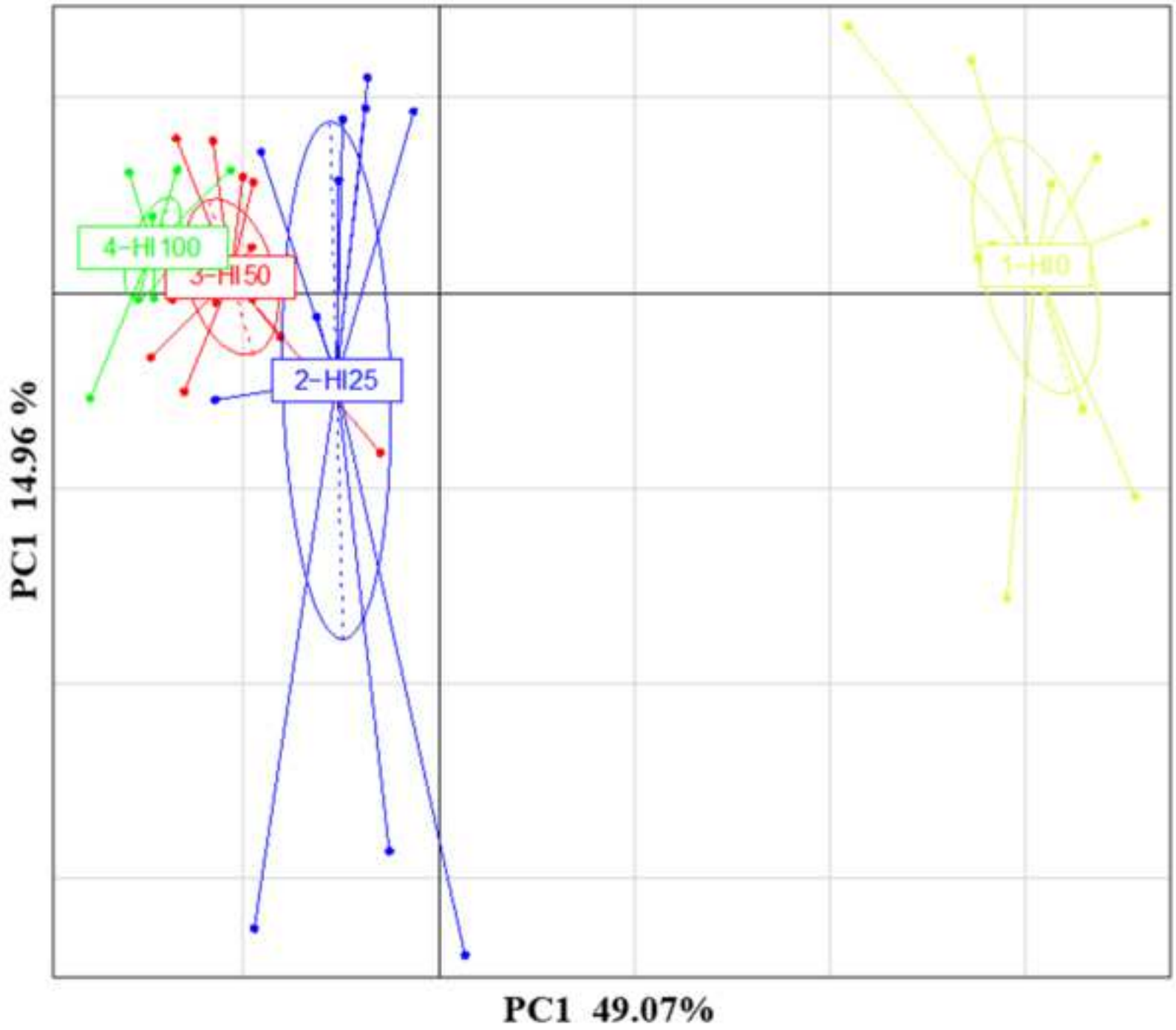

Figure 4A

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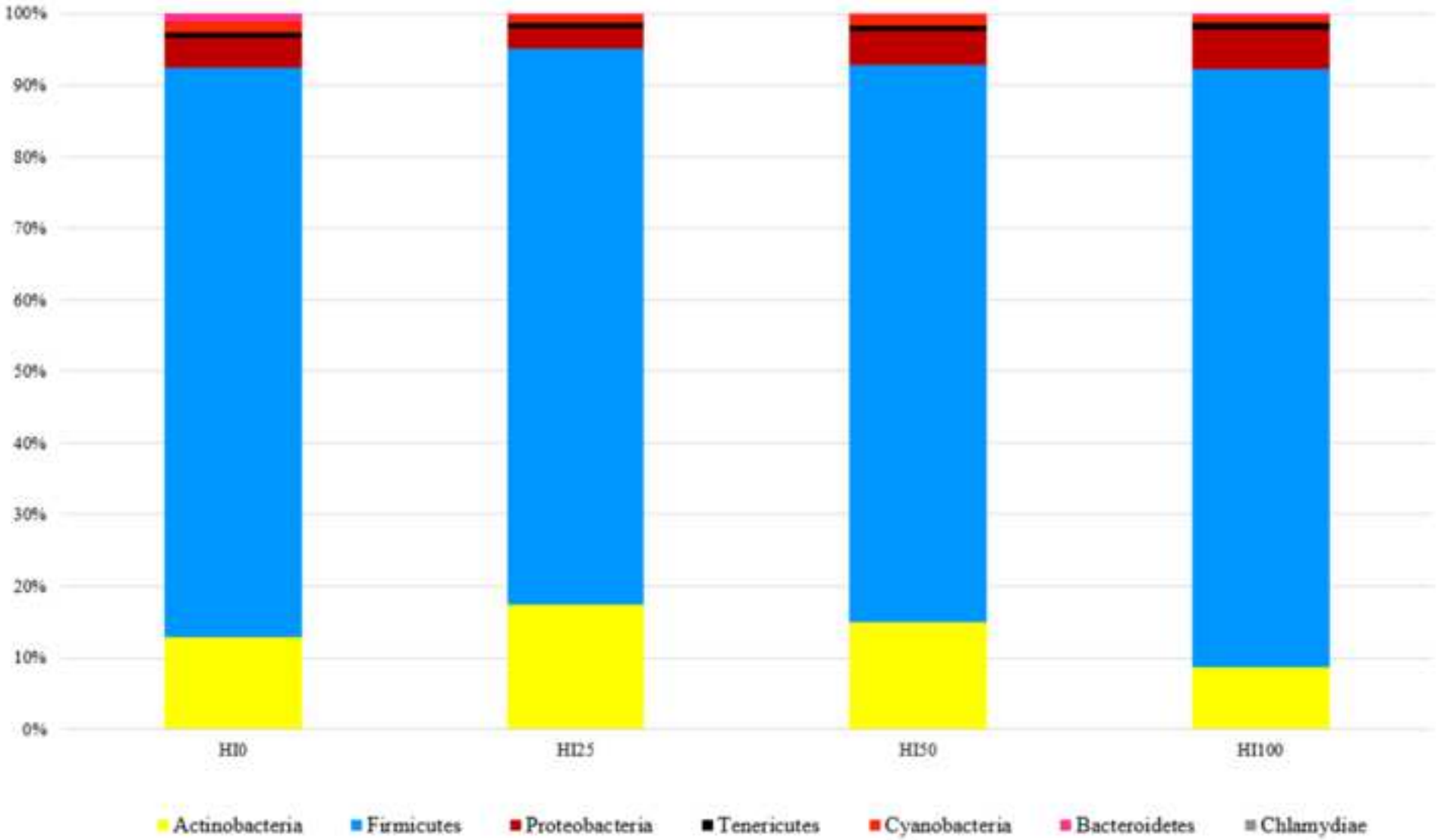

Figure 4B

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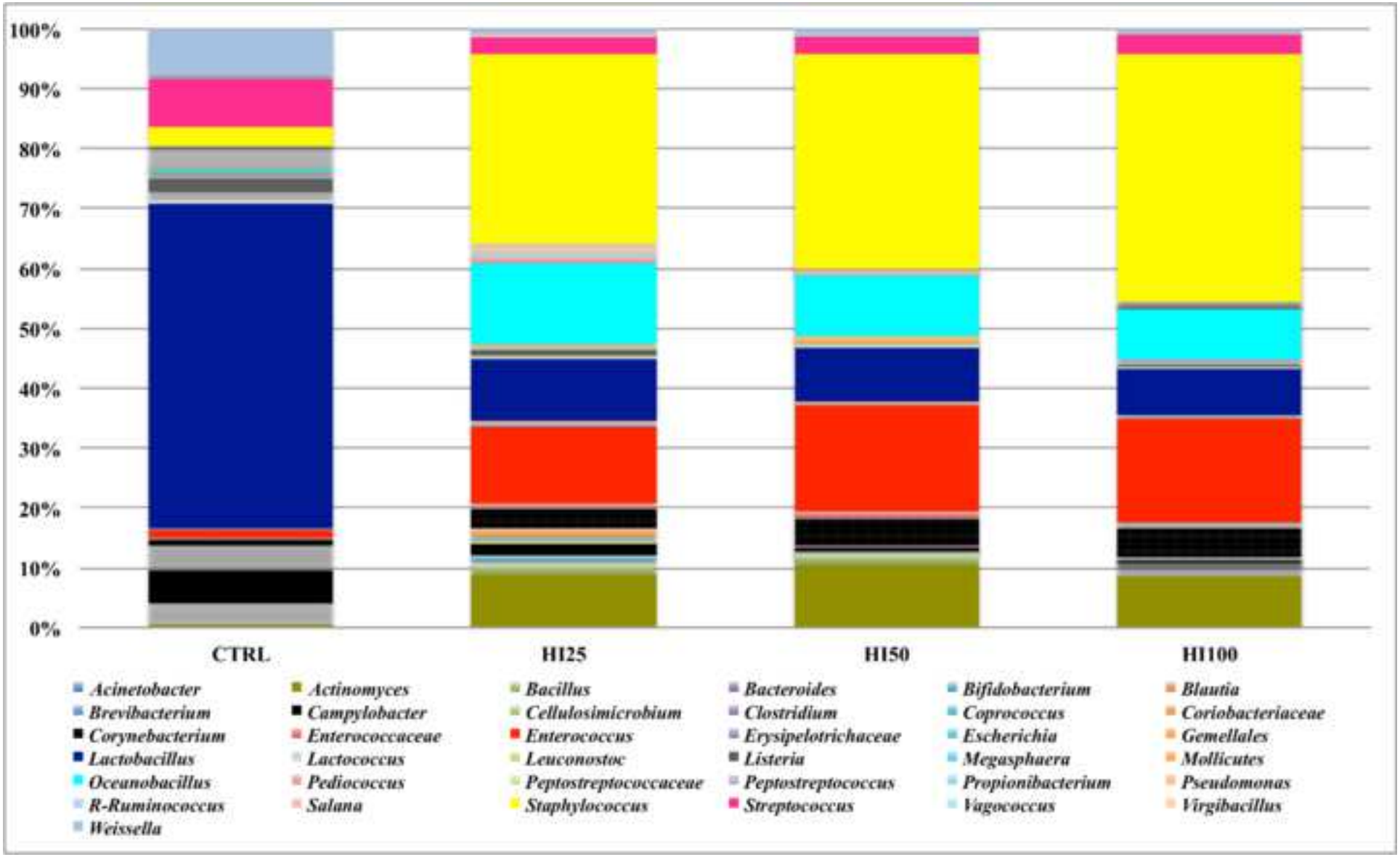

Figure 5

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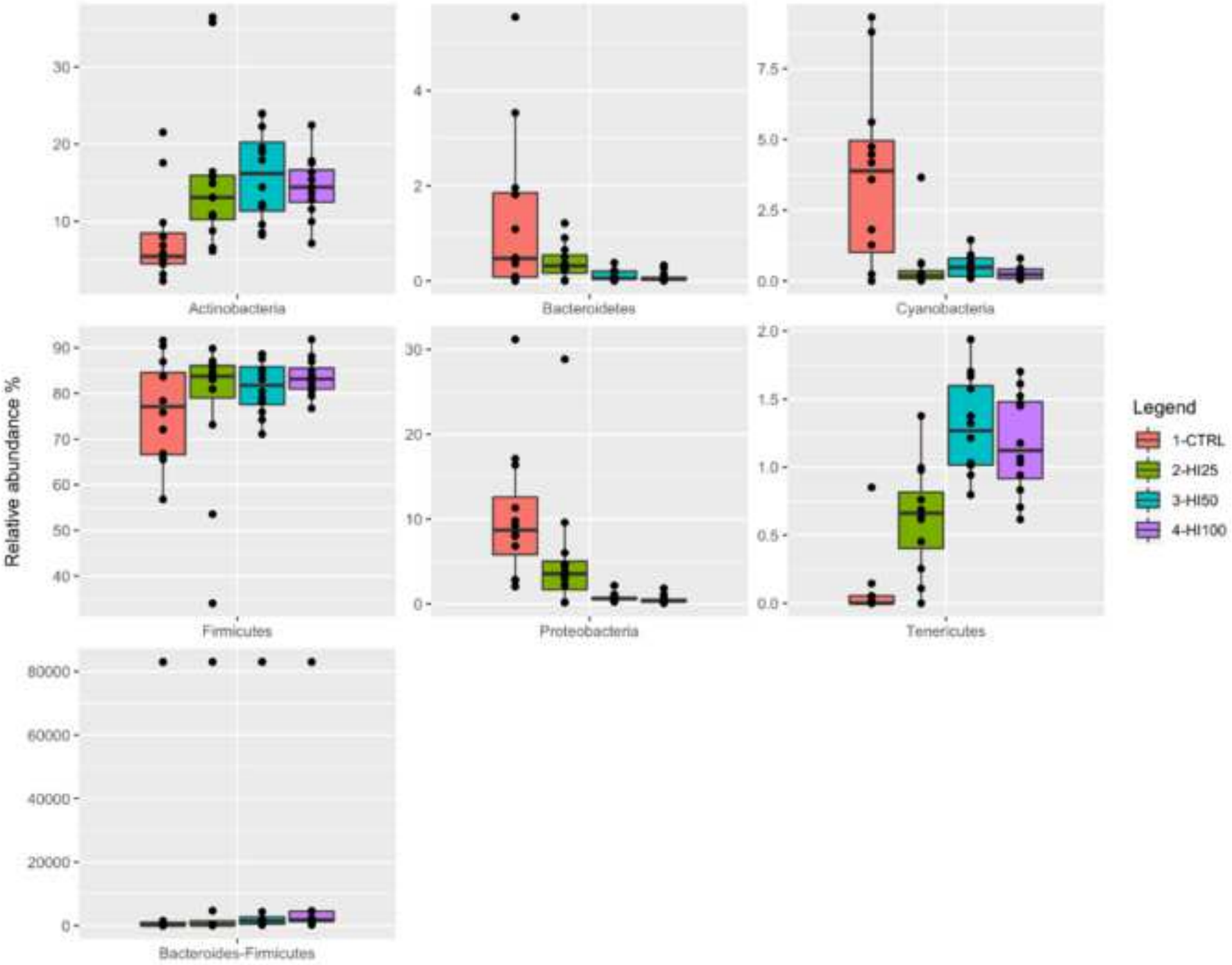

Figure 6

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