Effect of two insect diets on the intestinal commensal microbiome of healthy *Salmo trutta* vr. *trutta*

Agata Józefiak (agata.jozefiak@up.poznan.pl)
University of Life Sciences in Poznań
Silvia Nogales-Mérida
Animal Health Research Center (CISA-INIA)
Mateusz Rawski
University of Life Sciences in Poznań
Zuzanna Mikołajczak
University of Life Sciences in Poznań
Bartosz Kierończyk
University of Life Sciences in Poznań
Jan Włodarek
University of Life Sciences in Poznań
Jan Mazurkiewicz
University of Life Sciences in Poznań

Research Article

Keywords: sea trout, next generation sequencing, NGS, microbiome, metagenome, fish, mealworm, superworm

Posted Date: March 16th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-234130/v1

Abstract

Background The balance of the intestinal commensal microbiome of fish and other animals plays an important role in the physiological processes of healthy animals, contributes to the defense against pathogens, stimulates the immune system and facilitates nutrient metabolism. In the last decade, the use of insects in fish nutrition has been increasing exponentially, although little is known regarding the effects of insect meals on the fish gastrointestinal tract. The aim of this study was to evaluate the effect of two insect diets containing mealworm (MW) and superworm (SW) on the microbiome of the digesta of sea trout fingerlings and the relative abundances of different taxa among communities under controlled conditions.

Results The insect meals produced a similar weight gain and survival rate to sea trout fed fishmeal. The most abundant bacterial phylum in all the treatment groups was Firmicutes followed by Proteobacteria and Actinobacteria, and significant differences in the amount of Cyanobacteria were observed in the SW group.

Conclusions The insect meals did not produce differences in the three most abundant phyla in the sea trout digesta. However, the effect of each type of meal on the lower taxonomic levels was evident, particularly in the case of the superworm meal. These microbiome differences indicated that mealworm meal was more related to fishmeal than superworm meal. Our results highlight the potential effects of insect meals, such as mealworm and superworm meals, on the microbiota of sea trout.

Background

Fish, as well as other animals, must maintain the microbiome (bacteria, archaea, fungi, and viruses) in their intestinal tract in a balanced state, preserving the mutualistic relationship along their life cycles. The microbiome contributes to the defense against pathogens, stimulates the immune system and assists with nutrient metabolism [1]. Recently, several studies have related the importance of maintaining a balanced gut microbiota to the maintenance of a healthy status, and related a higher alpha diversity with healthy fish, which leads to improving the studies of the gut microbiota to develop methods to improve fish health [1], [2], [3], [4].

In the last decade, the use of insects in fish nutrition has been increasing exponentially, although little is known about the effects of insect meals on the fish gastrointestinal tract. Moreover, the use of different species of insects to feed a fish species, as well as different methods of insect meal preparation, are in most cases also unknown. Several studies have analyzed the fish microbiota using the next generation sequencing (NGS) technique. The microbial communities are affected by certain factors such as the species, the stage of development, the type of food consumed, and the intestinal morphology [5], [6]; environmental and physiological factors also modify the gut microbiota of fish [7]. The type of microbiome will also be conditioned by the feeding habit of the species; in salmonids such as rainbow trout, the predominant phyla are Proteobacteria, Firmicutes, Bacteriodetes, Fusobacteria and

Actinobacteria [8], [9]. However, Rimoldi et al. (2018) found that rainbow trout fed higher levels of plant meals and rendered animal meals, and lower levels of fishmeal exhibited more Fusobacteria and Bacteroidetes, and this difference was related to the lower growth performance [10]. Furthermore, Huyben et al. (2019) observed a variation in the abundance of a group of bacteria present in the gut of the rainbow trout according to the stage of development of the insect meal (larvae, prepupae, and pupae) [11]. The Proteobacteria and Firmicutes have been detected as dominated phyla in all gut regions of brown trout (*Salmo trutta* L.) [12]. Moreover, Michl et al. (2019) observed significantly increased abundances of Proteobacteria and Fusobacteria following the consumption of fishmeal, whereas plant-derived proteins increased the abundance of Firmicutes and Bacteriodetes [8].

In general, plant-based protein meals markedly modify the microbiome, as Kononova et al. (2019) showed the effects of soybean protein and carbohydrates, which are associated with some anti-nutritional factors, on the autochthonous microbiota, provoking inflammatory processes in the intestine of salmonids [13]. Regarding insect meals, Antonopoulou et al. (2019) found that rainbow trout fed 0 and 60% mealworm meal did not differ in the bacterial species or their amounts [14]. A possible explanation for this finding is that insects are part of the natural diet of this species.

Because insects are also part of the natural diet of sea trout, at least in the first stages of development, the aim of this study was to evaluate the effects of two insect meal diets on the microbiome of the digesta of sea trout fingerlings and the abundances of different taxa among communities.

Results

Growth performance

At the end of the experimental period, no significant differences in body weight gain and survival rates were observed among groups fed the different experimental diets, as shown in Fig. 1.

Microbiota diversity

In the sea trout gut microbiota, significant differences were observed in the number of bacteria and archaea among treatment groups, as fish fed the SW diet presented the lowest amount of bacteria and the highest amount of archaea (Fig. 2). In general, 99.95% of the microbiota was constituted by bacteria, 0.03% by archaea and 0.01% by viruses and 0.02% of the microorganisms were not identified. Considering bacteria, 24 phyla were identified, and 20 were represented with an abundance less than 1%, whereas the most predominant bacteria among the remaining phyla were Firmicutes (Fig. 3). In general, significant differences in the abundances of Actinobacteria, Proteobacteria, and Firmicutes were not observed, although the abundance of Cyanobacteria exhibited significant differences, as the SW group presented the lowest content of this phylum as well as the combined data for the 20 phyla ($p \leq 0.05$).

Table 1. ANOVA results of most dominant classes, orders, and families of sea trouts' digesta fed with the three experimental diets: control diet (CON), mealworm diet (MW), and

superworm diet (SW)

Identifications	CON	MW	SW	EE	P-value
Classes					
Actinobacteria	7.99	9.12	10.32	1.71	0.6415
Alphaproteobacteria	2.05	3.37	0.41	0.81	0.0826
Bacilli	41.57^{a}	50.27^{ab}	65.69^{b}	5.47	0.0350
Clostridia	32.92^{b}	26.77^{b}	13.53^{a}	2.75	0.0022
Gammaproteobacteria	5.43	2.97	7.43	3.74	0.7054
Nostocophycideae	8.24^{b}	6.24^{b}	1.80^{a}	1.07	0.0060
40 classes*	1.79^{c}	1.26^{b}	0.78^{a}	0.10	0.0003
Order					
Actinobacteria	7.99	9.12	10.32	1.71	0.6415
Alphaproteobacteria	2.05	3.37	0.41	0.81	0.0826
Bacilli	41.57^{a}	50.27^{ab}	65.69^{b}	5.47	0.0350
Clostridia	32.92^{b}	26.77^{b}	13.53^{a}	2.75	0.0022
Gammaproteobacteria	5.43	2.97	7.43	3.74	0.7054
Nostocophycideae	8.24^{b}	6.24^{b}	1.80^{a}	1.07	0.0060
87 orders*	1.79^{c}	1.26^{b}	0.78^{a}	0.10	0.0003
Families					
Bacillaceae	3.05^{b}	2.00^{a}	1.74^{a}	0.25	0.0106
Clostridiaceae	27.70^{b}	23.39^{b}	11.22^{a}	2.74	0.0056
Corynebacteriaceae	0.58^{a}	1.85^{b}	2.58^{c}	0.13	0.0001
Enterobacteriaceae	5.26	2.77	7.31	3.73	0.6993
Enterococcaceae	8.02^{a}	25.84^{b}	17.07^{ab}	4.10	0.0395
Lachnospiraceae	1.87^{b}	1.00^{ab}	0.71^{a}	0.28	0.0391
Lactobacillaceae	12.76^{a}	8.52^{a}	24.62^{b}	1.90	0.0006

Leuconostocaceae	3.09[a]	2.63[a]	7.55[b]	0.74	0.0020
Microbacteriaceae	0.25	1.46	1.05	0.47	0.2370
Micrococcaceae	4.10	3.09	2.75	0.47	0.1608
Nocardioidaceae	1.13	1.82	2.21	1.07	0.7790
Peptostreptococcaceae	1.61	1.29	0.87	0.22	0.1073
Rickettsiaceae	1.30[b]	0.68[a]	0.20[a]	0.12	0.0005
Rivulariaceae	8.10[b]	6.16[b]	1.77[a]	1.05	0.0060
Ruminococcaceae	1.11	0.64	0.42	0.21	0.1072
Streptococcaceae	10.70	7.23	11.07	0.99	0.0431
203 families*	9.27	9.52	6.80	0.96	0.1435

Values in the same row having different superscript letters are significantly different at $P < 0.05$, (n=4).

* Grouped data of the rest of classes, orders and families of bacteria with values lower than 0.5% plus the unidentified groups.

After observing the class distribution in sea trout digesta, Bacilli and Clostridia were the most predominant classes in the groups treated with the three experimental diets, although the SW group exhibited the highest percentage of Bacilli, but the lowest percentages of Clostridia, Nostocophycideae and other classes compared to groups fed the CON and MW diets (Table 1). A similar trend was observed for the class distribution, where Bacilli and Clostridia classes were the most predominant and the SW group exhibited the highest percentage of Bacilli and the lowest percentage of Clostridia among all three treatment groups. Superworm meal also reduced the amount of Nostocophycideae and the grouped orders (Table 1). In terms of the family distribution, fish fed the SW diet exhibited lower percentages of Bacillaceae, Clostridiaceae, Lachnospiraceae, Rickettsiaceae, and Rivulariaceae than fish fed the CON and MW diets. In contrast, higher abundances of Corynebacteriaceae, Lactobacillaceae, and Leuconostocaceae were observed in the SW group than in fish fed the CON and MW diets. However, the percentage of Enterococcaceae was significantly lower in fish fed the CON diet than in fish fed the SW and MW diets ($p < 0.05$).

Table 2. ANOVA results of the most representative bacterial phylotypes (%) isolated from the gut digesta of sea trout fed with the three experimental diets: control diet (CON), mealworm diet (MW), and superworm diet (SW).

Phyla	Species	CON	MW	SW	EE	P-value
Firmicutes	*Alkaliphilus crotonatoxidans*	4.04b	3.38b	1.75a	0.47	0.0186
Cyanobacteria	*Calothrix parietina*	8.10b	6.15b	1.77a	1.05	0.0060
Firmicutes	*Clostridium cadaveris*	15.47b	12.96b	5.94a	1.69	0.0085
Firmicutes	*Enterococcus avium*	4.49	5.58	2.90	0.87	0.1455
Firmicutes	*Enterococcus casseliflavus*	0.66	0.69	0.51	0.10	0.4619
Firmicutes	*Enterococcus durans*	0.30a	1.16a	5.78b	0.63	0.0004
Firmicutes	*Enterococcus gallinarum*	0.05a	0.25a	1.08b	0.10	0.0001
Firmicutes	*Enterococcus gilvus*	0.51a	0.64a	2.20b	0.23	0.0010
Firmicutes	*Enterococcus lactis*	0.18a	2.69b	2.60b	0.50	0.0095
Firmicutes	*Enterococcus mundtii*	0.004	2.10	0.04	0.90	0.2225
Firmicutes	*Enterococcus silesiacus*	0.01	7.39	0.07	3.00	0.1909
Proteobacteria	*Escherichia albertii*	1.53	0.77	2.10	1.08	0.6909
Firmicutes	*Lactobacillus antri*	1.35b	1.14b	0.52a	0.14	0.0064
Firmicutes	*Lactobacillus delbrueckii*	2.41b	0.11a	0.30a	0.41	0.0054
Firmicutes	*Lactobacillus oris*	2.32b	1.94ab	0.80a	0.30	0.0156
Firmicutes	*Lactococcus garvieae*	1.36a	1.23a	3.93b	0.48	0.0049

Firmicutes	*Pediococcus acidilactici*	0.06a	0.12a	2.24b	0.21	0.0001
Firmicutes	*Pediococcus pentosaceus*	0.15a	0.32a	8.80b	0.84	0.0001
Firmicutes	*Pediococcus stilesii*	0.04a	0.08a	1.89b	0.16	0.0001
Firmicutes	*Streptococcus gallinaceus*	2.80b	1.83ab	1.56a	0.30	0.0380
Firmicutes	*Weissella cibaria*	1.52a	1.42a	5.39b	0.55	0.0009
Firmicutes, Cyanobacteria, Proteobacteria, and Actinobacteria	1307 spp	20.39	20.33	18.48	1.51	0.6123

Five hundred forty-one genera were identified, which represented the 85.10 ±3.51% of the total samples. After comparing the most predominant genera present in fish digesta (Fig. 3), sea trout fed the CON diet presented higher amounts of *Clostridium* and *Lactobacillus*, and the highest content was observed for *Enterococcus* followed by *Clostridium* in the MW group, but the most representative genera in the SW group were *Pediococcus* and *Enterococcus*.

The total amount of bacterial species identified varied among treatment groups. In the CON diet group, only 67.75 ± 4.63% of the bacteria were identified, whereas the MW diet group exhibited the highest percentage of identification of species at 73.25 ± 3.36%, followed by the SW diet group at 70.07 ± 7.30%. When observing the most predominant species in each treatment group (Table 2), the fish fed the CON diet presented the highest percentage of *Clostridium cadaveris* and *Calothrix parietina.* In the case of the MW group, the most abundant species were also *C. cadaveris* and *C. parietina* along with *Enterococcus silesiacus*. Moreover, the highest abundances were observed for *Pediococcus pentosaceus*, *C. cadaveris*, and *Enterococcus durans*. In addition, significant differences were detected in the SW group, with the lowest levels observed for *Alkaliphilus crotonatoxidans, C. parietina, C. cadaveris, Lactobacillus antri, L. delbrueckii*, and *Streptococcus gallinaceus*, and the highest values observed for *E. durans, E. gallinarum*, *E. gilvus*, *Lactococcus garvieae, P. pentosaceus, P. acidilactici, P. stilesii* and *Weisella cibaria* ($p \leq 0.05$).

Regarding the species relations (Fig. 4), 40.26% of the species were shared among treatments and 11.96 to 13.17% of the species are unique to each treatment; a lower number of shared species were observed between two treatments. Additionally, the amount of lactic acid bacteria (LAB) increased significantly in the fish fed the insect meals, namely, 36.16% and 47.98% in the MW and SW groups, respectively, compared to fish fed the control diet at 27.29% ($p \leq 0.05$).

Table 3. Alpha diversity indexes in sea trout gut microbiota species, comparison among treatments

	CON	MW	SW	EE	P-value
Individuals	73.50	74.00	74.50	0.78	0.6760
Dominance (D)	0.15	0.12	0.12	0.01	0.3782
Simpson 1-D+	0.85	0.88	0.88	0.01	0.3781
Shannon (H)	3.05	3.15	3.16	0.06	0.3632
Evenness e^H/S	0.04	0.05	0.05	2.1^{-3}	0.1354
Brillouin	1.17	1.30	1.36	0.05	0.0966
Menhinick	51.05	49.28	50.80	1.69	0.7314
Margalef	118.58	114.28	117.63	4.09	0.7441
Equitability (J)	0.49	0.51	0.51	0.01	0.2154
Berger Parker	0.32	0.27	0.30	0.03	0.4398
Chao-1	510.50	492.75	508.00	16.88	0.7314

Values in the same row having different superscript letters are significantly different at $p \leq 0.05$, (n=4).

Regarding species richness, 1328 species were identified, and the species richness was 905 species for the CON group, 879 species for the MW group, and 905 species for the SW group. At the same time, the Margalef index (D_{Mg}) showed no significant differences among treatments. The alpha diversity indexes, such as Simpson Diversity Index (D), Menhinick index, and Shannon index (H), revealed that insect meals did not affect the species richness. Meanwhile, the Evenness index (e^H/S), and equitability Brillouin index also showed no significant differences among treatments. The dominance index (Berger-Parker) and Dominance D displayed similar values among meal-fed groups, and the dominance was low among groups. Regarding the abundance estimator, the Chao1 calculation showed no significant differences among treatments as well (Table 3).

The Bray-Curtis analysis of beta diversity is presented in Table 4. Additionally, the nonmetric multidimensional scaling (NMDS) analysis (Fig. 5) and the clusters showed that CON and MW groups were much more related, with 76.6% similarity, than the SW group (61.2%) (Fig. 6).

Table 4. ANOSIM and Bray-Curtis results

ANOSIM	Bray-Curtis
Permutation N:	9999
Mean rank within:	14.67
Mean rank between:	40.56
R:	0.787
p (same):	0.003

Discussion

In the last several decades, the importance of the gut microbiota has been documented in numerous studies showing that growth performance and fish health are closely related to the microbiota. As Butt and Volkoff (2019) commented, feeding habits influence the structure and composition of the gut microbiota [15]. Additionally, plant-based proteins change the content and structure of the autochthonous microbiota of carnivorous species (Kononova et al. 2019) such as sea trout; in contrast, the use of a natural source of protein such as insect meals may play a role in maintaining the amount of these types of microorganisms that are part of the gut environment of the fish and enhance fish health [13]. Furthermore, insect meals would be able to modulate the microbiota of these animals due to the chitin and antimicrobial peptide contents [16], [17].

In this trial, more than 40% of FM (fish meal) was replaced with insect meals in the two diets, although the insect meals produced similar growth and survival rates when observing the weight gain. When analyzing the bacteria present in the digesta, the dominant phylum in all the treatment groups was Firmicutes, in contrast to brown trout fed a commercial diet, in which the dominant phylum was Proteobacteria, ranging from 88.4 to 92.6% [12]. However, Michl et al. (2019) reported a reduction in the amount of Proteobacteria and an increase in the amounts of Firmicutes and Bacteriodetes in the same species fed diets with more plant-based meals [8]. In contrast, Rimoldi et al. (2019) detected a gradual increase in the abundance of Tenericutes and a reduction in the abundances of Proteobacteria and Firmicutes in n rainbow trout fed different amounts of black soldier fly meals [18]. Moreover, Kononova et al. (2019) affirmed that Proteobacteria is more abundant in carnivorous species and Firmicutes is more abundant in herbivorous species [13]. The results from the present trial showed that the abundance of Firmicutes would be conditioned by the amount of plant meal in the three diets, which was approximately 47% of the total, but not the inclusion of insect meals.

After performing a detailed analysis of the classes present in the digesta, Bacilli was the most abundant in all treatment groups, followed by Clostridia, both of which belong to the Firmicutes phylum, but the sum of Alphaproteobacteria and Gammaproteobacteria, which belong to the Proteobacteria phylum, presented similar amounts in all treatment groups, indicating that MW and SW meals exerted the same effect as FM on the digesta of sea trouts. In addition, that the phylum Firmicutes and class Clostridia

have been repeatedly identified in the digestive tracts of herbivorous fish, and as described above, the higher abundance of this class would be related to the higher amount of plant meal present in the diets [6].

The order and family distribution followed a similar trend as the class distribution. Although the bacterial genera exhibited changes based on the type of protein meal source, the most representative genera in the CON group were *Clostridium* and *Lactobacillus* and those in the MW group were *Enterococcus* and *Clostridium*, but the most representative genera in the SW group were *Pediococcus* and *Enterococcus*. With the exception of *Pediococcus*, the other genera are used as probiotics in aquaculture, increasing bacterial diversity [15], which probably occurred in fish fed these insect meals. The type of meals exerted a direct effect on the abundance of different genera in the intestinal digesta.

An analysis of the species abundance showed that fish fed the diet with SM exhibited a decrease in the relative abundance of *C. cadaveris* compared to the CON and MW groups; this species is known as a component of the normal fecal flora of humans and animals, which affects people with a poor overall condition of immunosuppression [19]. On the other hand, *C. cadaveris* is one of the most prominent bacterium present during the decay of dead bodies [19]. Moreover, *C. cadaveris* might trigger bacteremia that is related to a high mortality rate in humans. In the present study, the relative abundance of the commensal species *C. cadaveris* was decreased in the SW group, but significant differences were not observed between the CON and MW groups. Therefore, the reduction in the relative abundance *C. cadaveris* in the gastrointestinal tract of fish induced by the diet containing SW should may considered as a positive effect on public health.

C. parietina belongs to phylum Cyanobacterium and was previously detected in alkaline and oxygenated freshwaters [20]. The growth of Cyanobacteria is stimulated by the hypoxia of water reservoirs. Moreover, the contamination of dry food and feed with Cyanobacterium is considered a risk of toxin prevalence. Moreover, *C. parietina* is the bacteria with a higher potential for endotoxin production. The diet containing SW caused a decrease in the abundance of the bacterial species *C. parietina* in the fish GIT, which may reduce possible cyanobacterial toxin reservoirs in the fish GIT.

The diet containing SW improved the commensal probiotic microbiome in intestinal digesta of *Salmo trutta* vr. *trutta*. The SW diet increased the abundance of some bacterial genera, such as *Pediococcus* that is considered a positive fish GIT bacteria. *Pediococcus* is a genus of gram-positive lactic acid-producing bacteria belonging to the family Lactobacillaceae. In the SM group, an increase in the abundance of pediococci was observed, with the most abundant species identified as *P. pentosaceus* in the SW group. The bacterial species *P. pentosaceus* exerts bacteriocynogenic effects on *Staphylococcus aureus* and *Escherichia coli* [21]. *P. pentosaceus* is mostly associated with food fermentation; it produces pendocins that are safe for food preservation and is used as a starter culture in the fermentation of meat products.

Enterococcus is a key component of the intestinal flora of humans and is widespread in the intestines of most animals, including fish. Some species belonging to the *Enterococcus* genus, such as *Enterococcus faecalis* from fish intestine, are use as aquatic probiotics [22]. The SM diet increased the abundance of

some Enterococcus species in the fecal digesta, among which *E. durans* may be considered a possible probiotic, because it potentially produces bacteriocins, namely, durancins [23]. Another species with probiotic potential that have been isolated from fish is *Enterococcus gallinarum* that regulates the innate immune response [24]. An increase in the abundance of *Enterococcus gilvus* was also observed in the fecal digesta of the analyzed SW group. The analysis of gene expression in *Enterococcus gilvus* has identified novel carotenoid biosynthesis genes that improve the multistress tolerance of *Lactococcus lactis* and promotes their activity toward methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci (VRE) [25]. Additionally, *W. cibaria*, which was more abundant in the SW group, has shown to be an effective probiotic in hybrid surubim [26]. Although significant differences among certain groups of bacteria were observed, the composition of the most representative species shows that they are part of the digestive tract flora, the environment, or part of the protein sources with probiotic properties that help the fish to thrive and achieve target growth and survival rates. In addition, Gajardo et al. (2016) commented that LAB are more abundant in salmon fed a plant-based diet than in fish fed a fishmeal-based diet [27], although, Ringø and Gatesoupe (1998) commented that LAB, such as the *Lactobacillus, Carnobacterium*, and *Streptococcus* genera, are also commonly detected in healthy fish microbiota of different fish species, including salmonids [28]. However, insect meals also increase the amount of LAB, as observed in the present study.

Our research diet containing SW decreased the relative abundance of *Streptococcus gallinaceus* in the intestinal digesta. *S. gallinaceus* was first described in 2002 and was isolated from clinical samples of chickens. In 2003, *S. gallinaceous* was isolated from an outbreak of septicemia associated with a high prevalence of endocarditis in a flock of broiler parents. Chadfield et al. (2005) reported an association of this species with septicemia and endocarditis in chickens [29]. The decrease in the relative abundance of *S. gallinaceus* detected in the intestinal digesta might be considered a positive dietary effect of SW.

Furthermore, when comparing alpha diversity parameters, the inclusion of insect meal in the diet did not modify the different parameters measured, such as richness, evenness, and dominance. The Shannon H values were similar to those obtained in rainbow trout fed only FM and greater than 60% of MW meal [14]. Additionally, the Chao1 values obtained in the present study were similar to those observed in the digesta of the proximal intestine of salmon fed 45% FM and 38% plant meals [27]. These authors obtained a higher Shannon H index than observed in our results. Moreover, in brown trout fries fed three experimental diets, 100% FM, 50% and 90% plant-based diets followed by a crossover feeding design, plant-based diets produced higher Chao1 and Shannon indexes than the FM diet, although the Chao1 values were lower than the values reported for sea trout in this experiment [8]. In general, the diversity among treatments was similar. Additionally, the NMDS analysis and the similarities of the clusters showed that the microbiome of the MW group is more similar to the FM group than the SW group, which would be more useful for salmonid nutrition, as described by Antonopoulou et al. (2019) [14].

As mentioned above, the two insect meals exerted a similar effect to FM on maintaining the alpha diversity, and the values of dominance, equitability, and evenness were similar between all treatment

groups, showing a balanced microbiome population that varied in abundance among bacterial classes, orders, genus and species as a natural consequence of the type of protein sources used. Nevertheless, the different meals that the fish consumed exerted positive effects on the microbiome, growth and survival performance of the sea trout, although the predominance of phylum Firmicutes in all treatment groups would be a consequence of the amount of plant meals, which were higher (47.17%) than animal meals (33.5%) in the diets, particularly for soybean meal, as highlighted by Kononova et al. (2019) and Michl et al. (2019) [8], [13]. Nevertheless, we cannot forget that plant meals are part of all commercial diets because of their availability and lower prices than fishmeal, and they are used to study the effects of alternative meals, such as insect meals.

Conclusion

Insects are part of the sea trout diet in nature, at least in the first stages of development, before these fish feed on more diverse prey, including other fish. To conclude, this finding may explain why the main phyla present in the digesta were similar in all the treatment groups. However, the effect of each type of meal on the lower taxonomic levels was evident, particularly in the case of superworm meal. These differences were highlighted through the NMDS and the clusters, where fish fed mealworm meal were more related to fish fed fishmeal than fish fed superworm meal. Nevertheless, further studies are necessary to corroborate the finding that insect meals are one of the best alternatives to replace fishmeal in the diets of carnivorous fish.

Methods

Fish rearing conditions and experimental diets

Living insects were provided by HiProMine S.A (Robakowo, Poland). The larvae were euthanized by freezing at -20°C for 24 h, after which the insects were oven-dried at 50°C for 24 h and finely ground. Then, two commercial (*Bacillus amyloliquefaciens*) endopeptidases COROLASE® 7090 (AB Enzymes GmbH, Darmstadt Germany) were incubated with 1.5 g/kg protein for 5 h at 50°C. Afterwards, 0.75 g kg^{-1} of the fungal protease enzyme FLAVOURZYME® (endopeptidase and exopeptidase from *Aspergillus oryzae* supplied by Novozymes A/S, Denmark) was applied to hydrolyze the dried larvae meals for 3 h in two subsequent steps. The hydrolyzed meals were stored at 4°C until diet preparation.

Table 5. Chemical composition of the three experimental diets: fishmeal diet (CON); enzyme hydrolysed mealworm diet (MW); enzyme hydrolysed superworm diet (SW).

A control diet (CON) and two experimental diets were formulated. A fixed 10% of hydrolyzed insect meal was included in both experimental diets, corresponding to 42% and 44% of fishmeal replacement by hydrolyzed mealworm (MW) and superworm (SW) diets, respectively. The diets were manufactured at the Aquaculture Experimental Station in Muchocin, Poland using a semi-industrial single-screw extruder

Ingredients (g kg^{-1})	Diets		
	CON[a]	MW[b] (42%)	SW[c] (44%)
Fish meal	250	145	140
Mealworm meal[a]	-	100	-
Superworm meal[b]	-	-	100
Soybean meal	100	100	100
Wheat flour	219	220	226
Corn gluten	150	150	150
Blood meal	70	100	100
Brewer yeast	35	35	35
Fish oil	164	143	140
Dicalcium phosphate	7.2	0.8	2.1
Premix[c]	1.5	1.5	1.5
DL-Methionine	1.2	2.2	2.4
L-Lysine HCL	1.1	1.8	2.0
L-Threonine	0.6	0.6	0.7
Proximate analysis (% DM)			
Dry matter	93.0	93.7	93.5
Crude protein	48.0	51.1	49.8
Crude lipid	16.3	14.6	15.3
Ash	6.5	5.4	5.1
Crude fibre	1.7	1.7	1.7
Chitin[d]	0	9.3	4.8
NFE[e]	35.0	33.8	35.0
Gross energy (MJ kg^{-1})	22.18	22.77	22.55

[a] Mealworm meal (dry matter: 95.58%, crude protein: 47.0%, crude lipid: 29.6%)

[b] Superworm meal (dry matter: 96.32%, crude protein: 49.3%, crude lipid: 33.6%)

[c] Polfamix BASF Poland Ltd. (Kutno, Poland) (g kg^{-1}): vitamin A, 1 000 000 IU; vitamin D_3, 200 000 IU; vitamin E, 1.5 g; vitamin K, 0.2 g; vitamin B_1, 0.05 g; vitamin B_2, 0.4 g; vitamin

B_{12}, 0.001 g; nicotinic acid, 2.5 g; D-calcium pantothenate, 1.0 g; choline chloride, 7.5 g; folic acid, 0.1 g; methionine, 150.0 g; lysine, 150.0 g; Fe, 2.5 g; Mn, 6.5 g; Cu, 0.8 g; Co, 0.04 g; Zn, 4.0 g; J, 0.008 g; carrier > 1000.0 g.

[d] Calculated based on chitin content of insect meals.

[e] Nitrogen-free extract = 1,000 – (crude protein + ether extract + crude fibre + ash).

(Metalchem S-60, Gliwice, Poland) at 110°C to obtain pellets with 1.5-mm and 2.5-mm diameters. After extrusion, pellets were dried in an oven for 48 h at 40°C, and then fish oil was added to the mildly heated pellets. The nutritional values are shown in Table 5.

Sea trout were transported from the Feed Production Technology and Aquaculture Experimental Station in Muchocin, Poland, to the Division of Inland Fisheries and Aquaculture laboratory was experiment were conducted. At the beginning of the experimental period, 225 sea trout fingerlings with an average body weight of 5.08 ± 0.9 g were weighed individually and distributed randomly into nine tanks. The fiberglass tanks with a 60-L capacity were supplied with water from the reservoir in an open-flow system at a rate of 2 L min^{-1}. Water parameters were recorded daily. The temperature was 14.7 ± 0.6°C, the dissolved oxygen content was maintained at a constant value of 7.5 ± 0.3 mg L^{-1} and the photoperiod was maintained at 16:8 (light:dark) during the entire experiment.

The fish were housed in the experimental tanks for 60 days, after which the animals were sacrificed by an overdose of clove oil, according to the EU (no 2010/63/EU) regulation for experimental animals. Under sterile conditions, the animals were dissected and the digesta from the distal part of the intestine were collected. The samples were pooled by 4 fish and immediately packed, sealed in sterilized plastic bags, frozen, and stored at −80 °C for analyses of the microbial populations by next-generation sequencing (NGS).

Bacterial DNA Extraction and 16Sr RNASequencing

The research was conducted in accordance with the methodology of the Authors' previous research [30], [31].

DNA was extracted with a commercial kit (Sherlock AX, A&A Biotechnology, Poland) according to the manufacturer’s instructions. Samples were mechanically lysed on FastPrep - 24 on Zirconia beads (A&A Biotechnology, Poland) and additionally lysed enzymatic towards bacteria. The presence of bacterial DNA in the samples was confirmed using Real-Time PCR on termocycler Mx3000P (Stratagene, USA) with SYBR Green as fluorochrome. In the reaction for amplification of 16S rDNA, the following universal reaction primers were used: 1055F 5'-ATGGCTGTCGTCAGCT-3' and 1392R 5'-ACGGGCGGTGTGTAC-3'. The temperature profile of reaction was: 95 °C, 3 min; 95 °C, 15 s; 58 °C, 30 s; 72 °C, 30 s; Tm 65 °C -> 95 °C.

DNA was quantified using the NanoDrop and standardized at 5 ng/µl. Microbial diversity was studied by sequencing the amplified V3-V4 region of the 16S rRNA gene by using primers 16S Amplicon PCR Forward Primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG 16S Amplicon PCR Reverse Primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC. PCR conditions: 95 °C for 3 min; 25 cycles of: 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, 72 °C for 5 min, hold at 4 °C. The expected size on a Bioanalyzer trace after the Amplicon PCR step is ~550 bp. The PCR products were cleaned up step uses AMPure XP beads. The libraries were sequenced running 2 × 300 bp paired-end reads. The PCR products were cleaned, and the library was combined with the sequencing adapters and the dual indices using the Nextera XT Index Kit (Illumina, San Diego, CA, USA), according to 16S Metagenomic Sequencing Library Preparation instruction (Illumina, San Diego, USA). The PCR with Nextera XT Index Primers was carry out in following conditions: 95 °C for 3 min; 8 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, 72 °C for 5 min, hold at 4 °C. The PCR products were cleaned up again with AMPure XP beads. The library was validated to the expected size on a Bioanalyzertrace for the final library of ~630 bp. The libraries were quantified using a fluorometric quantification method using dsDNA binding dyes. Individual concentrations of DNA libraries were calculated in nM, based on the size of DNA amplicons, as determined by an Agilent Technologies 2100 Bioanalyzer.

For sequencing, the individual libraries were diluted for 4 nM, denaturated with 10 mM Tris pH 8.5, and spiked with 20% (*v*/*v*) of PhiX. Aliquots with 5 µl of diluted DNA were mixed for pooling libraries preparation for MiSeq (Illumina, San Diego, CA, USA) run. Then, >100,000 reads were performed per sample.

Metagenomic Analysis

The research was conducted in accordance with the methodology of the Authors' previous research [30], [31].

The microbiome sequences were classified according the V3 and the V4 amplicons and analyzed using a database of 16S rRNA data. Specific sequences, 341F and 785R, were used for the amplification and the libraries preparation. For the table PCR reaction with Q5 Hot Start High-Fidelity 2X Master Mix available, reaction conditions were performed in accordance with the manufacturer’s requirements. Sequencing took place on the MiSeq sequencer in paired-end (PE) technology at 2 × 250 nt using Illumina v2 kit. Automatic initial data analysis was performed on the MiSeq apparatus using the MiSeq Reporter (MSR) v2.6 software. The analysis consisted of two stages: automatic demultiplexing of samples and generating fastq files containing raw reads. The output of sequencing was a classification of reads at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. Quality analysis of the sequence was conducted with quality control and filtration to obtain high-quality sequences. Valid sequences were screened from samples according to the barcode at both ends of the sequence and were corrected for the direction by the primer sequences. All valid and filtered sequences were clustered into

operational taxonomic units (OTUs) based on a 97% 16S rRNA gene sequence identity level. The obtained sequences were checked with BLAST

(Basic Local Alignment Search Tool) and searched against the Greengenes database (http://greengenes.lbl.gov) to determine the phylogeny of the OTU. The results were classified at several taxonomic levels: kingdom, phylum, class, order, family, genus, and species. Relative abundance profiles of cecal microbiota were established according to OTU abundance of different groups.

Statistical Analysis

The research was conducted in accordance with the methodology of the Authors' previous research [30], [31].

Bioinformatic analysis ensuring the classification of readings by species level was carried out with the free Infostat software was used for the one-way ANOVA, and if significant differences were observed among treatment groups, data were further analyzed using Tukey's post hoc test.

The beta diversity measure was calculated based on the Bray–Curtis method [32].

The Kolmogorov-Smirnov test was used to determine the normality of the data distribution and equality of variances. Data are presented as the means ± standard errors of the means (SEM). Statistical significance was declared at $p \leq 0.05$.

Abbreviations

NGS: next generation sequencing

CON: control diet

MW: mealworm diet

SW: superworm diet

FM: fish meal

DM: dry matter

NMDS: nonmetrics multidimensional scaling

Declarations

Availability of data and materials

Raw data for calculation of tables and figures are available from the corresponding author upon request.

Acknowledgements

Not applicable

Funding

This work was funded two sources: the project TEAM TECH no. POIR.04.04.00-00-204E/16-00, entitled Insects as Novel Protein Sources for Fish and Poultry, financed by the Foundation of Polish Science (POIR 4.4); Faculty of Veterinary Medicine and Animal Science Poznan University of Life Sciences, Poland; Department of Veterinary Medicine and Animal Science no. 506.514.05.00.

Availability of data and materials

All data are included in this published article. The raw datasets are available from the corresponding author on reasonable request.

Authors' contributions

AJ: preparation of manuscript and data analysis. SNM, MR, BK, ZM, AJ: running of laboratory and statistical analysis. JW: prepared figures. SNM prepared the draft of manuscript. JM, MR: conducted and designed the experiment. All authors contributed to the analysis of the data, discussion of results and implications and commented on the manuscript at all stages. All authors read and approved the final manuscript.

Ethics approval

All animal handling protocols and methods complied with the recommendations of Directive 2010/63/EU of the European Parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes, the Polish law of 15 January 2015 on the protection of animals used for scientific purposes (Dz.U.2015 poz. 266), and the good practices and recommendations of the National Ethics Committee for Animal Experiments and the Local Ethics Committee for Animal Experiments of Poznan University of Life Sciences. The trial was performed in the Division of Inland Fisheries and Aquaculture, a unit of the Faculty of Veterinary Medicine and Animal Science of Poznan University of Life Sciences with certify for animal experiments (approved unit no. 0091) by the National Ethics Committee for Animal Experiments (based on authorization by the Ministry of Science and Higher Education). The study was carried out in compliance with the ARRIVE guidelines. All methods were carried out in accordance with relevant guidelines and regulations.

According to Polish law and the EU directive (no 2010/63/EU), the experiments conducted within this study did not require the approval of the Local Ethical Committee for Experiments on Animals in Poznan.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

References

1. Gagliardi A, Totino V, Cacciotti F, et al (2018) Rebuilding the Gut Microbiota Ecosystem. International Journal of Environmental Research and Public Health 15:1679. https://doi.org/10.3390/ijerph15081679
2. Xiong J-B, Nie L, Chen J (2019) Current understanding on the roles of gut microbiota in fish disease and immunity. Zoological research 40:70–76. https://doi.org/10.24272/j.issn.2095-8137.2018.069
3. Austin B (2011) Taxonomy of bacterial fish pathogens. Veterinary Research 42:20. https://doi.org/10.1186/1297-9716-42-20
4. Rimoldi S, Gini E, Koch JFA, et al (2020) Effects of hydrolyzed fish protein and autolyzed yeast as substitutes of fishmeal in the gilthead sea bream (Sparus aurata) diet, on fish intestinal microbiome. BMC Veterinary Research 16:118. https://doi.org/10.1186/s12917-020-02335-1
5. Ye L, Amberg J, Chapman D, et al (2014) Fish gut microbiota analysis differentiates physiology and behavior of invasive Asian carp and indigenous American fish. The ISME journal 8:541–551. https://doi.org/10.1038/ismej.2013.181
6. Egerton S, Culloty S, Whooley J, et al (2018) The Gut Microbiota of Marine Fish. Frontiers in Microbiology 9:873. https://doi.org/10.3389/fmicb.2018.00873
7. Dehler CE, Secombes CJ, Martin SAM (2017) Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (Salmo salar L.). Aquaculture 467:149–157. https://doi.org/https://doi.org/10.1016/j.aquaculture.2016.07.017
8. Michl SC, Beyer M, Ratten J-M, et al (2019) A diet-change modulates the previously established bacterial gut community in juvenile brown trout (Salmo trutta). Scientific Reports 9:2339. https://doi.org/10.1038/s41598-019-38800-7
9. Michl SC, Ratten J-M, Beyer M, et al (2017) The malleable gut microbiome of juvenile rainbow trout (Oncorhynchus mykiss): Diet-dependent shifts of bacterial community structures. PLOS ONE 12:e0177735
10. Rimoldi S, Terova G, Ascione C, et al (2018) Next generation sequencing for gut microbiome characterization in rainbow trout (Oncorhynchus mykiss) fed animal by-product meals as an alternative to fishmeal protein sources. PLOS ONE 13:e0193652
11. Huyben D, Vidaković A, Werner Hallgren S, Langeland M (2019) High-throughput sequencing of gut microbiota in rainbow trout (Oncorhynchus mykiss) fed larval and pre-pupae stages of black soldier fly (Hermetia illucens). Aquaculture 500:485–491. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.10.034
12. Al-Hisnawi A, Ringø E, Davies SJ, et al (2015) First report on the autochthonous gut microbiota of brown trout (Salmo trutta Linnaeus). Aquaculture Research 46:2962–2971.

https://doi.org/10.1111/are.12451

13. Kononova S V, Zinchenko D V, Muranova TA, et al (2019) Intestinal microbiota of salmonids and its changes upon introduction of soy proteins to fish feed. Aquaculture International 27:475–496. https://doi.org/10.1007/s10499-019-00341-1
14. Antonopoulou E, Nikouli E, Piccolo G, et al (2019) Reshaping gut bacterial communities after dietary Tenebrio molitor larvae meal supplementation in three fish species. Aquaculture 503:628–635. https://doi.org/https://doi.org/10.1016/j.aquaculture.2018.12.013
15. Butt RL, Volkoff H (2019) Gut Microbiota and Energy Homeostasis in Fish. Frontiers in Endocrinology 10:9. https://doi.org/10.3389/fendo.2019.00009
16. Gasco L, Finke M, van Huis A (2018) Can diets containing insects promote animal health? Journal of Insects as Food and Feed 4:1–4. https://doi.org/10.3920/JIFF2018.x001
17. Józefiak A, Engberg RM (2017) Insect proteins as a potential source of antimicrobial peptides in livestock production. A review. Journal of Animal and Feed Sciences 26:. https://doi.org/10.22358/jafs/69998/2017
18. Rimoldi S, Gini E, Iannini F, et al (2019) The Effects of Dietary Insect Meal from Hermetia illucens Prepupae on Autochthonous Gut Microbiota of Rainbow Trout (Oncorhynchus mykiss). Animals 9:143. https://doi.org/10.3390/ani9040143
19. Schade RP, Van Rijn M, Timmers HJLM, et al (2006) Clostridium cadaveris bacteraemia: Two cases and review. Scandinavian Journal of Infectious Diseases 38:59–62. https://doi.org/10.1080/00365540500388792
20. Rinkel BE, Manoylov KM (2014) *Calothrix* — an evaluation of fresh water species in United States rivers and streams, their distribution and preliminary ecological findings. Proceedings of the Academy of Natural Sciences of Philadelphia 163:43–59. https://doi.org/10.1635/053.163.0108
21. Bajpai VK, Han J-H, Rather IA, et al (2016) Characterization and Antibacterial Potential of Lactic Acid Bacterium Pediococcus pentosaceus 4I1 Isolated from Freshwater Fish Zacco koreanus. Frontiers in Microbiology 7:2037. https://doi.org/10.3389/fmicb.2016.02037
22. Mao Q, Sun X, Sun J, et al (2020) A candidate probiotic strain of Enterococcus faecium from the intestine of the crucian carp Carassius auratus. AMB Express 10:40. https://doi.org/10.1186/s13568-020-00973-0
23. Hanchi H, Mottawea W, Sebei K, Hammami R (2018) The Genus Enterococcus: Between Probiotic Potential and Safety Concerns—An Update. Frontiers in Microbiology 9:1791. https://doi.org/10.3389/fmicb.2018.01791
24. Román L, Padilla D, Acosta F, et al (2015) The effect of probiotic Enterococcus gallinarum L-1 on the innate immune parameters of outstanding species to marine aquaculture. Journal of Applied Animal Research 43:177–183. https://doi.org/10.1080/09712119.2014.928635
25. Hagi T, Kobayashi M, Kawamoto S, et al (2013) Expression of novel carotenoid biosynthesis genes from Enterococcus gilvus improves the multistress tolerance of Lactococcus lactis. Journal of Applied Microbiology 114:1763–1771. https://doi.org/10.1111/jam.12182

26. Mouriño JLP, Pereira G do V, Vieira F do N, et al (2016) Isolation of probiotic bacteria from the hybrid South American catfish Pseudoplatystoma reticulatum×Pseudoplatystoma corruscans (Siluriformes: Pimelodidae): A haematological approach. Aquaculture Reports 3:166–171. https://doi.org/https://doi.org/10.1016/j.aqrep.2016.03.001
27. Gajardo K, Rodiles A, Kortner TM, et al (2016) A high-resolution map of the gut microbiota in Atlantic salmon (Salmo salar): A basis for comparative gut microbial research. Scientific Reports 6:30893. https://doi.org/10.1038/srep30893
28. Ringø E, Gatesoupe F-J (1998) Lactic acid bacteria in fish: a review. Aquaculture 160:177–203. https://doi.org/https://doi.org/10.1016/S0044-8486(97)00299-8
29. Chadfield MS, Bojesen AM, Christensen JP, et al (2005) Reproduction of sepsis and endocarditis by experimental infection of chickens with Streptococcus gallinaceus and Enterococcus hirae. Avian Pathology 34:238–247. https://doi.org/10.1080/03079450500112252
30. Józefiak A, Benzertiha A, Kierończyk B, et al (2020) Improvement of cecal commensal microbiome following the insect additive into chicken diet. Animals 10:. https://doi.org/10.3390/ani10040577
31. Drażbo AA, Juśkiewicz J, Józefiak A, Konieczka P (2020) The Fermentation Process Improves the Nutritional Value of Rapeseed Cake for Turkeys—Effects on Performance, Gut Bacterial Population and Its Fermentative Activity. Animals 10:1711. https://doi.org/10.3390/ani10091711
32. Bray JR, Curtis JT (1957) An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecological Monographs 27:325–349. https://doi.org/10.2307/1942268

Figures

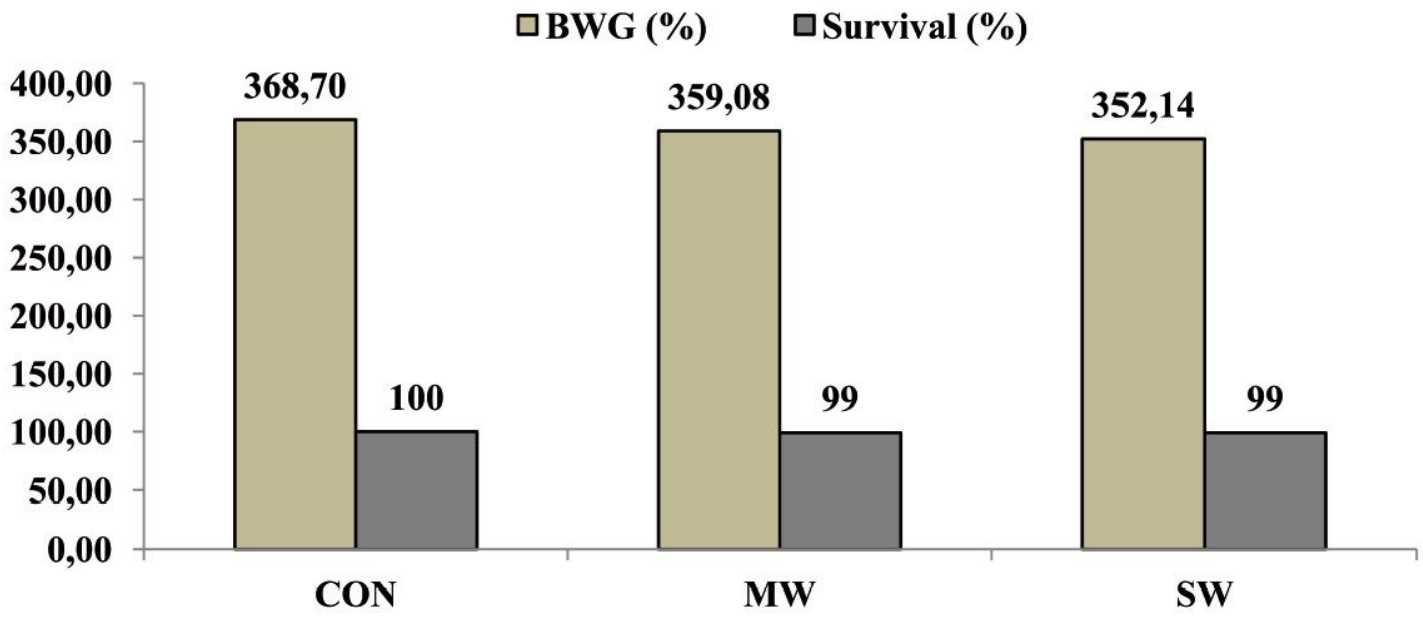


Figure 1

Bodyweight gain (BWG) and Survival rate of sea trout at the end of the experiment. Experimental diets: fishmeal diet (CON), mealworm diet (MW), and superworm diet (SW)

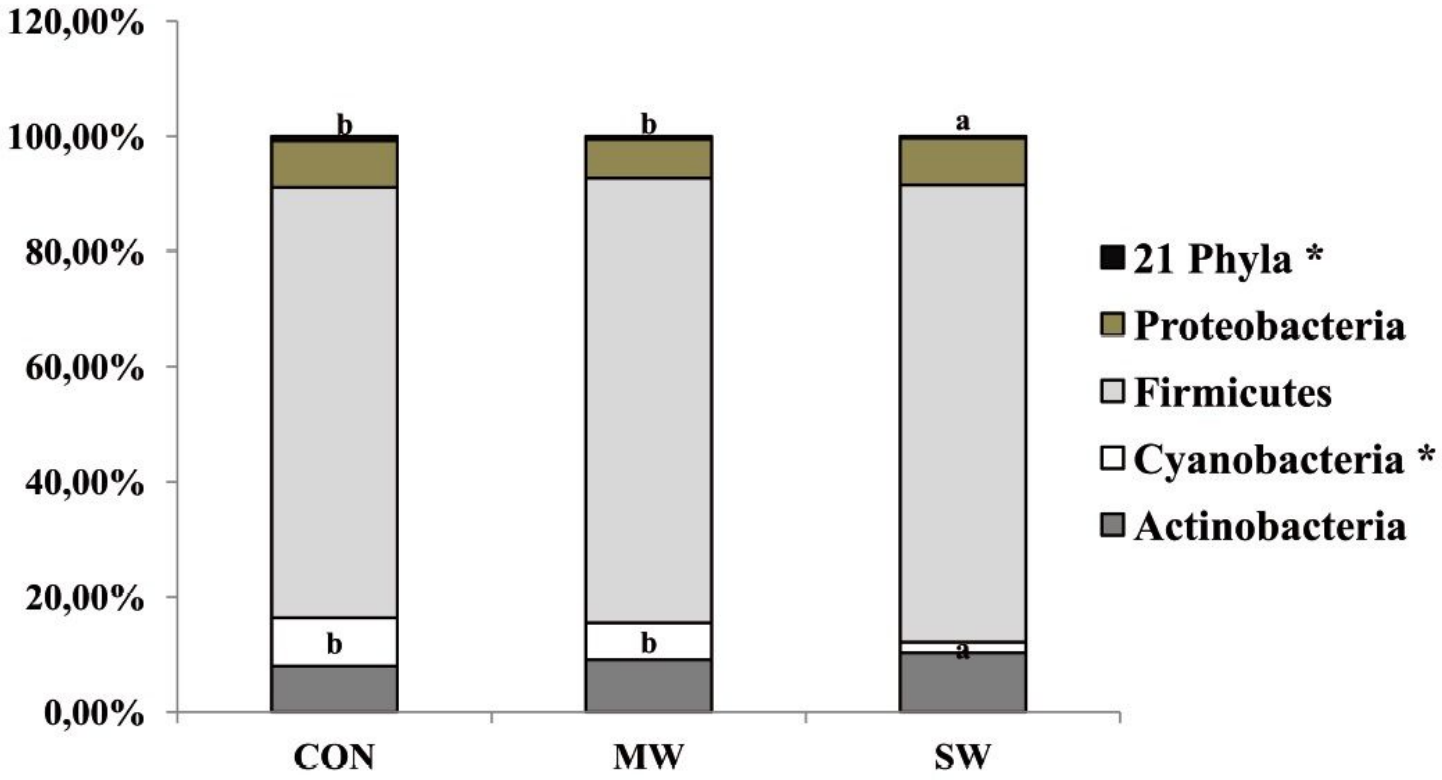


Figure 2

The most representative phyla of microbioma of sea trout guts fed with the three experimental diets; fishmeal diet (CON), mealworm diet (MW), and superworm diet (SW).

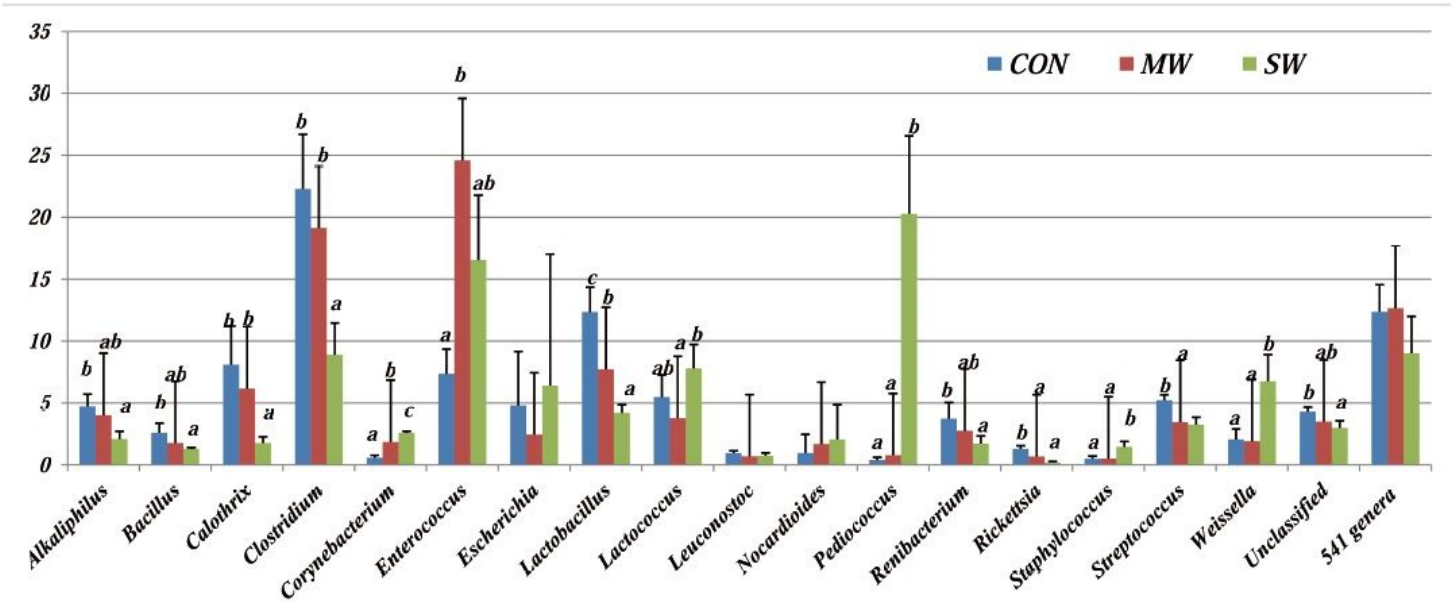


Figure 3

The relation between the genera proportions of sea trouts' gut digesta in the different treatments: control (CON), mealworm meal (MW), and superworm meal (SW).

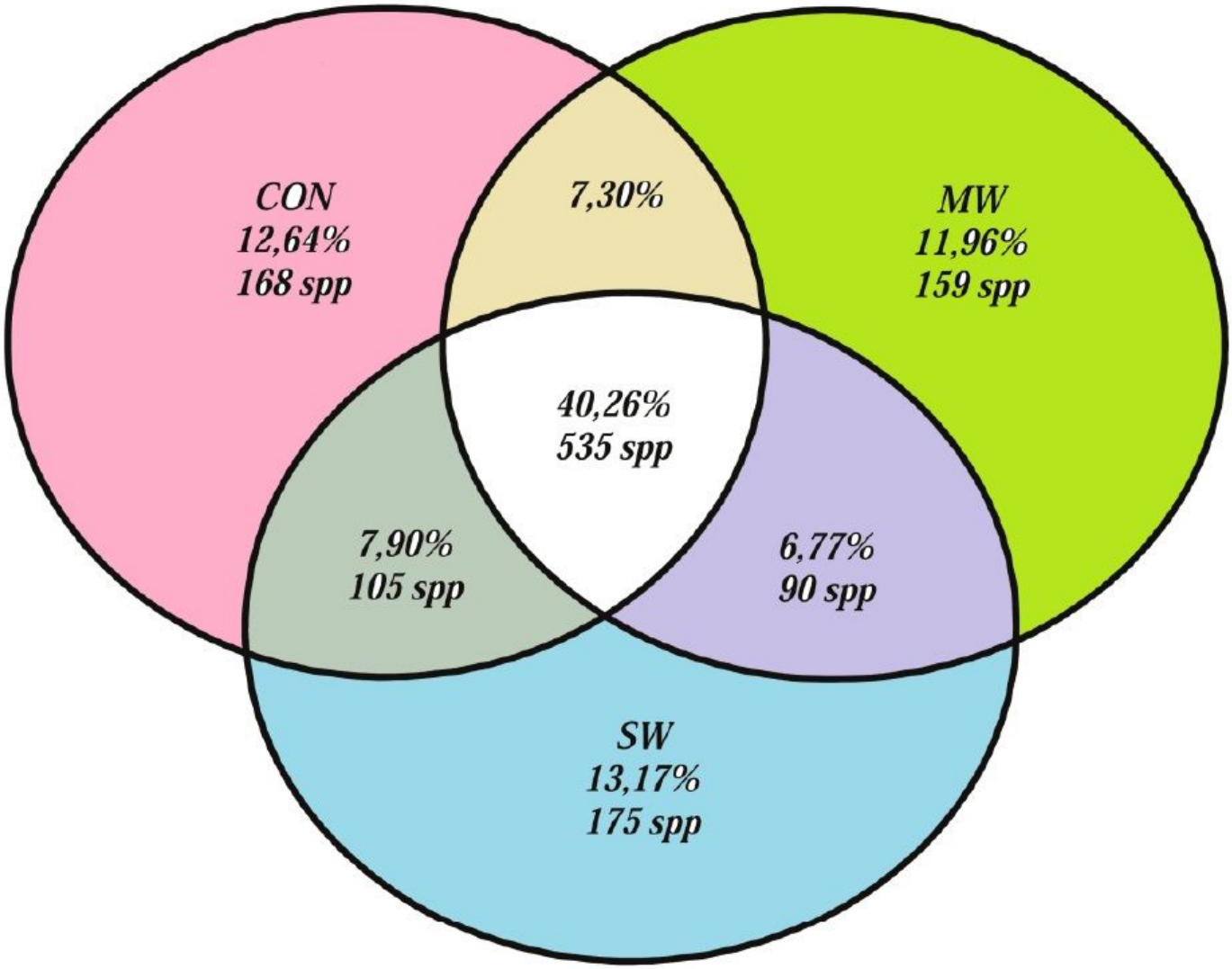


Figure 4

Relation of the number of species per treatment as well as those shared among treatments: Control diet (CON), mealworm diet (MW), and superworm diet (SW). The values are expressed in percentage and amount of species identified.

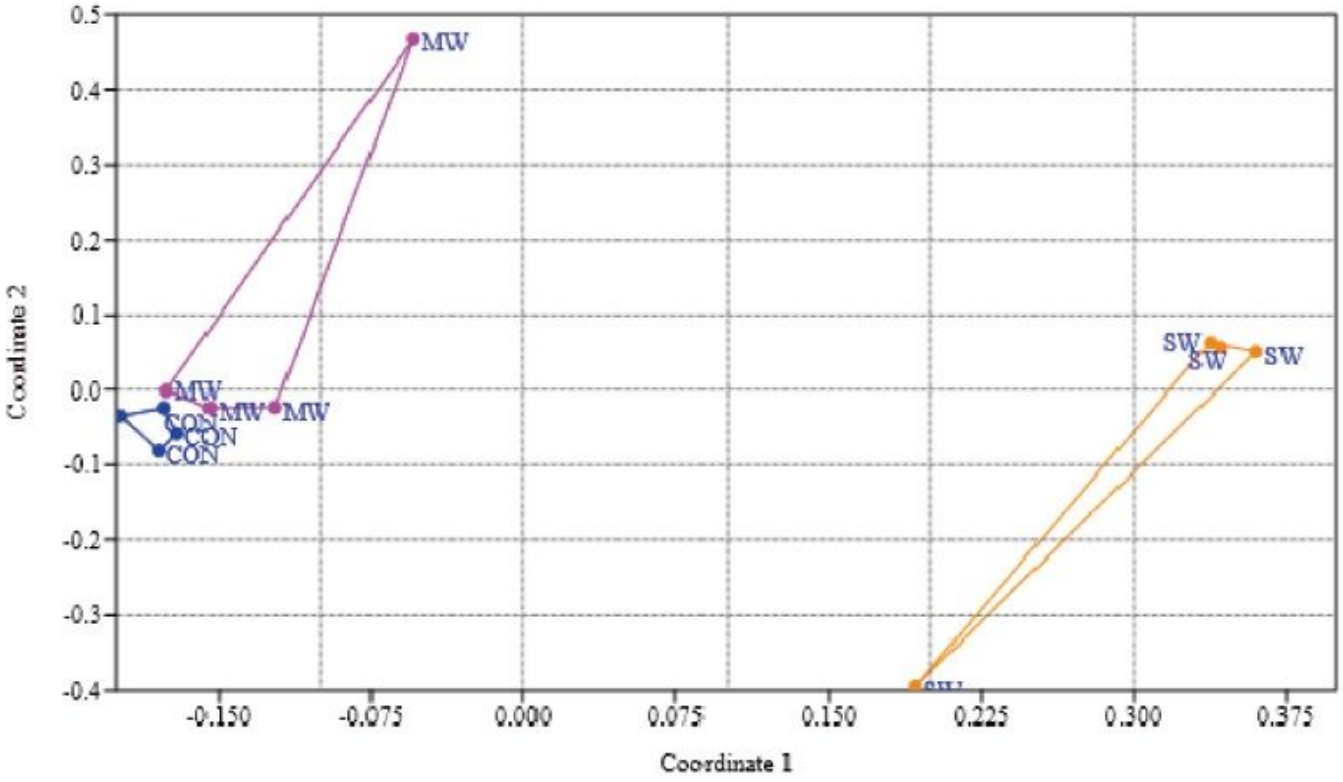
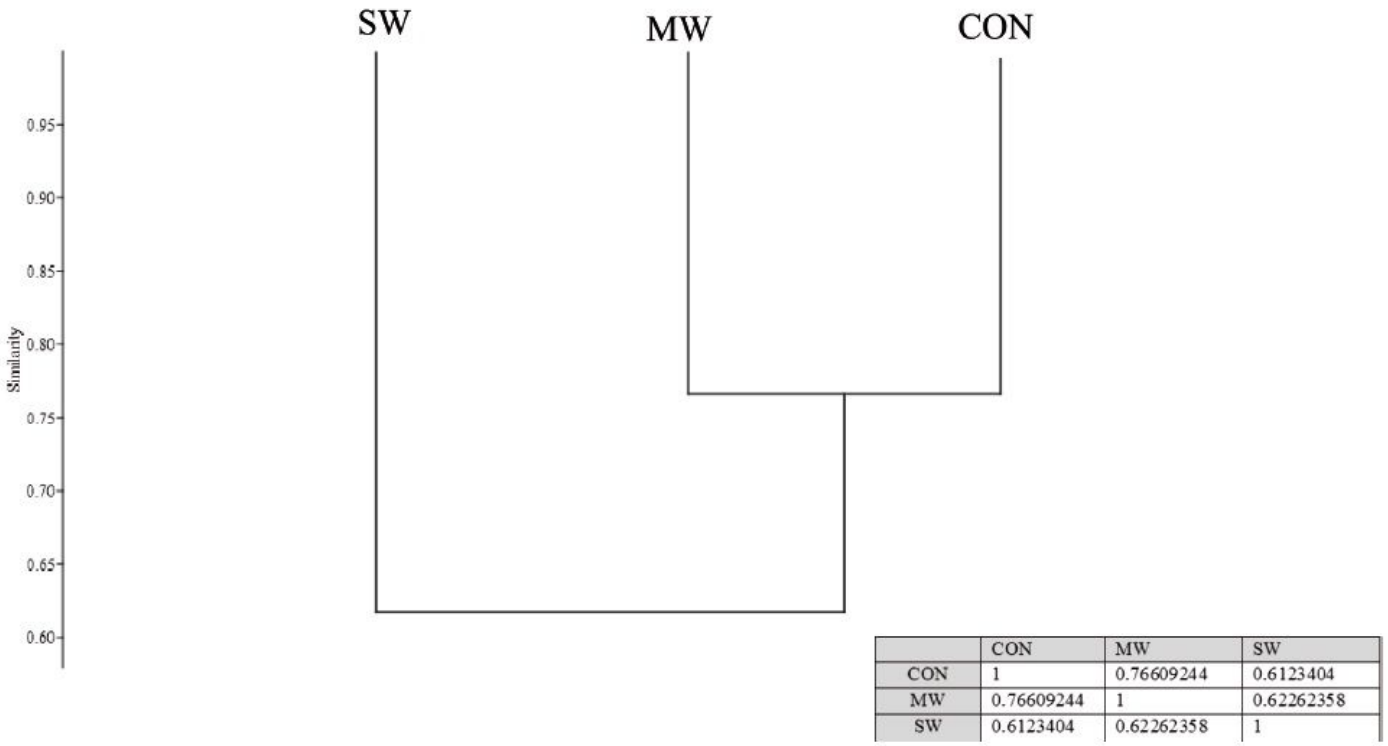


Figure 5

Non-metric multidimensional scaling (NMDS) analysis plot of sea trouts' microbiota fed with three experimental diets: CON (control diet), MW (mealworm diet), and SW (superworm diet).



	CON	MW	SW
CON	1	0.76609244	0.6123404
MW	0.76609244	1	0.62262358
SW	0.6123404	0.62262358	1

Figure 6

Sea trouts' microbiota clusters similarities and dissimilarities between treatments; CON (control diet), MW (mealworm diet), and SW (superworm diet).