Reshaping gut bacterial communities after dietary Tenebrio molitor larvae meal supplementation in three fish species

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Abstract

The aquaculture industry is currently looking for alternative, sustainable diets that provide similar or better growth for the reared species. We investigated whether replacing fishmeal with insect meal (Tenebrio molitor) in the supplied diets of Sparus aurata, Dicentrarchus labrax and Oncorhynchus mykiss causes similar shifts in the bacterial gut communities of these farmed fish species. The diversity of the gut bacterial 16S rRNA gene revealed the presence of most major phyla known to exist in the gut of these three fish species. However, there was a differential shift in the gut bacterial community structure of each species before and after the dietary meal replacement. S. aurata and D. labrax had more pronounced changes compared to O. mykiss, based on analysis of the most dominant and/or the shared vs. unique phylotypes before and after the replacement, suggesting that insect meal replacement resulted in new nutritional niches in the gut of these two fish compared to O. mykiss. Our results indicate that the most desirable fish diet substitution differentially affects the gut microbiota in different hosts, implying that a species-specific tailor-made approach in diet manipulations should be considered in the future.

Keywords	Bacteria; diet; fish; gut; insect meal; replacement; aquaculture
Taxonomy	Animal Science, Agricultural Products, Microbiology, Microbiome
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- Insect meal could be an alternative for fishmeal replacement in aquaculture.
- There is little information on the effect of such replacement to the fish gastrointestinal microbiota (GITM).
- We concomitantly investigated the impact of fishmeal replacement by insect meal in two marine and one freshwater farmed fish species.
- Our results showed that a similar amount of diet replacement results in more pronounced GITM changes in the marine fish than in the freshwater depicting, thus, the need for considering gut ecophysiology in diet replacement practices.

Reshaping gut bacterial communities after dietary *Tenebrio molitor* larvae meal supplementation in three different fish species

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Keywords: Bacteria; diet; fish; gut; insect meal; replacement; aquaculture

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ABSTRACT

The aquaculture industry is currently looking for alternative, sustainable diets that provide similar or better growth for the reared species. We investigated whether replacing fishmeal with insect meal (*Tenebrio molitor*) in the supplied diets of *Sparus aurata*, *Dicentrarchus labrax* and *Oncorhynchus mykiss* causes similar shifts in the bacterial gut communities of these farmed fish species. The diversity of the gut bacterial 16S rRNA gene revealed the presence of most major phyla known to exist in the gut of these three fish species. However, there was a differential shift in the gut bacterial community structure of each species before and after the dietary meal replacement. *S. aurata* and *D. labrax* had more pronounced changes compared to *O. mykiss*, based on analysis of the most dominant and/or the shared vs. unique phylotypes before and after the replacement, suggesting that insect meal replacement resulted in new nutritional niches in the gut of these two fish compared to *O. mykiss*. Our results indicate that the most desirable fish diet substitution differentially affects the gut microbiota in different hosts, implying that a species-specific tailor-made approach in diet manipulations should be considered in the future.

INTRODUCTION

The aquafeed production industry relies mainly on raw ingredients, such as fish meal supplied from wild fish, as protein sources. However, the replacement of fish meal in fish diets is a major objective towards achieving sustainability in both sectors, fisheries and aquaculture. Recently, much attention has been paid to insect meals due to their interesting nutritional value and sustainability (Henry et al., 2015; van Huis, 2013), and recent researches have highlighted their potential in fish feeds as substitute for conventional protein sources (Belforti et al., 2015; Gasco et al., 2016, Lock et al., 2016; Piccolo et al., 2017; Iaconisi et al., 2017; Renna et al., 2017). Such substitutes need to at least retain the growth and health features of the host unchanged, if not improving them.

Fishmeal replacement can affect the growth and health of the reared fish (e.g. Estruch et al. 2015, Parma et al. 2017, Schmidt et al 2016). However, whether these effects are induced by changes in the probiotics and the gastrointestinal microbiota (GITM) remains elusive and understudied compared to humans (Li et al. 2016, Mu e al. 2016). Such replacements can result in the intake of feed with dubious digestibility and absorption by the host (e.g. Santigosa et al. 2011, Baeza-Ariño et al. 2016) with largely unknown effects on its GITM in relation to the attachment, development and function of microbial symbionts. While the major beneficial roles of GITM on their hosts have been recognized (Mu et al. 2016, Sanchez et al. 2017), the origin, distribution, cosmopolitanism and host-specificity of these microorganisms remain largely unknown for most of the animals. Nonetheless, knowing these parameters is crucial to unraveling the exact mechanisms underlying their symbiotic relationships with the host. Although fish harbour less diverse gastrointestinal tract (GIT) bacterial communities than endothermic animals and humans (Ringø et al. 1995), these communities can form complex biological relationships (Sullam et al. 2012, Givens et al. 2013, Llewelyn et al. 2014, Ghanbari et al. 2015, Wang et al. 2017) that critically influence the host's nutrition, growth and health (Ringø et al. 2016).

Due to the complexity of the induced changes in the microbial community structure and the interactions of the potential metabolic benefits, many studies of these diet replacements tend to assess only major growth and/or health-related factors. One of the most understudied factors, is the effect of the new diets on the fish GITM (Ringøet al. 2016, Wang et al. 2017). There are even fewer studies that investigate the ecological features of the GITM in hosts undergoing feed

substitution such as co-occurrence and distribution patterns. Some of these features, such as abundance distributions, are pivotal for explaining niche partitioning, environmental selection, fitness, etc., (Magurran2004, Kirchamn2012) and could reflect the suitability and importance of a specific GITM for a particular host. Specifically, for the gut environment, the microbiota remains largely a deterministic characteristic driven by the nutritional background of the gut and the interplay between the members of the GITM (Pereira & Berry 2017).

In this paper, we hypothesized that the replacement of fishmeal with alternative proteins of insect origin is not equally beneficial to all fish species as far as the community structure of their GITM is concerned. To test this hypothesis, the major shifts in the GITM of three commercially important farmed fish species were evaluated after substituting the fish meal to achieve ≥50% *Tenebrio molitor* larvae meal (TM) inclusion in the feed. The changes in the GIT bacterial community structure were investigated by analyzing the 16S rRNA gene diversity by next generation sequencing analysis in the midgut of sea bream (*Sparus aurata*), sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) individuals before and after the fish meal substitution in feeds.

MATERIALS AND METHODS

Experimental growth of fish and sampling. All experimental protocols applied in this work were designed according to the guidelines of the current European Directive (2010/63/EU) on the protection of animals used for scientific purposes. For all trials, the same full-fat TM larvae meal was used and purchased from the Gaobeidian Shannong Biology CO. LTD (Shannong, China). TM larvae meal was imported by the Department of Agricultural, Forest, and Food Sciences (DISAFA) of the University of Torino (Italy) (DGSFA 0019960-P (02/11/2012)) and used for the preparation of three distinct experimental diets based on the nutritional needs of each experimental fish species used in the present study. Thus, three independent dietary experiments were performed. The rainbow trout (*Oncorhynchus mykiss*) trial was carried out in the experimental facility of DISAFA (Torino, Italy) (DM n. 182/2010). The European sea bass (*Dicentrarchus labrax*) trial was conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Center for Marine Research (Crete, Greece) (EL91-BIOexp-04). The gilthead sea bream (*Sparus aurata*) trial took place at the Department of

Veterinary Medicine and Animal Production (University of Naples Federico II, Italy). The European sea bass and gilthead sea bream trials protocols were also evaluated and approved by the Aquaexcel Ethic committee (Ref 0013/03/05/15B and Ref. 0125/08/05/15/TNA).

In each trial, diets were formulated to cover the fish nutritional requirements which differed due to fish species and age. Nevertheless, within the species, diets were formulated to be isonitrogenous, isolipidic and isoenergetic. Therefore, comparisons of the GITM are based between control and their respective replacement treatment for each species separately. *S. aurata* juveniles (105.2±0.17g initial body weight) were fed two isoenergetic and isoproteic diets for 163 days: a control diet (TM0) contained 100% fishmeal (FM) and a 50% partial substitution of fishmeal with TM (TM50). *D. labrax* juveniles (initial body weight: 5.2±0.82g) were fed two isonitrogenous, isolipidic and isoenergetic diets (TM0 and TM50) for 70 days. *O. mykiss* (115.2±14.21g initial body weight) were fed two experimental diets for 90 days, having 0% or 60% of TM. Fish were fed daily to apparent satiation for 90 days and reared in triplicate tanks per treatment, for all trials. Detailed description of experiments can be found in Piccolo et al. (2017) and Gasco et al. (2016).

For all trials, fish were fed to apparent satiation every day. The TM composition is reported in Table 1. Moreover, TM was used as partial substitute of FM that represented the main protein source in the control diet (0% TM). Ingredients and proximate composition of experimental diets are reported in Table 1. To keep the diets isoproteic and isoenergetic, the quantities of the other ingredients used in diets formulation were slightly modified (Table 1). Since the used TM had a high fat content, the level of fish oil was dramatically reduced in TM diets.

At the end of each growth trial fish were starved for 24 hrs and 10 healthy fish from each dietary group of the examined species were removed and sacrificed by aneasthesia overdose (tricaine methanesulfonate-MS222, Sigma Aldrich, St. Louis, MO, USA). After fish body weight measurements, the midgut was removed under sterile conditions. As most of the studies don't consider sample preparation for distinguishing indigenous (resident) from transient gut bacteria (Ghanbari et al. 2015), the digesta -if present- was removed by gentle mechanical force with flat forceps. The emptied midgut samples were rinsed thrice in sterile particle free distilled water, were flash-frozen in liquid nitrogen and kept at -80^{o}C. Stable rearing conditions were kept during the trials, thus, no or little effect was expected from the recirculating water (Meziti et al. 2012,

Estruch et al. 2015, Borsodi et al. 2017) and the effect of water microbiota on the midgut
142 bacterial community composition was not assessed as the origin of the GITM was out of the scope of this paper

144 **Molecular and sequencing analysis**. Bulk DNA was extracted from each individual fish gut sample with the PowerMax Soil DNA Isolation kit (MoBio, Carlsbad, CA, USA) following
146 the manufacturer's protocol. Tag pyrosequencing was performed on the Roche 454 FLX titanium platform, by targeting the V3–V4 region of the 16S rRNA gene with the primer pair S-DBact-
148 0341-b-S-17 (5'-CCTACGGGNGGCWGCAG-3') and S-D-Bact-0785-a-A-21 (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth et al. 2013) according to Dowd et al. (2008)
150 (MRDNA Ltd., Shallowater, TX, USA). Raw pyrosequencing data were processed by the MOTHUR platform (v. 1.35.0) (Schloss et al. 2009). Quality control of data analysis included
152 denoising of the flowgrams by PyroNoise software (Quince et al. 2009); sequences with ≥250 bp and no homopolymers of ≥ 8 bp were excluded for further analysis. The remaining sequences
154 were aligned in the SILVA 126 database (Pruesse et al., 2007), binned into operational taxonomic units (OTUs) and clustered based on averageneighbour algorithm at 97% the sequence
156 similarity cut-off (Kunin et al. 2010, Stackebrandt& Goebel 1994). The unique OTUs were taxonomically classified by using the SILVA 126 database (Pruesse et al., 2007). All sequences
158 from this study are available in the Short Reads Archive (http://www.ncbi.nlm.nih.gov/sra) with accession number SRR5161931.

160

RESULTS AND DISCUSSION

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In this paper, multiple pieces of evidence are provided showing that a high(≥50%)
164 replacement of fishmeal with insect (*Tenebrio molitor*) meal has a differential effect on the structure and possibly on the subsequent gut ecophysiology of gilthead sea bream (*Sparus*
166 *aurata*), European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*). As the level of TM inclusion and consequent substitution of fishmeal substitution was high (71.5 –
168 85.7%), we hypothesize that any observed shifts in the gut bacterial communities would be attributed mainly to insect meal as the major dietary ingredient.

170 Individual variability has occurred in similar studies (Desai et al. 2012) mostly due to the feeding practices of massively reared fish where not all individuals consumed the same amount

172 of food. The majority of the replicated samples per treatment in our study were variable with low similarity regarding the relative abundances of the found operational taxonomic units (OTUs)
174 (Fig. S1); individual variability has been proposed for the human gut microbiota (Bashan et al. 2016) but has not yet been shown for fish gut microbiota. Since a comparable number of
176 sequence reads per treatment (Tab. 2) were obtained, we used the average sequence reads per treatment for our analyses.

178 The first evidence for the differential effect of fishmeal replacement comes from the increased number of OTUs after the replacement for *S. aurata* and *D. labrax*, which contrasts
180 with the decreased number of OTUs for *O. mykiss* (Tab. 2). In gut microbial communities that undergo some kind of perturbation, more novel species are introduced due to the novel niches
182 introduced to the ecosystem (Zelezniak et al. 2015, Pereira & Berry 2016). The insect meal replacement is most likely a deviation for the standard GIT microbiota, at least for *S. aurata* and
184 *D. labrax* and their fishmeal containing diet. The newly created nutritional niches in the *S. aurata* and *D. labrax* gut after the replacement are also evidenced by the fact that the majority of the
186 newly appearing OTUs represent unique bacteria (62.2% and 60.0% for *S. aurata* and *D. labrax*, respectively), i.e., species that were not detected in the 0% insect meal diet (Fig. 1). For *O.*
188 *mykiss*, only 33.0% of novel OTUs were detected after the replacement, indicating that the nutritional background in this species was altered to a lesser degree than the two other species.

190 The meal replacement also impacted the dominant OTUs. When fed on a specific diet each species is usually characterized by a gut bacterial community with usually a few dominant
192 species (Ringø et al. 2016, Wang et al. 2017) but this changes when there is an increased number of bacterial species associated with more diverse diets, i.e., more nutritional niches or changing
194 environmental conditions (Givens et al. 2015); these changes have also been observed in comparisons of farmed and wild fish GITM (Kormas et al. 2014, Ramírez & Romero 2017a, b).
196 The dominance by a few bacterial species is indicative of a more specialized habitat (e.g. Lowrey et al. 2015, Lyons et al. 2017). In this study, although 598 OTUs were detected in total, only 8 –
198 20 of them composed >80% of the community's population in the 100% fishmeal diet; after fishmeal replacement, the dominant OTUs dropped to 5 – 12 in the three fish species (Tab. 2).
200 However, not only very few of the shared OTUs were found before the replacement but also they were found after the replacement for *S. aurata* and *D. labrax*, but the shared OTUs between the
202 two treatments had incomparable relative abundances (Fig. 2). For *S. aurata*, five of the eight

dominant OTUs were detected after the replacement, but the most dominant one OTU (OTU-
204 0004) before the replacement (26.8%) was not detectable in the insect meal treatment. The most dominant OTU in *D. labrax*, OTU-0002, decreased from 17.9% to 3.6% dominance, while a total
206 of eight out of 14 dominant OTUs appeared after the replacement in *D. labrax*, when OTU-0009 was dominant (13.6%). In the *O. mykiss* midgut, 11 of the 21 dominant OTUs found before the
208 replacement were also found afterwards. The dominant OTU-0009 (10.0%) before the replacement was the second most dominant OTU (33.3%) after the replacement, indicating that it
210 represents a bacterium that is probably favoured by the insect meal addition. This OTU is related to a Tenericutes-associated species originating from chicken caeca (Tab. S1). The Tenericutes,
212 are amongst the protagonists of gut symbionts in fish (Lowrey et al. 2015, Gatesoupe et al. 2016, Lyons et al. 2016, Dehler et al. 2017, Ringø et al. 2016, Wang et al. 2017) and other aquatic
214 animals with a chitin exoskeleton (Demiri et al. 2009, Meziti et al. 2012, Givens et al. 2013, Hakim et al. 2016), indicating that they are possibly related to the metabolism of this compound.
216 OTU-0009 was also found to be the most abundant in the insect meal fed to *D. labrax* but not in the *S. aurata*, where it might be outcompeted in the latter by another Tenericutes related
218 bacterium, OTU-0001, which is also associated with fish GIT (Tab. S1). The higher affinity of the *O. mykiss* midgut bacteria to the insect meal diet is also supported by the fact that the relative
220 importance of the abundant, common and rare OTUs showed a different response in the three species after the replacement (Fig. S2), with only *O. mykiss* again showing increased abundant
222 OTUs and the lowest change in new rare OTUs. Recently, it has been proposed that the transition from rarity to abundance in bacterial populations could be related to substrate availability and
224 lability (Newton& Shade 2016) and, thus, such gut bacterial community changes might impose some effect on fish nutrition that remains to be investigated.

226 The dominant and/or shared OTUs could be considered to be true GIT residents as their inferred phylogeny depicts fast growers that could take over the GIT habitat; additionally, in this
228 study, most of the midgut faecal material was removed and, thus, almost exclusively the epi- and endobionts of the GIT tissue were detected. The fast-growing capability of these bacteria is
230 suggested by their high 16S rRNA gene copy number (Tab. S1) which corresponds to high maximum growth rates (μ_{max}) according to Roller et al. (2016). However, as not all OTUs can be
232 safely assigned to known taxa and available genomes do not equally cover all taxa, this speculation requires further confirmation.

234 The differential response of the three fish gut communities is not only shown in the structural changes (above) but also in their inferred ecophysiological roles as dictated by the
236 different major taxonomic groups. Although all major bacterial phyla known to occur in other fish gut (Sullam et al. 2012, Llewellyn et al. 2014,Estruch et al. 2015,Ringø et al. 2016, Tarnecki
238 et al. 2017, Wang et al. 2017) were also found in our study (Fig. 3, Tab. S2), the lowest number of novel taxa (families) appearing in the gut after the meal replacement occurred in *O. mykiss*
240 (25.0% of all families before and after the meal substitution), compared to *S. aurata* (48.4%) and *D. labrax* (44.0%) (Fig. 4). Although functional redundancy is common among bacterial taxa, it
242 is expected that the accumulation of new taxa appearing in a community are due to an increase in the novel niches that are available after a perturbation (Maguran2004). The prevalence of
244 differently expected ecophysiologies is also shown by using the ratios between the major phyla to which the OTUs belong (e.g. Desai et al. 2012, Givens et al. 2015, Zhu et al. 2015, Dehler et al.
246 2017), that occurred in the different treatments (Fig. 4). Based on the ratios of Proteobacteria:Firmicutes, Proteobacteria:Bacteroidetes and Firmicutes:Bacteroidetes, only in the
248 case of *O.mykiss* did these ratios remained practically unchanged, suggesting that the changes caused by the insect meal replacement affected the dominant phyla in this animal less compared
250 to the other two species and, therefore, fewer differences are expected. However, even in *O. mykiss,* the ratio of Proteobacteria:Actinobacteria was nearly halved. In the human gut, for
252 example, the dominance of Proteobacteria vs. Firmicutes and/or Bacteroidetes has been suggested to be an indication of gut dysbiosis (Shin et al. 2015). In fish GITM, Proteobacteria usually
254 dominate (Llewellyn et al. 2014, Wang et al. 2017). However, the observed changes in their relative abundance in *S. aurata* and *D. labrax* but not in *O. mykiss*, might indicate gut imbalances
256 that need to be further investigated by comparing omics approaches and physiological assays.

The prevalence of a "natural" gut microbiota is of crucial importance for the host (Pereira
258 & Berry 2016). Our reported imbalanced gut microbiota in *S. aurata* after fish meal replacement corroborates the findings by Piccolo et al. (2017) who in the same experiments as ours, they
260 found a lower digestibility coefficient in fish with 50% TM inclusion (78.46% for dry matter digestibility coefficients in the TM50 group compared to 87.02% in the TM0 fish group),
262 although TM50 fish showed a similar final weight and growth performance compared to the control group. Additionally, it is known that fish meal replacements with other ingredients
264 (Estruch et al. 2015, Parma et al. 2016, Rico et al. 2016) or other diet changes (Silva et al. 2011,

Cerezuela et al. 2013) induces gut microbiota changes in *S. aurata*, which can be reflected on fish growth parameters. Kormas et al. (2014) have recently shown that the gut of organically reared *S. aurata* more closely resembles that of wild populations than the guts of conventionally reared fish. All of the above findings, lead to the conclusion that seabream is sensitive to dietary fish meal replacements and more research is needed to elucidate the effect of dietary ingredient on gut microbiom as has also been suggested by Estruch et al. (2015) and Parma et al. (2016).

Similarly, for *D. labrax*, Gasco et al. (2016) reported that for the same experiment as in our study, *D. labrax* fed with 50% insect meal inclusion resulted in statistically significantly lower dry matter intake, weight gain, specific growth rate and protein efficiency ratio, as well as a higher mortality (Table 4 of Gasco et al. 2016). In this experiment the dominant microbes from the control (100% fish meal) treatment were not detected in the 50% insect meal inclusion treatment (Fig. 3) and the different taxonomic groups of bacteria observed between the two treatments (Fig. 4) indicate different metabolisms. Even if functional redundancy is the case, more time might be required for the selection of the new (unique) microbes to be established and form fully functional communities. Torrecillas et al. (2017) reported gut microbiota alterations after the exclusion of fish meal and the sensitivity of the *D. labrax* gut microbiota to diet alterations was also shown by Carda-Diéguez et al. (2014).

A different picture is presented for *O. mykiss,* as this species showed the fewest changes in all investigated GITM parametersafter dietary MT inclusion. Most of the known bacterial taxa found in this species' gut (Navarrete et al. 2012, Ingerselv et al. 2014, Lowrey et al. 2015, Lyons et al. 2017) were also present in the current study. Similarly, Lyons et al. (2017) found an almost invariable gut microbiota before and after microalgal material feed supplementation in the same species, coupled with increased growth effects. However, fish meal replacements by soy bean based meals have impacted the gut microbiota of *O. mykiss* either negatively, i.e. more novel phylotypes (Heikkinen et al. 2006, Desai et al. 2012) or positively, i.e. fewer novel bacteria appearing after the replacement (Dimitroglou et al. 2009). In our study, the lower number of novel OTUs appearing in the *O. mykiss* after meal replacement, could be related to the animal's natural diet. A chitin-enriched diet -like the one used in the present study- probably imposes little gut microbiota changes because this species' natural diet includes insects (Power 1990).

In conclusion, the differential impact of the fish meal replacement by *Tenebrio molitor* meal on the gut bacterial community structure of the three commercially important fish species of

296 *S. aurata, D. labrax* and *O. mykiss*, suggests that any such feed substitutions should include an assessment of the GITM, as these symbiotic microorganisms are inseparable from the growth-and
298 health-related metabolic features of the species. Our data also depicts that insect meal, at least in terms of GITM changes, is more suitable for species whose natural diet includes such ingredients.
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AUTHOR CONTRIBUTIONS

LG, GP, FG, SC, EA: performed the fish growth experiments; EN: contributed to molecular and bioinformatics analysis, EM: contributed to data analysis, KAK: analysed bacterial diversity data, wrote the first draft of the summary on which the rest of authors contributed.

CONFLICT OF INTEREST

All authors declare no financial/commercial conflicts of interest.

Table 1. Ingredients and proximate composition of *Tenebrio molitor* larvae meal (TM) and experimental diets using ≥50% TM meal inclusion in *Sparus aurata*, *Dicentrarchus labrax* and *Oncorhynchus mykiss*.

		***S. aurata*[1]**		***D. labrax*[2]**		***O. mykiss*[3]**	
	TM	**TM0**	**TM50**	**TM0**	**TM50**	**TM0**	**TM60**
Ingredients (g kg^{-1})							
Fish meal		500	130	700	200	700	100
Tenebrio molitor larvae meal		0	500	0	500	0	600
Corn gluten meal		150	130	0	0	0	37
Wheatglutenmeal		0	0	50	150	0	0
Wheat bran		0	0	55	25	57	50
Wheat meal		0	0	92	80	40	40
Gelatinized starch		180	150	0	12	33	100
Fish oil		140	60	90	20	150	53
Vit-min		20	20	4	4	20	20
Amino acids		0	0	9	9	0	0
Carboximethylcellulose		10	10	0	0	0	0
Chemical composition[3]							
DM (g kg^{-1})	939	951	952	920	917	956	949
Ash (g kg^{-1}, as fed)	47	89	50	115	57	107	82
CP (g kg^{-1}, as fed)	519	438	430	548	546	424	413
EE (g kg^{-1}, as fed)	236	193	194	152	157	213	211
Gross Energy (MJ kg^{-1}, as fed)	24.4	21.81	21.10	21.29	22.62	22.82	23.13

Abbreviations: DM, dry matter; CP, crude protein; EE, ether extract

[1]: from Piccolo et al., 2017; [2]: from Gasco et al., 2016; [3] Values are reported as mean of duplicate analyses

Table 2. Cumulative bacterial sequence reads and operational taxonomic units (OTUs) in the midgut of *Sparus aurata*, *Dicentrarchus labrax* and *Oncorhynchus mykiss* after a dietary inclusion of 50% or 60% of *Tenebrio molitor* larvae meal (TM).

TM	***No. of reads***	***No. of OTUs***	***Average relative abundance (%) of the most abundant OTU***	***No. of dominant OTUs****
S. aurata				
0%	1977	28	26.8	8
50%	2093	55	30.2	5
D. labrax				
0%	4114	28	17.9	10
50%	4923	54	13.6	12
O. mykiss				
0%	832	69	10.0	20
60%	1411	54	46.0	8

* Cumulative relative abundance ≥80% per treatment.

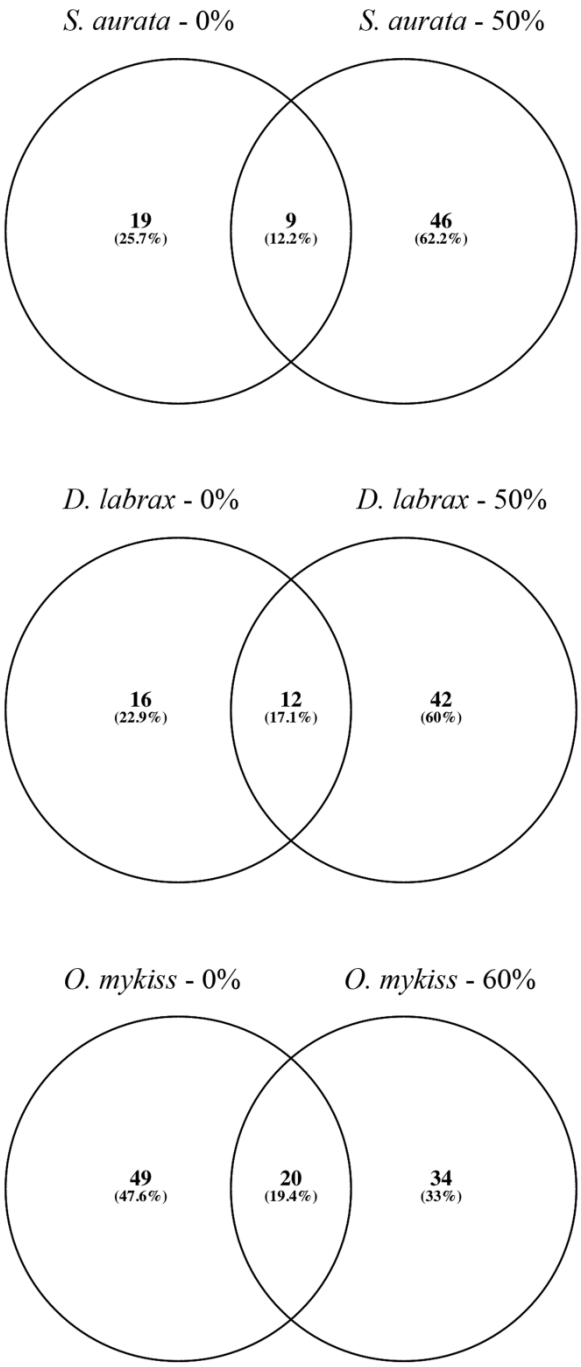

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Figure 1. Number of shared operational taxonomic units in the midgut of *Sparus aurata*,

538 *Dicentrarchus labrax* and *Oncorhynchusmykiss* after a dietary inclusion of 50% or 60% of *Tenebriomolitor*larvae meal.

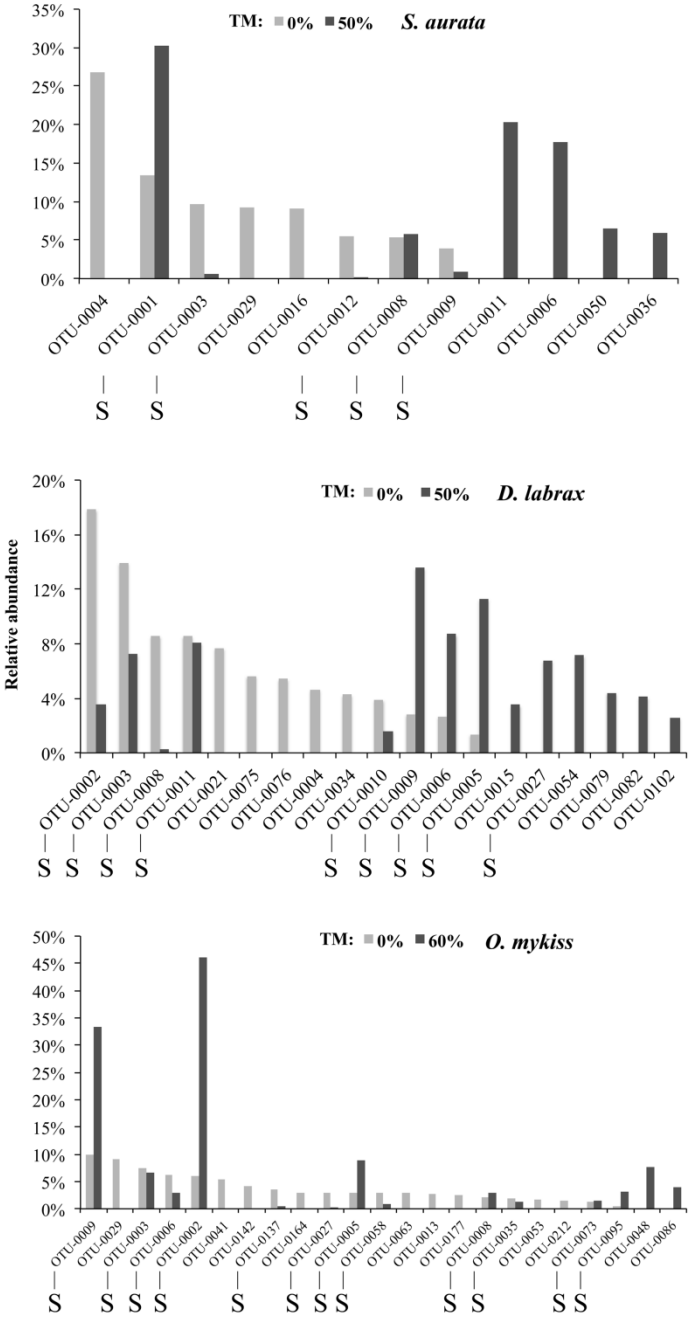
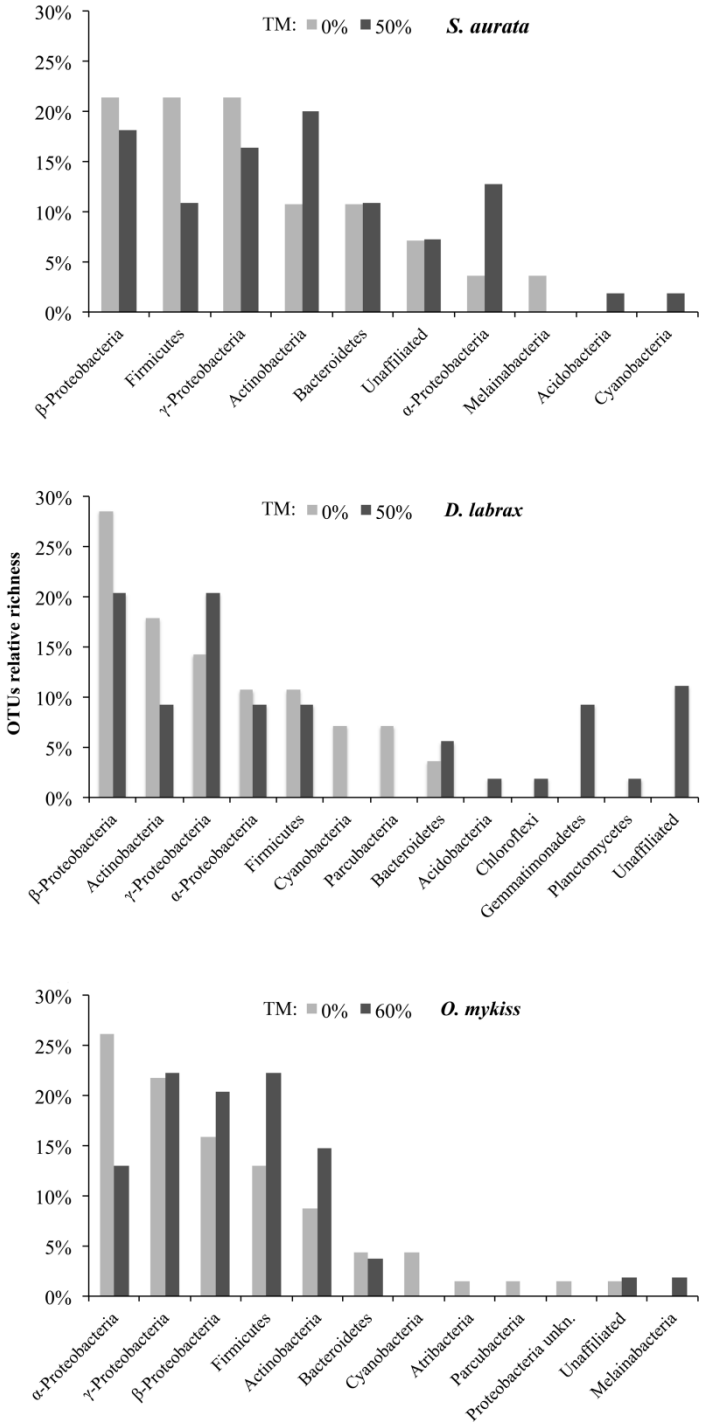
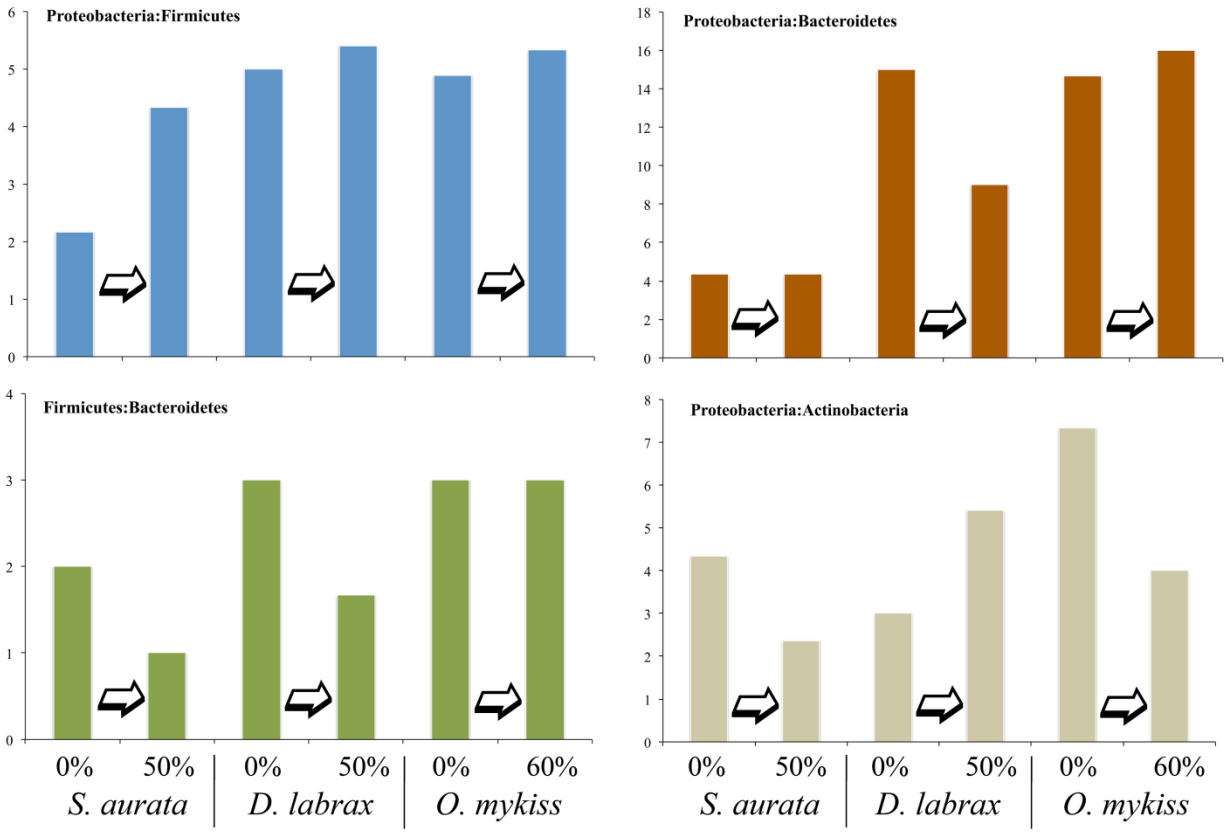


Figure 2. Changes of the most abundant (cumulative abundance ≥80% per treatment) operational taxonomic units (OTUs) in the midgut of *Sparus aurata*, *Dicentrarchus labrax* and *Oncorhynchusmykiss* after a dietary inclusion of 50% or 60% of *Tenebriormolitor* larvae meal (TM). S: shared between the two treatments. Note that OTUs are placed in decreasing order of relative abundance of the 0% TM treatment.



548

550 **Figure 3**. Taxonomic affiliation of the operational taxonomic units (OTUs) found in the midgut of *Sparus aurata*, *Dicentrarchus labrax* and *Oncorhynchusmykiss* after a dietary inclusion of
552 50% or 60% of *Tenebriormolitor* larvae meal (TM). Note that phyla are placed in decreasing order of their relative abundance of the 0% TM treatment.



554

556 **Figure 4**. Change of the most abundant bacterial phyla occurring in the midgut of *Sparus aurata*, *Dicentrarchus labrax* and *Oncorhynchusmykiss* after a dietary inclusion of 50% or 60% of

558 *Tenebiormolitor* larvae meal (TM).

Reshaping gut bacterial communities after dietary *Tenebrio molitor* larvae meal supplementation in three different fish species

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Supplementary information

Table S1. Most abundant (cumulative relative abundance per treatment >80%) operational taxonomic units (OTU) found in the midgut of commercially reared *Sparus aurata* (*Sa*), *Dicentrarchus labrax* (*Dl*) and *Oncorhynchus mykiss* (*Om*).

OTU	Found in	Closest relative	Similarity (%)	GenBank accession No	Habitat of origin	Average ± SD number of 16S rRNA gene copies*
0001	*Sa*	Clone TP-2 (Tenericutes)	95.8	DQ340193	*Gillichthys mirabilis* (mudsucker, estuarine fish) gut	-
0002	*Dl/Om*	TTGE gel band N123 (≈Firmicutes)	100.0	JN185158	*Oncorhynchus mykiss* gut	-
0003	*Sa/Dl/Om*	*Pseudomonas* sp. (Pseudomonadales)	100.0	KF366100	*Danaus plexippus* (overwintering butterflies) midgut	*Pseudomonas* spp. (N=208) ?? = 4.9 ± 1.25
0004	*Sa*	Clone 25	100.0	DQ889971	Juvenile Atlantic salmon (*Salmo salar*) digestive tract	-
0005	*Dl/Om*	*Cetobacterium somerae* 23 (Fusobacteriales)	97.9	HG326498	*Siganus canaliculatus* (rabbitfish, coral reef) gut	*Cetobacterium* spp. (N=2): *??* = 1 ± 0
0006	*Sa/Dl/Om*	Bio-material L100	99.6	HG966676	*Pisum sativum* subsp. *elatius* (wild pea) chloroplast	-
0008	*Sa/Dl/Om*	*Weissella confuse* (Lactobacillales)	100.0	LC127180	Human faeces	*Weissella* spp. *??* = 7.6 ± 1.94
0009	*Sa/Dl/Om*	Clone T-RFLP_clone_K44 (≈Tenericutes)	100.0	KP780113	Chicken caeca	-
0010	*Dl*	*Pseudomonas brenneri* (Pseudomonadales)	100.0	KU750791	Rhizosphere from *Lepidium meyenii* (maca)	*Pseudomonas* spp. (N=208) ?? = 4.9 ± 1.25
0011	*Sa/Dl*	Clone Sch1000_2	99.6	HE586962	Freshwater fish gut	-
0012	*Sa*	Clone FecI096 (Lactobacillales)	100.0	KM244870	Faecal matter of pigs under indoor system	-
0015	*Dl*	Clone OTU0162 (Pseudomonadales)	100.0	KM059059	*Bactrocera minax* (Chinese citrus fly) gut and reproductive organ	-
0016	*Sa*	*Plesiomonas shigelloides* (Enterobacteriales)	100.0	DQ822763	Intestinal bacteria of freshwater salmon *Salmo salar* and sea trout *Salmo trutta trutta* and diet	*P. shigelloides* NCTC10360 *??* = 11

0021	*Dl*	*Streptococcus equinus* (Lactobacillales)	100.0	LC145574	Cow faces	*Streptococcus* spp. (N=220) ?? = 5.1 ± 1.26
0027	*Dl/Om*	Clone A292_NCI	100.0	FJ456668	*Notothenia coriiceps* (Southern Ocean fish) intestinal content	
0029	*Sa/Om*	*Streptococcus oralis* (Lactobacillales)	100.0	CP019562	*Homo sapiens* blood	*Streptococcus oralis* (N=1) *??* = 4
0034	*Dl*	*Acinetobacter johnsonii* (*γ*-Proteobacteria)	99.7	AB859672	Human duodenum	*Acinetobacter johnsonii* (N=1) *?? = 7*
0035	*Om*	*Diaphorobacter polyhydroxybutyrativorans* (*β*-Proteobacteria)	100.0	KU041595	*Holotrichia serrata* gut	-
0036	*Sa*	*Bacillus circulans* (Firmicutes)	100.0	LT223624	Human stool	*Bacillus* spp. *??* = 10.5 ± 2.46
0041	*Om*	Clone YZ19	100.0	KJ457337	Intestinal tract of three spotted seahorse	-
0048	*Om*	Clone SEV1CE011	100.0	JQ407962	Horizontal subsurface flow constructed wetland treating domestic wastewaters	-
0050	*Sa*	*Saccharopolyspora gloriosae* (Actinobacteria)	99.7	JX007996	Marine sponge	*Saccharopolyspora erythraea* (N=1) *??* = 1
0053	*Om*	*Bacillus niabensis* (Firmicutes)	100.0	LT223631	Human stool	*Bacillus* spp. (N=292) *??* = 10.5 ± 2.46
0054	*Dl*	Clone C77	100.0	KC633566	Activated sludge of a full-scale wastewater treatment plant	-
0058	*Om*	*Acidovorax* sp. (*β*-proteobacteria)	100.0	KF003188	Grass carp gut mucus	*Acidovorax* spp. (N=6) ?? = 3 ± 0.00
0063	*Om*	*Chryseobacterium pallidum* (Flavobacteriales)	99.6	KU362282	Soil	*Chryseobacterium* spp. (N=4): ?? = 6.3 ± 0.50
0073	*Om*	*Brevundimonas naejangsanensis* (*α*-Proteobacteria)	100.0	KX223755	Sludge of an anaerobic digestion reactor	*Brevundimonas* spp. (N=4) ?? = 2 ± 0.00
0075	*Dl*	Clone AquaspiC	100.0	AY322153	Micromanipulated cells from activated sludge	-

0076	*Dl*	Clone T0-An-20C-25	100.0	JX105530	Ornamental fish aquaria	-
0079	*Dl*	Clone TX2_4J13	92.7	JN178241	Extreme saline-alkaline soil of the former lake Texcoco	-
0082	*Dl*	Clone BF2E04	100.0	JN820212	ferromanganese deposit	-
0086	*Om*	Clone RII-AN118	99.7	JQ580497	Sediments from Rodas Beach polluted with crude oil	-
0095	*Om*	Clone ELU0062-T425-S-NIPCRAMgANa_000345	99.5	HQ768070	*Homo sapiens* gastrointestinal pecimens	-
0102	*Dl*	Clone TX5A_63	100.0	FJ152771	Alkaline saline soils of the former lake Texcoco	-
0137	*Om*	*Hyphomicrobium* sp. (*α*-Proteobacteria)	99.4	FJ536930	Waste-activated sludge from municipal waste water treatment plant	*Hyphomicrobium* spp. (N=4) *??* = 1 ± 0.00
0142	*Om*	Clone nby369a03c1	100.0	HM810474	Back swab from shaved skin or wound of mouse deficient in the leptin receptor	-
0164	*Om*	Clone SINI595	99.2	HM126967	Chaerhan Lake	-
0177	*Om*	Clone D32	99,5	KJ808142	Activated sludge	-
0212	*Om*	Clone ML711O1eO6	95.6	JN615992	White microbial mat from lava cave wall	-

* From Microbial Genome Resources (https://www.ncbi.nlm.nih.gov/genomes/MICROBES/microbial_taxtree.html) or *rrn*DB (https://rrndb.umms.med.umich.edu/), accessed, 31/03/2017.

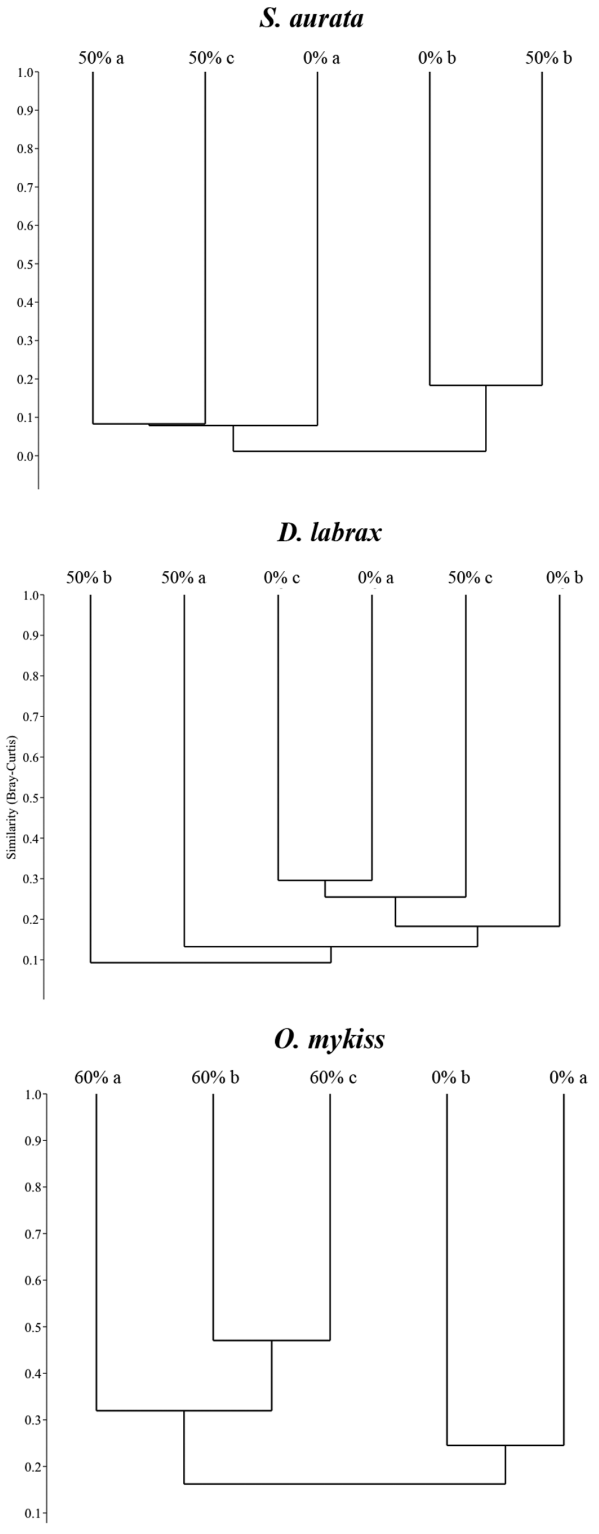

Figure S1. Percentage change of the midgut operational taxonomic units number from 0% to 50% (*Sparus aurata, Dicentrarchus labrax*) or 60% (*Oncorhynchus mykiss*) insect meal inclusion. Rare: <1%, common:1-10%, abundant: >10% relative abundance.

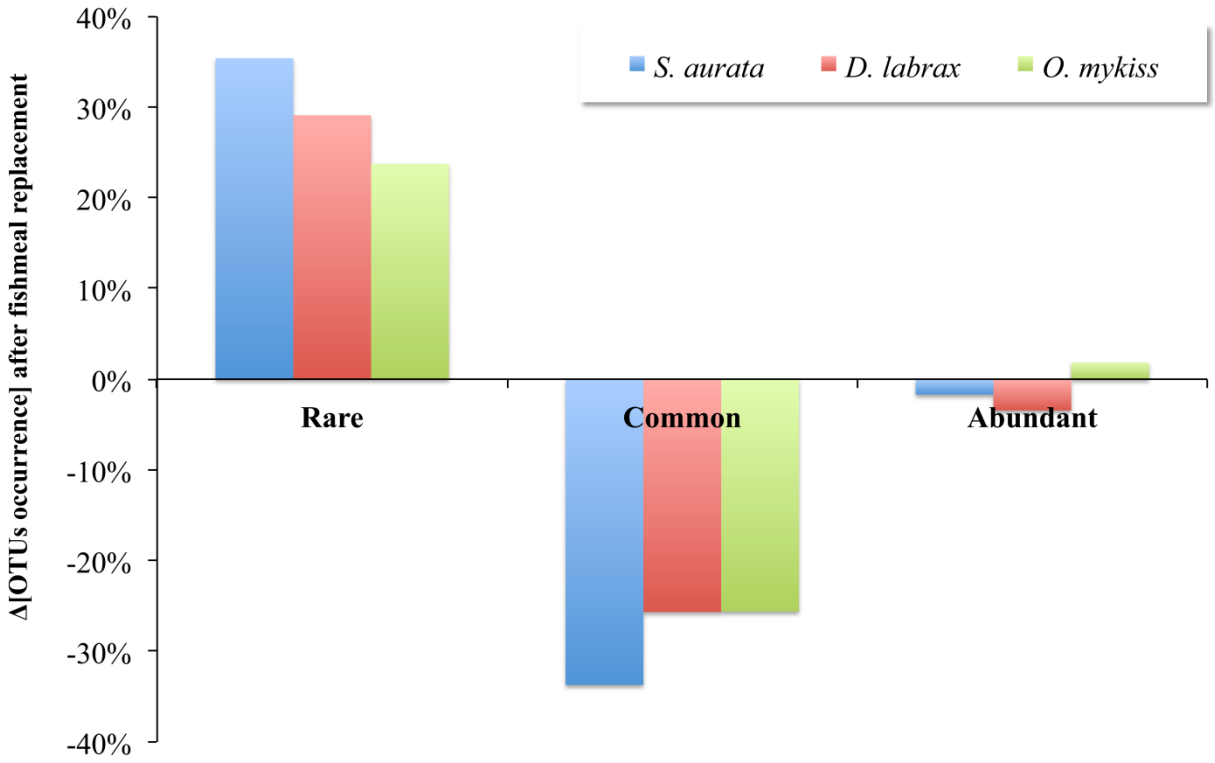

Figure S2. Cluster analysis of the operational taxonomic units relative abundance in midgut individual samples of *Sparus aurata*, *Dicentrarchus labrax* and *Oncorhynchus mykiss* after a dietary inclusion of 50% or 60% of *Tenebior molitor* larvae meal (TM).. a, b, c: replicates.