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RESEARCH ARTICLE

Pyrosequencing survey of intestinal microbiota diversity in cultured sea bass (*Dicentrarchus labrax*) fed functional diets

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Abstract

The routine use of chemotherapy to control bacterial diseases in aquatic populations has resulted in the development and spread of antibiotic resistance. The inclusion of immunostimulants in fish diets (functional diets) is one of the main strategies to solve this threat. This study aimed to analyse the intestinal microbiota of cultured European sea bass (*Dicentrarchus labrax*) fed two functional diets applying pyrosequencing of PCR-amplified 16S rRNA gene. Quality-filtered reads were assigned to family and genus taxonomic levels using the Ribosomal Database Project classifier. The autochthonous intestinal microbiota of sea bass consisted of two dominant bacterial genera: *Dysgonomonas* (Bacteroidetes) and *Ralstonia* (*Beta-proteobacteria*), but effects of diet on this dominance were observed. In fact, the genus *Dysgonomonas* significantly decreased in samples from fish fed functional diets, recovering control levels at the end of the study. However, *Ralstonia* proportion significantly raised in samples from fish fed diet C and maintained this high level along the study period. The developed protocol could be used to study the composition of bacterial communities in the fish intestine under different nutritional and environmental conditions and its impact on infection, immune system and general fitness of fish.

Introduction

Aquaculture is the food production sector growing fastest in last years (FAO, 2011), but infectious diseases are an important constraint for its advance (Hemmingsen 2008). The wide and frequent use of chemotherapy to control bacterial infections has resulted in the development and spread of antibiotic resistance (Defoirdt *et al.*, 2011). Moreover, vaccines for controlling parasitic and viral diseases are not available or are poorly developed. Under this scenario, novel strategies are needed for a sustainable development of aquatic cultures.

Quality aquafeeds should impart health benefits to the farmed organism. In fact, fish diets including growth- and health-promoting substances (functional feeds) can have positive effects on the fish immune system (Tacchi *et al.*, 2011). The most common additives used in aquaculture diets are probiotics, prebiotics, immunostimulants, vitamins and nucleotides. The inclusion of immunostimulants in fish diets (functional diets) is one of the main strategies to achieve a sustainable aquaculture. These enriched diets are administered during short periods typically, 4 weeks, when the risk of getting infections by aquatic animals is higher.

In some fish species, the effect of different additives on their growth has been proved (Martin-Antonio *et al.*, 2007; Jatobá *et al.*, 2008; Suzer *et al.*, 2008; Vieira *et al.*, 2008; Wang *et al.*, 2008; Ferguson *et al.*, 2010; Tapia-Paniagua *et al.*, 2010; Merrifield *et al.*, 2011). In the case of European sea bass (*Dicentrarchus labrax*), growth is improved by feeding with probiotics (Carnevali *et al.*, 2006). Reinforcement on mucus and epithelium of intestine has also been reported as other positive effect of functional diets (Barouei *et al.*, 2012). In fact, microbiota associated with gut epithelium has a crucial role in the interchange and assimilation of nutrients as well as defence barrier against pathogens (Bermudez-Brito *et al.*, 2012).

As most intestinal microbiota (IM) from fish cannot be cultured under laboratory conditions, 16S rRNA gene

fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) have frequently been used for determining diversity of bacterial communities in the fish intestine (Kim *et al.*, 2007; Ferguson *et al.*, 2010; Tapia-Paniagua *et al.*, 2010; De Schryver *et al.*, 2011; Jatoba *et al.*, 2011; Merrifield *et al.*, 2011; Silva *et al.*, 2011; Yang *et al.*, 2012). However, this methodology is labour-intensive, and its resolution is limited due to the low number of DNA fragments obtained as representative of a microbial community.

In the last years, high-throughput pyrosequencing has been developed and applied to 16S rRNA gene-based analysis of different microbial samples, the human gastrointestinal tract among them (Andersson *et al.*, 2008; Nakayama, 2010; Nam *et al.*, 2011). However, this second-generation sequencing technique has been poorly used to study the complex microbial community in the fish gut (Wu *et al.*, 2012b).

In this study, we fed fish the same base diet with the addition of β-glucans and essential vegetal oil that may have an immunomodulatory health effect. We analysed and compared the IM of cultured sea bass fed these different functional diets applying pyrosequencing of the 16S rRNA gene, with the aim of observing possible effects of the potential immunostimulants on its composition.

Materials and methods

Fish and diets

European sea bass (*Dicentrarchus labrax*) of 14.6 ± 1.27 g on average weight were maintained in tanks at 22 °C and 30% salinity in facilities at University of Valencia (Planta de Acuarios de Experimentación, PAE). Fish were fed during 8 weeks with a control diet (A) or functional diets (B and C). Food regime was once per day at 5% on a body weight basis. The basal composition was the same in the three diets, and purified β-1,3/1,6-glucans (Macrogard®) were used as immunostimulant at 0.1% (Table 1). Moreover, an essential oil from vegetal origin was added to functional diet C at a proportion of 0.1%.

Table 1. Composition of the fish diets

	Diet		
Ingredients	A	B	C
Wheat	12.73*	12.62	12.53
Hi-pro soya 12C	15.00	15.00	15.00
Wheat gluten 12C	15.09	15.09	15.09
SPC 12C	20.00	20.00	20.00
FM North-Atlantic 12C	20.00	20.00	20.00
Fish oil North-Atlantic	16.05	16.05	16.05
Vitamin–mineral premix	1.13	1.13	1.13
B-glucans	0.00	0.10	0.10
Essential oil	0.00	0.00	0.10

*Percentage of the ingredient in the general composition of diet.

Sample collection

Intestinal content and intestinal mucus were sampled following the method described by Kim *et al.*(2007). Briefly, the digestive tract was aseptically separated from the abdominal cavity with a scalpel, and its content was removed and collected. Then, after rinsing the digestive tract several times in sterile PBS, the mucus was scraped off with a sterilized scalpel and collected in sterile 1.5-mL tubes.

Four fish per group were sampled after four (time 1, T1) and eight (time 2, T2) weeks of diet administration. The average weight of fishes at the final of diet administration was 39.6 ± 3.78. Each sample was divided in two subsamples before DNA extraction. Moreover, in selected mucus samples, DNA was immediately extracted (fresh sample/nonfrozen sample) or extracted after storing at −80 °C (frozen sample).

DNA extraction

DNA was extracted using the MasterPure Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) following the manufacturer's instructions and adding a lysozyme (SIGMA) treatment (1 mg mL^{-1}, 37 °C for 30 min). The DNA concentration and quality was determined by agarose gel electrophoresis [1% wt/vol agarose in Tris-acetate-EDTA (TAE) buffer] and using NanoDrop ND-1000 spectrophotometer (Thermo Scientific).DNA extracted was stored at −20 °C until used for PCR amplification.

PCR and pyrosequencing

The first 300 bp of the 16S rRNA gene was amplified with the universal eubacterial primers 27F (5′-AGAGTTT GATCMTGGCTCAG-3′) and 300R (5′-GCTGCCTCCCG-TAGGAGT-3′) with an annealing temperature of 52 °C and 20 cycles to minimize PCR biases (Sipos *et al.*, 2007). A secondary amplification with equal conditions was performed using the purified PCR product as a template when the DNA concentration was insufficient. A nested PCR approach has been used to reduce the frequency of nonspecific amplification (Patin *et al.*, 2013). The 27F universal primer was modified to contain an 8-bp 'tag sequence' specific to each sample, following McKenna *et al.* (2008). Barcodes were different in at least two nucleotides from each other to minimize mistakes in sample assignments.

PCR products were purified using PCR Clean-up DNA Purification Kit (Mo Bio) or GelSpin DNA Extraction (Mo Bio).

The pyrosequencing was performed at the Center for Advanced Research in Public Health (CSISP; Valencia, Spain) using the 454 FLX sequencer (Roche, Basel, Switzerland) with Titanium chemistry. Selected DNA samples were amplified and pyrosequenced by duplicate to assess the reproducibility of this methodology (duplicate samples).

Sequences from all experiments have been deposited in the Sequence Read Archive of National Center for Biotechnology Information, submission number SRS38 6036.

Taxonomic analyses of sequence reads

Sequences of < 250 bp as well as those with an average quality score lower than 20 and sequences with more than one ambiguous base call were removed using Ribosomal Database Project (RDP; Wang *et al.*, 2007). The program was used to separate samples according to the barcode sequences tagged to each forward primer. The possible chimeras were detected using Mothur (Schloss *et al.*, 2009), and an average of 4.45% of sequences for each sample were filtered out as potential chimeras. As we observed high differences in the number of reads in T1 mucus subsamples, they were pooled for subsequent taxonomic analysis.

Taxonomic assignment of the sequences was made using the RDP classifier (Wang *et al.*, 2007), with an 80% confidence threshold.

To estimate bacterial diversity, the number of operational taxonomical units (OTUs) present in the samples was determined, and a rarefaction analysis was performed. Rarefaction curves were obtained by plotting the number of observed OTUs against the number of sequences. Equal numbers of sequences were used to minimize the biases caused by sequencing effort differences (Schloss *et al.*, 2011). A 97% sequence identity of the 16S rRNA gene was used to determine species-level phylotypes. Additionally, indexes of diversity (Shannon) and richness (Chao1) were calculated.

The overall composition of intestinal microbial communities was compared using principal coordinate analysis (PCoA) performed by Fast UniFrac (Lozupone *et al.*, 2006; Hamady *et al.*, 2010). This tool measures the similarity between bacterial communities based on phylogenetic distances, taking into account both taxonomically assigned and unassigned reads. After clustering sequences at a 97% sequence identity, a weighted and unweighted PCoA test was performed.

Statistics

Significance tests based on the phylogenetic UniFrac distances (Hamady *et al.*, 2010) were performed. The *P*-values reported for multiple comparisons were corrected by Bonferroni correction, which is performed by multiplying the raw *P*-value by the number of permutations (Roesch *et al.*, 2009).

Results

Diversity of sea bass IM

Microbiota present in intestinal content (allochthonous microbiota) and gut mucus (autochthonous microbiota) from fish fed the different diets was assessed by subjecting pyrosequencing reads to OTU determination and rarefaction analysis.

The number of reads (filtered and assigned) and genera observed in allochthonous samples was significantly lower than in autochthonous ones (Table 2).

When a rarefaction analysis was carried out, we observed that the microbiota from mucus samples is more diverse than the microbiota from intestinal contents (Fig. 1). Although the microbiota-based rarefaction curves failed to reach a saturation phase, the slope of the curve from allochthonous microbiota is less steep. Moreover, Shannon-Wiener index from autochthonous samples was higher than that from allochthonous samples. Interestingly, the average number of genera accounting

Table 2. Number of reads, assigned taxa and diversity/richness indexes of different samples

Samples	Reads filtered	Reads assigned (genus)	Number of genera	Number of families	Shannon-Wiener index	Chao1 index
Allochthonous	21823	13075	97	58	2.95	709.95
Autochthonous	118270	50752	156	78	3.13	1019.94
Time 1	76041	25295				
Diet A	42679	20185	106	61	2.76	797.49
Diet B	10012	1927	52	39	1.96	279.18
Diet C	23350	3183	62	42	2.58	333.68
Time 2	42229	25457				
Diet A	13976	7580	79	47	2.85	397.31
Diet B	10447	7597	85	52	2.80	420.63
Diet C	17806	10280	89	51	2.66	469.46

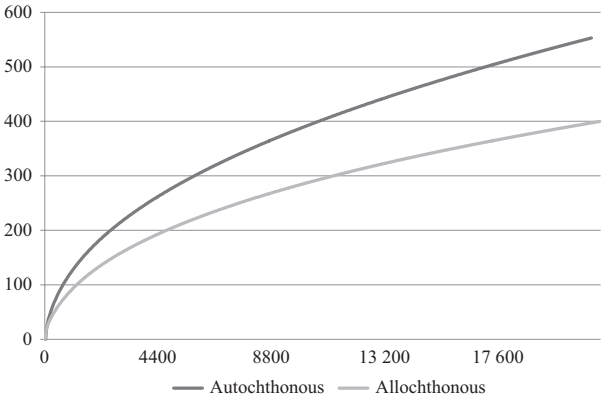

Fig. 1. Comparison of rarefaction curves between autochthonous and allochthonous intestinal microbiota. Curves represent the number of OTUs at 97% of sequence identity, which is used as an inference of the number of species, as a function of the sequencing effort.

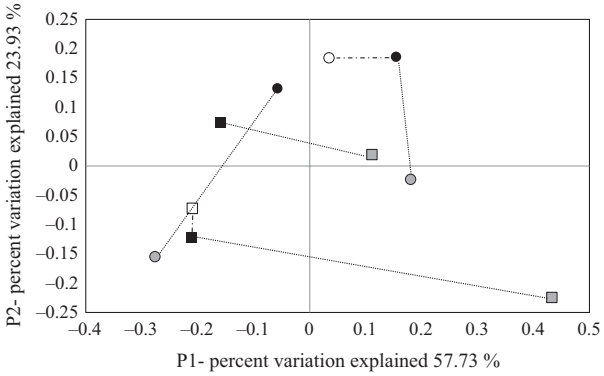

Fig. 2. Principal coordinates analysis (PCoA) from intestinal mucus, replicate and frozen samples according to intestinal microbiota composition. Length of dot lines is correlated with differences in the IM composition. Square, diet A; Circle, diet C; black, mucus sample; white, duplicate sample; grey, frozen sample.

for < 1.5% of the total autochthonous and allochthonous microbial population were 46 and 10.8, respectively, indicating a higher microbial diversity in rare OTUs in mucus-associated niches. Rarefaction curves of individual samples were not totally homogeneous and, in some of them, more sequencing effort would be required to reach saturation (Supporting information, Fig. S1).

Gut mucus subsamples from fish fed the same diet clustered together by PCoA. However, sequence reads from IC subsamples of fish fed the same diet appeared very distant from each other in the PCoA analysis (Fig. S2). This is in agreement with intestinal content samples being more variable depending on environmental factors and sampling protocols. In addition, the higher diversity of mucus samples indicates that intestinal content material lacks some of mucus-associated microbiota. For these reasons, analyses for assessing in-depth the microbial composition were only performed with mucus samples.

Microbial composition of mucus was significantly different (P-test ≤ 2.0e-03) depending on the storage conditions of samples (Fig. 2; Fig. S3). When DNA was obtained immediately after sampling fish fed the control diet, the major bacterial genera were *Dysgonomonas, Mycoplasma* and *Weissella* (Fig. 3). However, *Methylobacterium*, *Ralstonia* and *Bradyrhizobium* were the dominant genera found in the same samples maintained at −80 °C before DNA extraction (Fig. 3). In contrast, the replicate samples appeared together in a principal coordinates analysis (Fig. 2) and were not significantly different (P-test ≤ 0.5).

Influence of diets on the composition of autochthonous IM

Microbial community from gut mucus of sea bass showed differences depending on the diet and time of

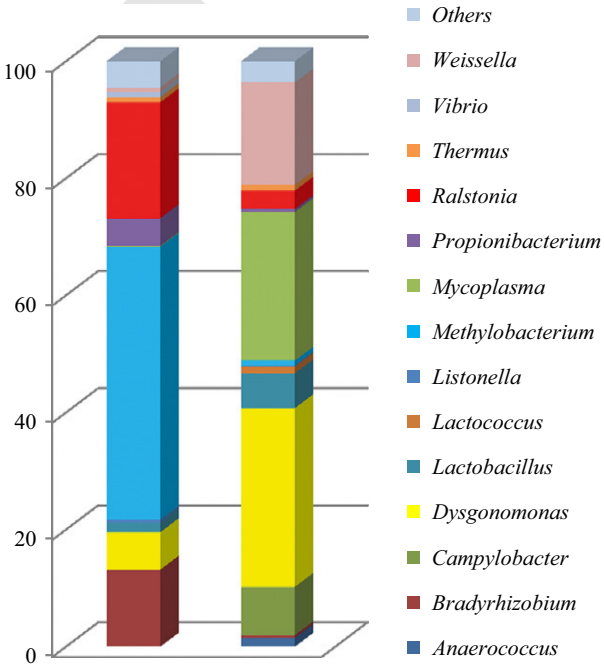

Fig. 3. Autochthonous community composition from control samples stored at different conditions. DNA from one sample was immediately extracted (nonfrozen) and the other was previously stored at −80 °C (frozen) before extraction.

administration. Interestingly, only two or three dominant taxa (more than 30%) were observed in most of the autochthonous microbiota samples.

At family taxonomic level, composition of autochthonous microbiota was different depending on the diet after 4 weeks of administration (Fig. 4). These differences disappeared after 8 weeks. The family *Bradyrhizobiaceae* was

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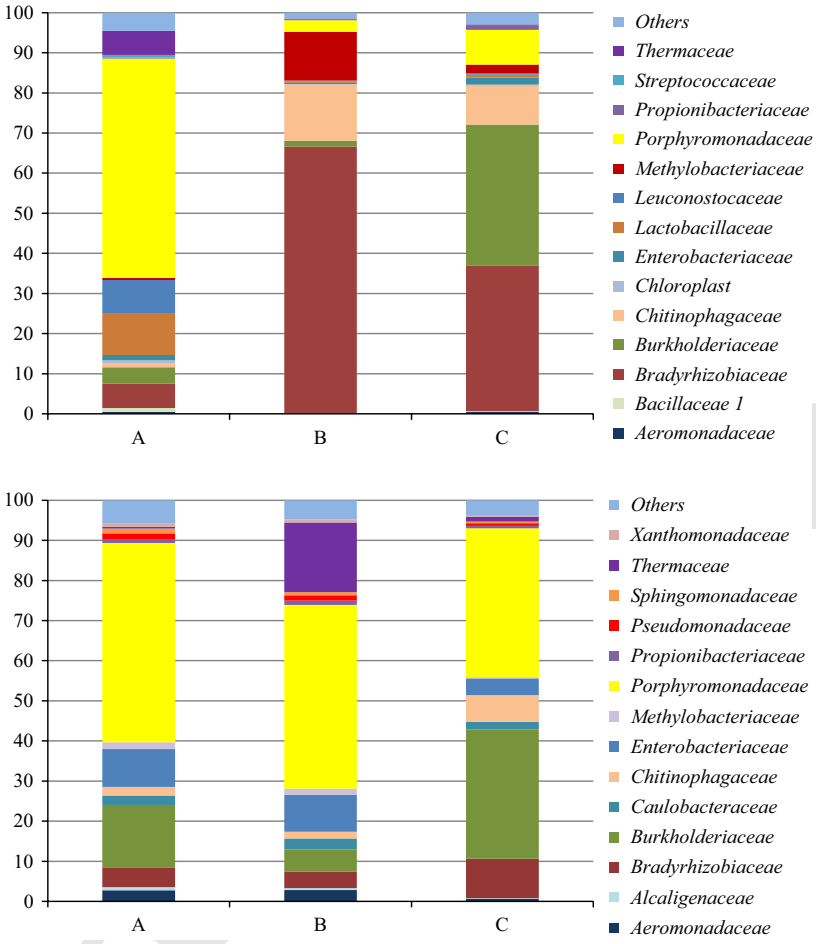

Fig. 4. Comparison of intestinal microbiota composition among control diet (A) and functional diets (B and C) during 4 (T1) and 8 (T2) weeks. Graphs show the percentage of 16S pyrosequencing reads assigned to different bacterial families.

overrepresented at 4 weeks of feeding functional diets, recovering the baseline levels in all samples, independently on the diet, after 8 weeks (Fig. 4). Furthermore, *Porphyromonadaceae* was the major group in microbiota from the control group only at time 1, reaching the same levels in communities from fish fed functional diets after 8 weeks. Finally, *Burkholderiaceae* family only showed a high proportion along the study in samples from fish fed functional diet C.

When the analysis was performed at genus taxonomic level, similar differences were found. The dominant genus in control samples after 4 weeks of diet administration was found to be *Dysgonomonas* (*Porphyromonadaceae* family). This genus reached the same proportions in microbiota from fish fed functional diets at 8 weeks. Moreover, *Methylobacterium* genus dominated the IM of fish fed diet B at T1, reducing the proportion after 8 weeks. Finally, *Ralstonia* genus (*Burkholderiaceae* family) also maintained an elevated proportion only in samples from fish fed functional diet C along the study (Fig. 5).

Principal coordinate analysis (PCoA) was used to cluster the mucus samples depending on the microbial community composition. Comparison of autochthonous microbiota composition among fish fed different diets showed distinct clusters after 4 weeks (Fig. 6). After 8 weeks of diet administration, samples from the different fish groups were clustered closer (Fig. 6), showing that bacterial composition homogenized with time. Moreover, replicate samples were highly similar, appearing very close in the PCoA plot (Fig. 6), thus indicating that variability due to sequencing bias was minimal.

Discussion

Pyrosequencing analysis was successfully applied to the study of microbial communities of the mucosal layer in the sea bass intestine. Moreover, the developed protocol showed to be a reliable approach to analyse the autochthonous IM of this species fed different diets. The results revealed that the transient microbial populations present in intestinal contents of fish are highly variable. Given that the low clustering by PCoA of IC samples from fish fed the same diet also supported this finding, performing comparative analysis of allochthonous microbiota from

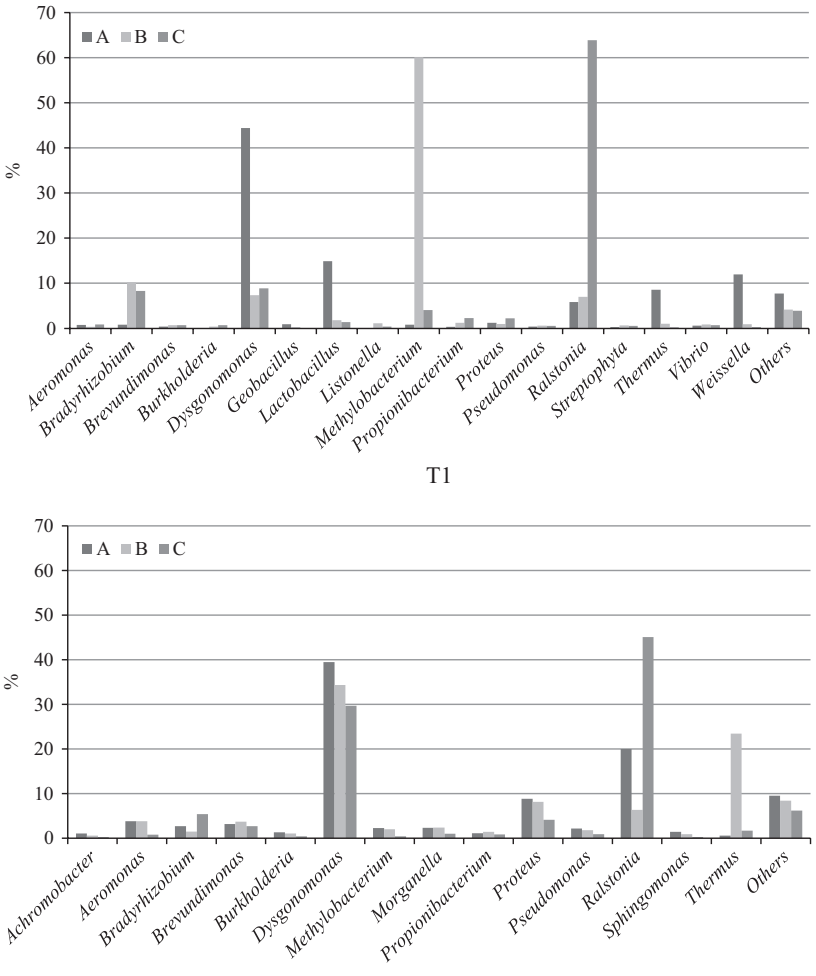

Fig. 5. Autochthonous intestinal microbiota from *Dicentrarchus labrax* fed ~~with~~ control diet (A) functional diets (B and C) during 4 (T1) 8 (T2) weeks. The abundance of each bacterial genus was determined with partial sequences of 16S rRNA genes using the Ribosomal Database Project (RDP). The genera which represent < 1.5% of the autochthonous community were ~~include~~ on 'Others'.

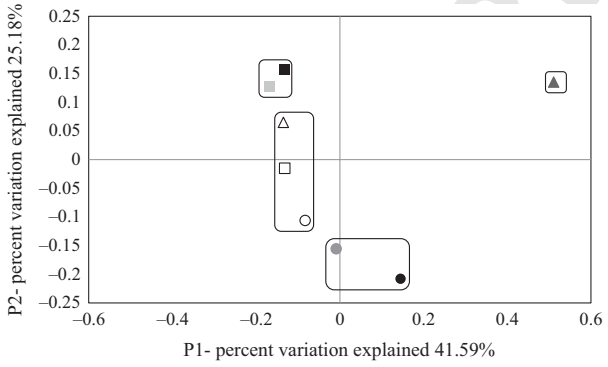

Fig. 6. Principal coordinates analysis (PCoA) from mucus samples according to IM composition. ~~IM from each diet was roughly~~ clustered by diet or time of administration. Square, diet A; Triangle, diet B; Circle, diet C; black, 4 weeks of administration; white, 8 weeks of administration; grey, duplicate sample.

gut could be inappropriate. The autochthonous microbiota appeared to be more diverse than allochthonous one, suggesting that faecal content may have a reduced diversity due to the lower presence of mucus-associated bacteria. Differences in diversity and abundance of bacterial populations in intestinal content and mucus have also been described in other fish species using 16S rRNA gene sequence-based comparison (Kim *et al.*, 2007; Wu *et al.*, 2010, 2012a, b). Similar results had been observed in studies on human IM (Durban *et al.*, 2011). These findings support the view that IC microbiota may not accurately represent the bacterial communities living in the intestinal tract of fish.

IM samples should be stored under the same conditions as our data clearly show that freezing at −80 °C caused significant qualitative and quantitative differences in bacterial composition. In fact, we found significant variation in genus proportions and also in the presence/absence of genera in the same sample depending on its previous storage at −80 °C. As other authors have also reported similar effects in studies of different microbial communities, an effort should be done to standardize sample preservation conditions and to minimize the time

between sampling and sample processing (Morgan *et al.*, 2010; Bahl *et al.*, 2012; Bai *et al.*, 2012; Cardona *et al.*, 2012). Among others, Bacteroidetes presence is adversely affected by the storage conditions of human faecal samples (Bahl *et al.*, 2012), and we have found this bacterial group abundant in autochthonous gut microbiota of sea bass. From our results, we would recommend to extract DNA immediately after obtaining samples to avoid biases due to freezing like those described above.

The present study shows that putative representatives of *Dysgonomonas* genus (*Porphyromonadaceae* family, Bacteroidetes group) are one of the more abundant groups in autochthonous microbiota from sea bass gut under the assayed conditions, but this proportion transitorily reduces in fish fed functional diets during 4 weeks. It has been reported that the tropical reef fish *Kyphosus cinerascens* contains mostly Bacteroidetes in its hindgut, being *Dysgonomas* one of the genus predicted by RDPII analysis of the 16S rRNA gene clones (Troy *et al.*, 2009). These bacteria are believed to digest algae consumed by *K. cinerascens*. Moreover, communities of microbial symbionts, among them *Bacteroides* and *Porphyromonas*, have been found in the hindgut of marine herbivorous fish, which depend on microbial fermentation of their food for nutrition (Ramirez & Dixon, 2003). *Dysgonomonas* could be one of the intestinal symbionts present in the sea bass gut involved in digesting the vegetal material of the tested diets, ingredients which represent more than 60% of their general composition. Members of the genus *Dysgonomonas* are abundant, diverse and widespread in the gut of termites, probably acting as an important player in the digestion of lignocellulose by these arthropods (Husseneder *et al.*, 2009). These authors observed that *Dysgonomonas* species were fastidious and grew very slowly, mainly as satellite bacteria, supporting that this dominant genus could be passed unnoticed unless a molecular approach to determine microbial community composition is performed.

The short-time administration of these diets containing essential oils causes other transitory changes on autochthonous IM, being the *Bradyrhizobiaceae* family (class *Alpha-proteobacteria*) the dominant bacterial group in these fish. Recently, enrichment with species from Rhizobiales has been documented on IM of omnivorous fish (Sullam *et al.*, 2012). Our results are consistent with those of other authors, who suggested that the most common bacteria from fish intestine were those affiliated with proteobacteria (Kim *et al.*, 2007; Wu *et al.*, 2010; Sullam *et al.*, 2012; Wu *et al.*, 2012b). Our findings also support the complexity of IM of fish suggested by other authors (Wu *et al.*, 2012b). The β-1,3/1,6-glucans addition could promote the higher proportion of *Methylobacterium*in mucus samples after 4 weeks of fed diet B. Interestingly, the high proportion of the genus *Ralstonia* (*Beta-proteobacteria* group) in the autochthonous microbial community from fish fed functional diet C was maintained along the study. The dominance of this bacterial group could be related to some specific growth-promoting ingredients (perhaps the essential oil) included in this diet. *Ralstonia* genus has been found in gut content of other cultured fish in low proportions: *R. picketii*, for instance, is a ubiquitous bacterium in water and soil, as well as in yellow catfish (Wu *et al.*, 2010), and unknown species have been documented in rainbow trout (Kim *et al.*, 2007).

Separation of IM samples from fish fed different diets in PCoA supports an effect of functional diets on the microbial composition at the mucosal layer, suggesting that functional diets can be a feasible method to modulate the intestinal bacterial communities. Moreover, the close clustering of duplicate IM samples in PCoA demonstrates that variability due to sequencing bias was minimal.

In summary, the present study shows that pyrosequencing of PCR-amplified rRNA genes is a useful tool to analyse the diversity of authocthonous microbiota of sea bass intestine and that diet plays a significant influence on the composition of this microbial community. In fact, administration of functional diets containing essential oils causes significant changes in the gut microbiota of sea bass. The potential of these diets to affect the mucosal immune response of the fish (e.g. antimicrobial peptides production, changes in gene expression) should be further studied.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Rarefaction curves of individual samples of intestinal content (C) or mucus (M) from diet A, B and C.
Fig. S2. Principal coordinates analysis (PCoA) from autochthonous and allochthonous intestinal microbiota samples according to their composition.
Fig. S3. Autochthonous community composition from samples stored at different conditions.

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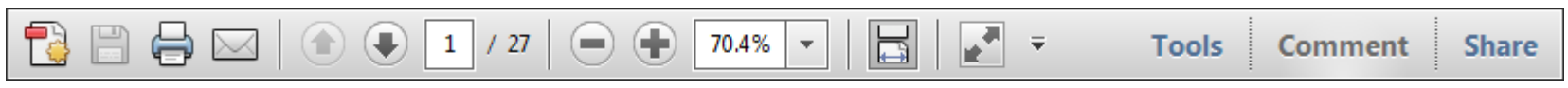

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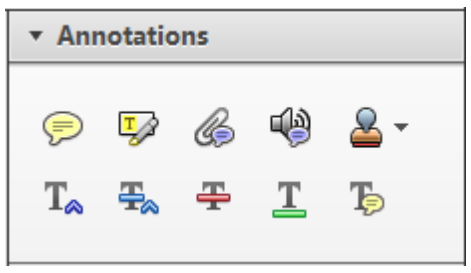
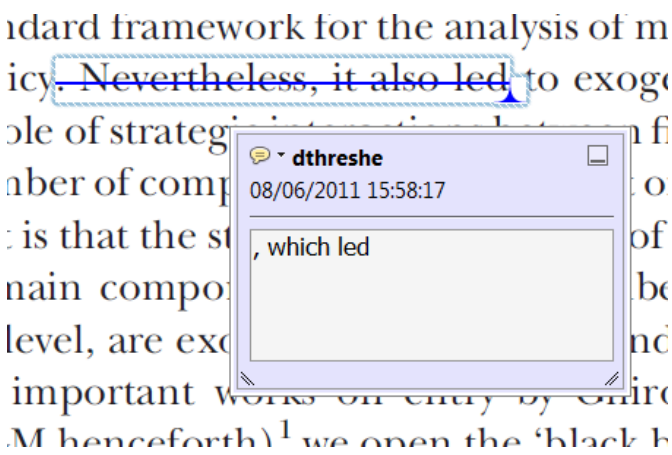


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

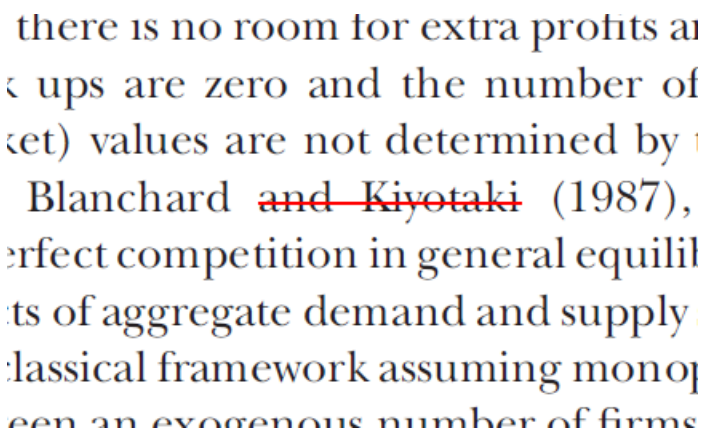


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.



3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
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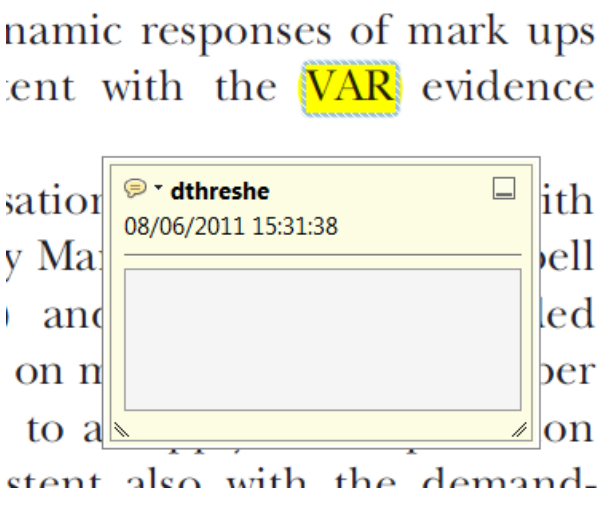
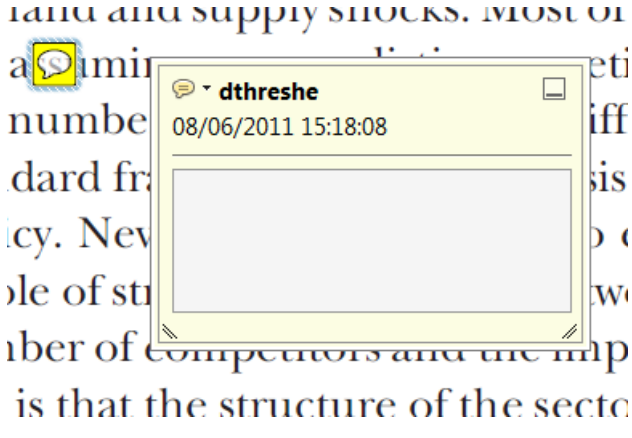


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

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- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
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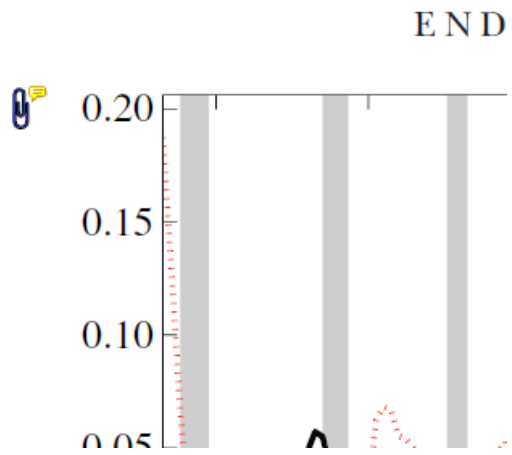

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Inserts a selected stamp onto an appropriate place in the proof.

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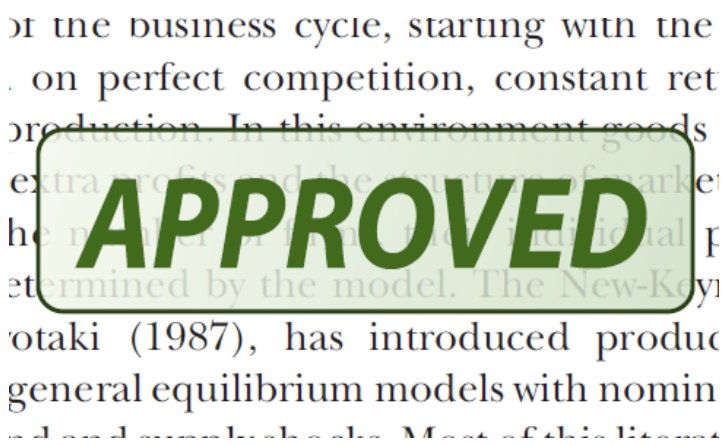

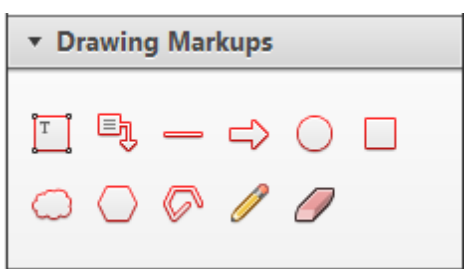

7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

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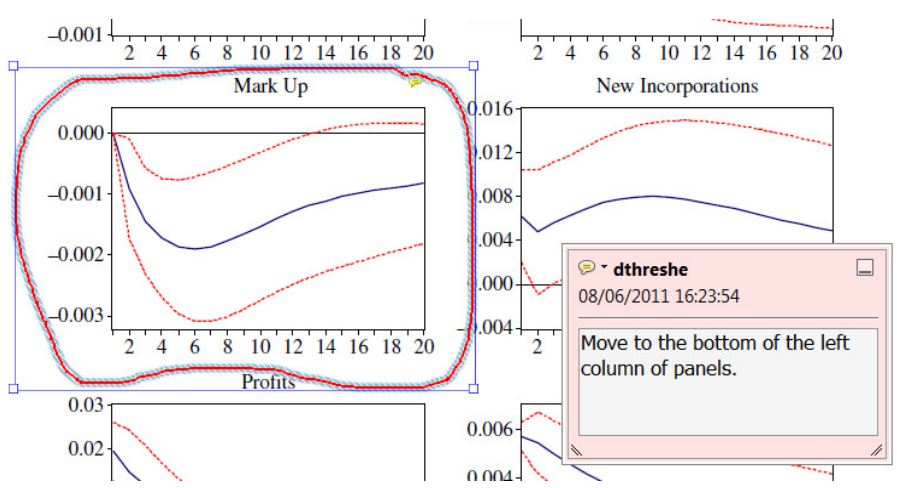

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: