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Research Article

Cultivable intestinal microbiota of yellowtail juveniles (*Seriola lalandi*) in an aquaculture system

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ABSTRACT. The yellowtail (*Seriola lalandi*) has been farmed for many years and is becoming a promising aquaculture species. Knowledge of the intestinal microbiota of this species is very limited. Thus, the aim of this study is to describe the bacterial populations associated with the intestinal tract of *Seriola lalandi* reared in Chile. The microbiota composition was analyzed at two growth stages distinguished by weight and parameters such as Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR). Juveniles (mean initial weight 7.33 ± 0.30 g) and pre-adults (81.7 ± 19.0 g) were fed with commercial diet for 33 and 50 days, respectively. The first intestinal samples were collected at the end of Trial 1 from specimens weighing approximately 50 g while the second samples were obtained at the end of Trial 2 from specimens weighing approximately 370 g. The microbiota composition was examined using conventional isolation in Tryptic Soy Agar (TSA) followed by 16S rRNA sequencing and identification. In total, 16 genera were identified. *Pseudomonas*, *Vibrio* and *Staphylococcus* were the predominant genera in fish at the 50 g stage, whereas *Microbacterium* and *Francisella* were the predominant genera in the 370 g stage. The microbiota composition showed different assemblages, depending on host size, with *Bacillus* and *Vibrio* being the only genera that were shared. Knowledge of the intestinal microbiota of *Seriola lalandi* is the first step in the exploration of microbiota management and the development of probiotics, as well as in the identification of the bacterial populations in healthy fish under cultured conditions.

Keywords: intestinal microbiota, 16S rRNA, *Seriola lalandi*, yellowtail, seawater, aquaculture, Chile.

Microbiota intestinal cultivable de juveniles de dorada (*Seriola lalandi*) en un sistema de acuicultura

RESUMEN. La dorada ("yellowtail", *Seriola lalandi*) ha sido cultivada durante los últimos años, y su expansión se ha debido al mayor conocimiento científico-técnico así como a la creciente demanda, convirtiéndose en una promisoria especie para la acuicultura. Dado que el conocimiento de la microbiota intestinal de esta especie es muy limitado, el objetivo de este estudio es describir las poblaciones bacterianas asociadas al tracto intestinal de *Seriola lalandi* cultivada en Chile. La composición de la microbiota fue analizada en dos etapas de crecimiento diferenciadas por peso y parámetros como SGR y FCR. Los juveniles (peso inicial promedio 7.33 ± 0.30 g) y pre-adultos (81.7 ± 19.0 g) fueron alimentados con dieta comercial por 33 y 50 días, respectivamente. Las primeras muestras fueron recolectadas al término del primer ensayo en ejemplares de 50 g app., y la segunda muestra fue tomada al término del segundo ensayo en peces de 370 g app. La composición de la microbiota fue examinada mediante aislamiento convencional en medio de cultivo de Agar Tripticasa Soya (TSA) seguido de secuenciación e identificación por 16S rRNA. Un total de dieciséis géneros fueron identificados, donde *Pseudomonas*, *Vibrio* y *Staphylococcus* fueron los géneros predominantes en peces de 50 g, mientras que *Microbacterium* y *Francisella* fueron los géneros predominantes en los peces de 370 g. Se determinó que la composición de la microbiota se ajustó a diferentes arreglos en función del tamaño del hospedero, donde los únicos géneros compartidos fueron *Bacillus* y *Vibrio*. El conocimiento de la microbiota intestinal de *Seriola lalandi* es el primer paso para explorar el adecuado manejo de la misma, así

como el desarrollo de probióticos y también obtener referencias de la microbiota de peces sanos en condiciones de cultivo.

Palabras clave: microbiota intestinal, 16S rRNA, *Seriola lalandi*, dorada, agua de mar, acuicultura, Chile.

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INTRODUCTION

Recently, the production of yellowtail from aquaculture has increased, driven by an expansion in biological knowledge and technological advances as well as by growing demand (http://www.worldwildlife.org/what/globalmarkets/aquaculture/WWFBinaryitem14170.pdf). As a way to use the competitiveness skills developed from the salmon farming industry, Chile has established a national policy to expand fish aquaculture and yellowtail (*Seriola lalandi*) has been selected as one of the four species upon which research and development resources will be focused (http://www.conicyt.cl/573/articles-31436 anexo.pdf). Thus, the Chilean Research and Development governmental agencies expect that the country face the challenge of a new yellowtail fish aquaculture industry in a way similar to that of the salmon industry, which started in the 1970s and now represents the fifth (http://www.bcentral.cl/publicaciones/estadisticas/sector.../ice012011.pdf) most important economic activity in the country, accounting for US$3.7 billion in exports during 2010 *FishStat Plus* (http://www.fao.org/fishery/statistics/software/fishstatj/en).

The gastrointestinal (GI) microbiota of fish consists of bacteria residing in the gut that can reach 10^7-10^{11} bacteria g^{-1} of intestinal content (Nayak, 2010), with the highest values observed in herbivorous tropical fish. The early view (1970-1980s) of the existence of a stable microbiota in the GI tract of fish was controversial (Cahill, 1990), principally because of the constant contact of this system with the aquatic environment. A significant number of studies have been performed during the past few decades to characterize the microbiota in a wide range of fish species, which demonstrate that a stable microbiota can be established after the first feeding stages (Navarrete *et al.*, 2010; Hovda *et al.*, 2012), and its major components can be derived from water and egg epibiota (Romero & Navarrete, 2006). The fish gut appears to contain less microbial diversity than the gut of homoeothermic animals. Aerobes and facultative anaerobes predominate in the bacterial microbiota, whereas strict anaerobes are less frequent. The low concentration of short-chain fatty acids in the salmonid gut revealed that bacterial fermentation is not an important process (Holben *et al.*, 2002). These observations can be explained by the consumption of a carbohydrate-poor diet, a short GI tract (in comparison to herbivorous fish), and a low ambient body temperature.

Currently, it is generally recognized that the GI microbiota of animals serves several functions, including nutrition, development, immunity and xenobiotic metabolism. Recent studies performed in model vertebrates, especially zebrafish, also provide insights into the microbial-host molecular dialogs that effect several functions of the host (Rawls *et al.*, 2006). These functions have been recently demonstrated in fish. An important study by Rawls *et al.* (2004) showed that the GI microbiota can regulate the expression of 212 genes in the digestive tract of zebrafish, some of which are related to the stimulation of epithelial proliferation and the promotion of nutrient metabolism and innate immune responses. An important aspect of these results was the specificity of the host response, which depends on the bacterial species that colonize the digestive tract (Rawls *et al.*, 2004). Hence, it is relevant to know the composition of this microbiota in fish.

The aim of this study is to describe for the first time the composition of the intestinal microbiota in *Seriola lalandi*, as well as to compare the changes in the diversity of the intestinal bacterial populations at two different stages during the initial grow out of the fish. Changes in microbiota composition have been described in marine fishes from non-feeding yolk sac to the larvae starting to feed (Verner-Jeffreys *et al.*, 2003). Considering that the marine fish in aquaculture are switched from live feed to dry feed, it is expected that the fish will face a change in the microbiota composition given that the type of food is important for the composition of the fish GI microbiota (Nayak, 2010).

To detect changes in the microbiota composition during initial juvenile growth, a first trial was performed when the fish were a few weeks from the change to dry feed and for a period of 33 days (Trial 1). A second trial was performed at a larger size when the dry feed had been supplied for several weeks and for a period of 50 days (Trial 2). Understanding some aspects of microbial ecology in aquaculture systems, such as knowing the types, number, and sources of

bacteria commonly associated with different developmental stages, could be useful for manipulating the microbiota as a strategy for preventing pathogenic infection or improving nutrition, especially at critical life stages.

MATERIALS AND METHODS

Fish and rearing conditions

Yellowtail fingerlings of 1 g were transferred from a production facility located in the north of Chile to an experimental facility (Universidad Catolica Santisima Concepcion) where the fish were acclimated for six weeks, until they reached approximately 7 g. The fish were located in three different tanks in the same recirculation system. During the first trial, an initial group of 50 fish by tank were loaded to achieve a final stocking stock density of 12.3 ± 0.9 kg m^{-3}. During the second trial, 6 fish were loaded by tank to achieve a final stocking stock density of 14.5 ± 0.3 kg m^{-3}. The fish were fed once per hour every 24 h per day with a commercial diet (SkrettingNutra Alpha Plus, 52% protein and 20-21% lipids, see Table 1). The proximal analysis of the feed diets was determined by the Laboratory of Nutrition and Physiology at the Universidad Catolica de Temuco using previously described methodology (Dantagnan *et al.*, 2009). The trials were performed in a recirculation system with full seawater at 33 ppt, a temperature of 21.2 ± 1.5°C and a 24-h day length (24L:0D).

In the first trial (Trial 1), the initial weight was 7.33 ± 0.30 g, and at the end of this trial, the weight was 52.2 ± 4.9 g. At an interval of 12 days, a second trial (Trial 2) was performed with fish coming from the same initial fish pool. This second trial was performed for 50 days, the initial weight was 81.7 ± 19.0 g and the final weight was 367.9 ± 50.6 g. During Trial 1, the feed rate averaged 4.37% of body weight per day, and during Trial 2, the fish were fed at an average feed rate of 4.46% of body weight per day.

Sample collection

Intestinal samples were collected at the end of day 33 (Trial 1) and at the end of day 50 (Trial 2). Five fish were taken arbitrarily at the end of Trial 1 and seven fish were taken arbitrarily at the end of Trial 2. Fish were anesthetized using benzocaine 20% (BZ 20®, Veterquimica) at a concentration of 3 mL 20^{-1} L water. Intestinal samples were obtained by aseptically dissecting the fish and carefully extracting the entire intestine. The intestinal contents (digesta) were collected aseptically. Every individual digesta was homogenized separately, weighed, and an equal amount of PBS was added.

Table 1. Approximate composition of the diets used in the two trials.

	Diet Trial 1	Diet Trial 1
Pellet size (mm)	1.8	2.3
Dry matter	91.62%	91.87%
Protein	56.51%	55.11%
Lipid	20.59%	21.37%
Fiber	1.34%	1.71%
Carbohydrates	12.10%	13.66%
Ashes	9.47%	8.14%
Energy (MJ kg^{-1})	24,130	24,485

Bacterial counts and isolation

Total viable counts of heterotrophic aerobic bacteria were performed through a spread plate method using Tryptic Soy Agar (TSA, Difco). Aliquots (100 µL) of appropriate dilutions of the homogenates were spread onto plates and incubated for 10 days at 17ºC. The total bacterial counts of the pooled samples were assessed by epifluorescence microscopy using acridine orange, as described previously (Navarrete *et al.*, 2010).

PCR amplification and restriction analysis

All colonies collected from plates seeded with the highest dilutions were analyzed for each sample. Then, 16S rRNA PCR amplification using 27F and 1492R primers, restriction fragment length polymorphism (RFLP) with AluI, and PAGE with silver nitrate staining were performed as described previously (Romero *et al.*, 2002).

Growth and FCR estimates

At the end of each trial, the weight and length of each fish were once again measured under anesthetic. The weight-specific growth rate (SGR) was calculated from the tagged fish as described previously (Hardy & Barrows, 2002). The feed conversion ratio (FCR) was estimated according to Hardy & Barrows (2002) methodology.

Ecological diversity indices

Richness has been measured as the simple count of total genera present at the end of every trial. To compare how different or similar the microbiota was for each trial, the β diversity was compared using the similarity coefficients of the Jaccard and Sorenson indices (qualitative) as well as the Sorenson quanti-

tative index (Magurran, 1988). Shannon's entropy or the Shannon index of diversity was used to compare the genera composition at the end of both trials. Using Simpson's index, dominance measures were determined because they are weighted towards the abundances of the commonest genera rather than providing a measure of genera richness. To evaluate how equally abundant the genera were, evenness (Buzas & Gibson's index) and equitability were calculated (Magurran, 1988).

Principal components analysis

Principal Components Analysis (PCA) was determined using Statgraphics Centurion XV (Statpoint Technologies Inc., Warrenton, Virginia, USA).

RESULTS

The average number of total bacteria was $2.6x10^7$ bacteria g^{-1} of intestinal contents. The average count of cultivable bacteria was $9.5x10^3$ CFU g^{-1} of intestinal contents, and in all samples, the cultivability was approximately 0.04% (ranging between 0.009% and 0.5%). In total, 50 strains recovered on TSA medium were analyzed by AluI digestion of PCR-amplified 16S rRNA.

The growth rates during the two trials exhibited different patterns. During Trial 1, the growth rate of the fish (SGR) was almost twice that of the growth rate of the fish during Trial 2 (Fig. 1, Table 2), even when the feed conversion ratio (FCR) was very close. The growth efficiency measured as the rate of SGR over FCR in Trial 1 was almost twice that of Trial 2, indicating that the fish grow better and use the feed better at smaller sizes than at larger sizes.

The analysis of the intestinal microbiota shows a different composition between the two trials. At the end of Trial 1(33 days), the intestinal microbiota was dominated by γ-Proteobacteria (73%, Fig. 2a), whereas at the end of Trial 2 (50 days), the intestinal microbiota was more heterogeneous in composition, being composed of Actinobacteria (43%), γ-Proteobacteria (39%) and Bacilli (18%) (Fig. 2b).

Analysis of the intestinal microbiota at the genus level shows that the composition at the end of Trial 1 (52 g and 33 days of feeding) is quite different from that at the end of Trial 2. At the end of Trial 1, the predominant taxa were *Pseudomonas* (32%), *Vibrio* (18%) and *Staphylococcus* (14%) (Fig. 3). At the end of Trial 2 (367.9 g and 50 days of feeding), the predominant taxa were *Francisella* (21%) and *Microbacterium* (21%); at lower proportions, Trial 2 also demonstrated the presence of *Arthrobacter* (14%), *Bacillus* (14%) and *Vibrio* (14%) (Fig. 3).

PCA of the intestinal microbiota (Fig. 4) shows a different composition that is consistent with the findings from the analysis of different taxa levels with respect to both class and genus. Furthermore, fish at the end of Trial 2 have a more homogeneous microbiota composition than fish at the end of Trial 1, which is consistent with the measurements of evenness and equitability (Table 3).

Even when the genus richness is the same between the sample points (Trials 1 and 2, see Table 3), a comparison of the diversity using the quantitative Jaccard and Sorenson indices and the Sorenson quantitative index shows that the composition is quite different (0.13, 0.22 and 0.20, respectively) because only two genera were shared by the fish from the two collection sample dates (Trial 1 and Trial 2). The analysis of the diversity indices showed that the dominance index was slightly higher in Trial 1 than in Trial 2. However, the Shannon's entropy index was slightly higher in Trial 2 than in Trial 1, while the evenness and equitability indices in Trial 2 were slightly higher than in Trial 1. None of these indices demonstrated statistically significant differences between the means at the 95% confidence level from a t-test, and the Kolmogorov-Smirnov test showed that there was no significant difference between the two distributions at the 95% confidence level.

DISCUSSION

Little information is currently available on the microbiota composition of the *Seriola* genus. The microbiota of *Seriola quinqueradiata* was reported by Sakata *et al.* (1978) using classic culture methods and phenotypic identification. To our knowledge, the current study is the first study of the intestinal microbiota of *Seriola lalandi*. The intestinal microbiota observed at different growth stages during this experiment was different even when richness was the same. The microbiota composition showed a similarity of approximately 20%, with only two genera shared at the end of the two trials, *Bacillus* and *Vibrio*. Regarding the difference in genera composition of the microbiota, neither trial showed a difference in the dominance Simpson's index, meaning that all genera were equally present, and the similarity of the evenness and equitability indices shows that there were no significant differences at the end of either trial, indicating that all genera were numerically equal in the fish intestinal microbiota. Shannon's entropy index is consistent with the

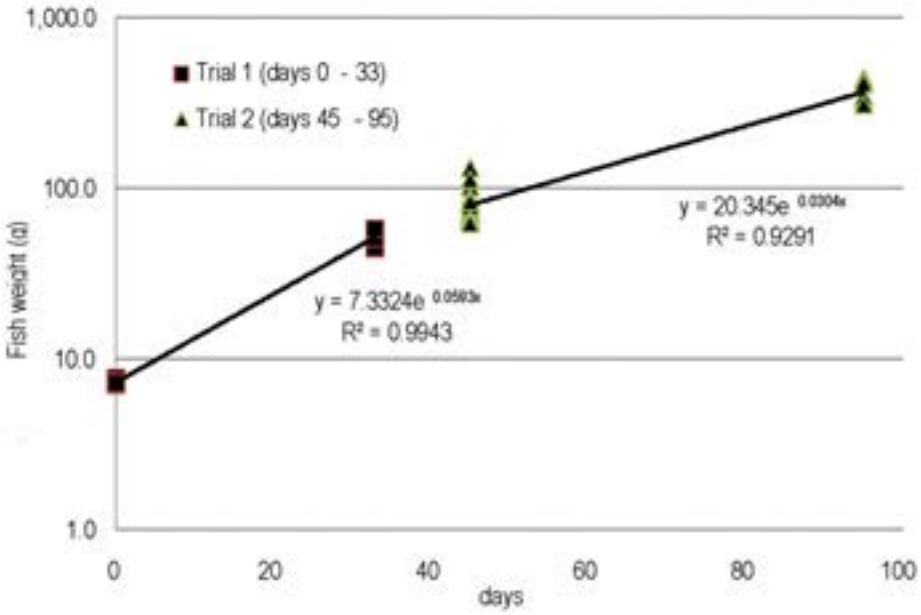

Figure 1. *Seriola lalandi* juvenile growth during the experiment. Growth from day 0 to day 33 is shown as well as the growth from day 45 to day 95. The samples were collected on day 33 (Trial 1) and day 95 (Trial 2). The weight is shown as a logarithmic scale to provide a better appraisal of differences in the slopes of the growth trends from both trials.

Table 2. Specific growth rate (SGR) and feed conversion ratio (FCR) of *Seriola lalandi* juveniles during the two trials.

Trial	1	2
Days of feeding	33	50
SGR	5.95	30.1
FCR	1.31	1.29
SGR/FCR	4.54	2.33

relatively low number of taxa detected in the present study.

The previous work on *Seriola quinqueradiata* GI microbiota (Sakata *et al.*, 1978) reported that the intestinal microbiota was mostly composed by *Vibrio* spp. Similarly, in the current study, *Vibrio* was reported as a secondary genus in terms of abundance in both small and large fish. However, other bacteria from the class γ-Proteobacteria, including *Vibrio*, *Pseudomonas* and *Acinetobacter*, were found. The carnivorous diet of yellowtail may partly explain the low number of taxa observed. Indeed, a recent study indicated that diet influences the bacterial diversity of the digestive tract. In that report, a more comprehensive analysis of vertebrate gut microbiota (although based primarily on mammalian data) indicates that bacterial diversity increases with the transition from carnivory to omnivory to herbivory (Ley *et al.*, 2008).

The aerobic cultivable isolates from the intestines of farmed yellowtail belonged to several genera of γ-Proteobacteria, which represented 73% of all isolates at day 33 (Trial 1). However, at the end of the experiment, at day 95 (Trial 2), the γ-Proteobacteria had become the second predominant taxa after the Actinobacteria. As γ-Proteobacteria have been shown to play an important role in animal nutrition and the ability to create conditions for the growth of other beneficial bacteria (Moya *et al.*, 2008; Neulinger *et al.*, 2008), the reduction in the abundance of this group at the end of Trial 2 may be a symptom of less favorable conditions for fish growth, as has been detected from the SGR and SGR/FCR outcomes. Remarkably, the Actinobacteria represent only 4% of the isolates at the end of day 33 (Trial 1) and become the predominant taxa at the end of the experiment (day 95, Trial 2). As the Actinobacteria are well known as secondary metabolite producers (Sanchez *et al.*, 2012), it is possible that the increase in the presence of this group is a response to some environment conditions or to some dietary needs that were not properly satisfied.

We propose that the changes in the growth trend between the two trials, as measured by SGR and the SGR/FCR rate, can be related to the microbiota composition, as has been described in humans (Ley *et al*., 2005, 2006), mice (Cani *et al*., 2008) and grouper (Sun *et al.*, 2009). In those hosts, important

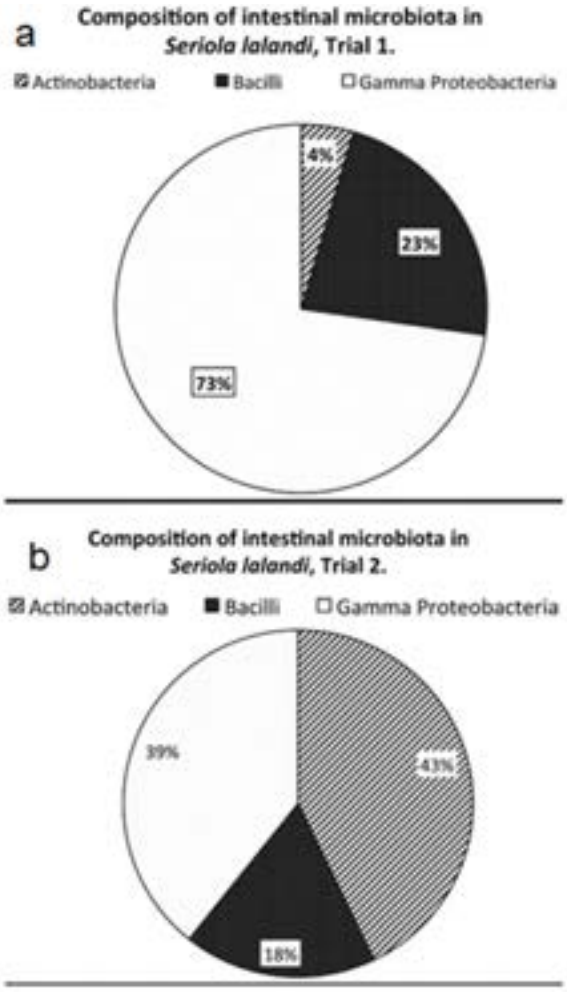

Figure 2. *Seriola lalandi* intestinal microbiota composition at day 33 (Trial 1, 2a) and at day 95 (Trial 2, 2b) at the class level. In Trial 1, the γ-Proteobacteria predominate the sample at 73%, whereas in Trial 2, the γ-Proteobacteria represent the second taxa after the Actinobacteria but at a close level (39% and 43%, respecttively).

differences in gut microbiota were found between normal individuals and obese ones. The obese individuals had a consistently larger proportion of Firmicutes than did normal-weight individuals. Similarly, fish from Trial 1 that exhibited better growth had a larger proportion of Firmicutes than did the fish from Trial 2 that had a lower growth rate. Importantly in mice, carbohydrate active enzymes were detected as being associated with the change of microbiota, which allowed obese individuals to enhance their metabolism (Turnbaugh *et al.,* 2009). In grouper, the authors suggested that fast growing fish harbor a favorable microbiota. The different microbiota observed between the trials was expected when considering previous reports in salmonids, where microbiota composition may change during different growing stages (Romero & Navarrete, 2006). This point should be further addressed in fish in the future.

There is evidence that some bacteria can play an important role in promoting the growth and feeding performance of freshwater fishes such as tilapia (Lara-Flores *et al.,* 2003; Wang *et al.,* 2008; Abd El-Rhman *et al.,* 2009), rainbow trout (Bagheri *et al.,* 2008) as well as marine fishes such as sea bass (Carnevali *et al.,* 2006). The finding of better growth and feeding performance during the first phase of the experiment, which showed *Pseudomonas* as the predominant genus, is consistent with the use of some strains of this genus as a probiotic agent and growth promoter in tilapia (Abd El-Rhman *et al.,* 2009). In contrast, during Trial 2, *Bacillus* was one of the predominant taxa; however, given the lower growth rate in Trial 2, it is possible that the *Bacillus* strains are different from those that have been used as growth promoters in other species (Yanbo & Zirong, 2006; Bagheri *et al.,* 2008; Mesalhy *et al.,* 2008).

More research is needed to modulate the intestinal microbiota to increase the proportion of beneficial bacteria that can allow improved growth and feeding performance, thus enhancing the promise of yellowtail as an aquaculture species. Furthermore, the results achieved in this current study are limited to fish raised in recirculation conditions, and further research is necessary to verify the intestinal microbiota composition of wild fish and fish raised in different aquaculture conditions.

The limitations of our study include the lack of a control for the effect of genetic variation within the study fish. In terms of the genetic composition of the study population, it is recognized that family effects on physiological performance (Johnson *et al.,* 2004) may influence gut microbiota (Cantas *et al.,* 2011). Moreover, there is evidence that the members of the genetic families share significant associations with certain bacterial groups, suggesting that the nature of the gut microbiota is influenced by the host (Navarrete *et al.,* 2012).

More research will be necessary to identify bacteria that cannot be cultured in aerobic TSA cultures because cultivable bacteria represent roughly 1% or less of the total bacteria present in a natural sample. Approaches using molecular techniques such as DGGE or TTGE may provide better and broader knowledge on yellowtail intestinal microbiota. This study presents an overview of the intestinal microbiota of *Seriola lalandi* that can be used as a reference for fish from production rearing conditions

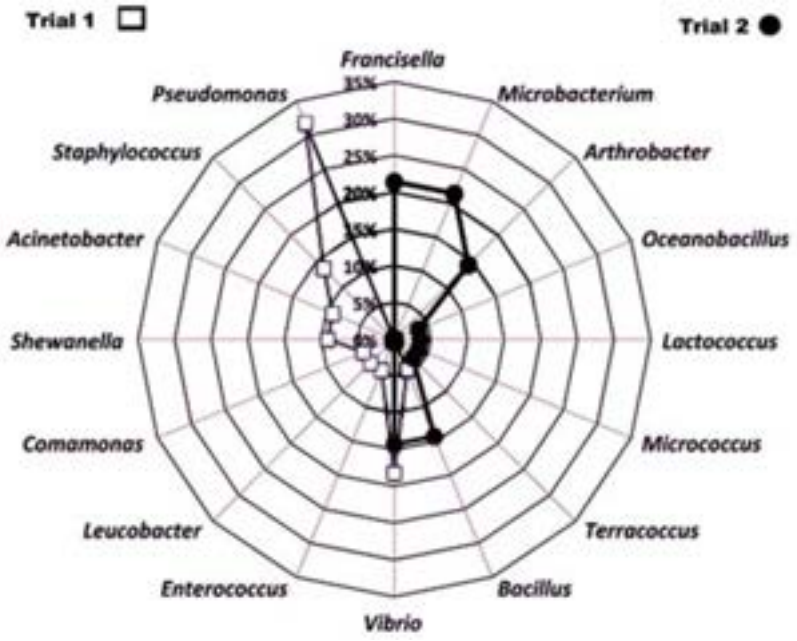

Figure 3. *Seriola lalandi* intestinal microbiota composition at day 33 (Trial 1) and at day 95 (Trial 2) at the genera level. In Trial 1, *Pseudomonas* is by far the most predominant taxa followed by *Vibrio* and *Staphylococcus*, whereas in Trial 2, *Francisella* and *Microbacterium* are the predominant taxa followed by *Vibrio*, *Arthrobacter* and *Bacillus*.

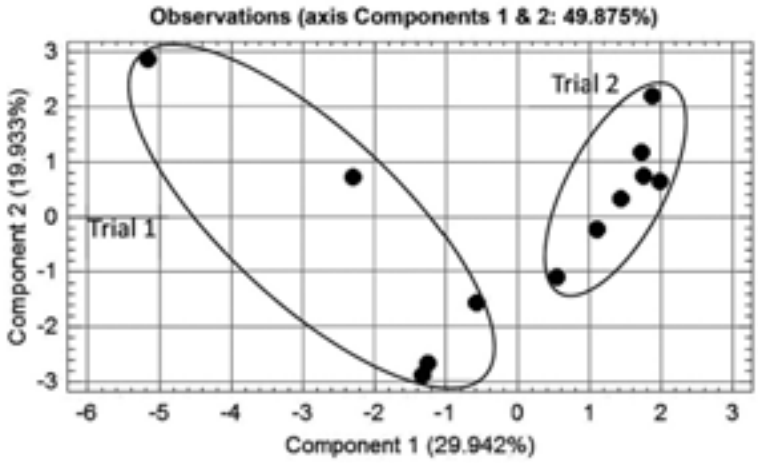

Figure 4. Comparison of the intestinal microbiota composition of *Seriola lalandi* at day 33 (Trial 1) and at day 95 (Trial 2). The Principal Components Analysis (PCA) plot based on the bacterial identification data from intestinal bacterial communities.

Table 3. Ecological diversity indices of the intestinal microbiota of *Seriola lalandi* juveniles at the end of the trials. ns: no significant difference at 95% confidence level t-test.

Trial	1	2
Days of feeding	33	50
Richness	9	9
Dominance Simpson's index	0.18 ± 0.05	0.16 ± 0.07 ns
Shannon's entropy	1.94 ± 0.01	1.97 ± 0.01 ns
Buzas & Gibson's evenness	0.78 ± 0.02	0.80 ± 0.01 ns
Equitability	0.88 ± 0.04	0.90 ± 0.01 ns
ß- Diversity		
Jaccard qualitative	0.13	
Sorenson qualitative	0.22	
Sorenson qualitative	0.20	

to allow the detection of environmental, nutritional or disease problems, especially when antibiotics are used (Navarrete *et al*., 2008; Romero *et al.*, 2012).

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