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Influence of the diet on microbial diversity of faecal and gastrointestinal contents in gilthead sea bream (*Sparus aurata*) and intestinal contents in goldfish (*Carassius auratus*)

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Abstract :

Fish intestinal microbiota changes with diet and this effect is of particular interest considering the increasing substitution of fish meal by plant protein sources. The objective of this work was to study the effects of partial substitution of fish meal with lupin and rapeseed meals on gut microbiota of the gilthead sea bream (*Sparus aurata*) and in goldfish (*Carassius auratus*). Faecal, gastrointestinal and intestinal contents were characterized by culture-based and molecular methods. *Vibrionaceae* was high in faeces and in the intestine of sea bream, while a more diverse microbiota was retrieved from the stomach, where *Bacillales* and *Flavobacteriaceae* appeared to be influenced by the diet. PCR-DGGE profiles revealed high diversity of the microbiota transiting in the sea bream digestive tract, with a shift between gastric and intestinal communities, specially in the group fed with lupin meal. The goldfish was different, with predominance of *Aeromonas* spp., *Shewanella putrefaciens* and *Staphylococcus* spp. among the aerotolerant cultivable bacteria. The culture-independent methods revealed the presence of anaerobes like *Cetobacterium somerae*, and that of *Vibrio* spp., likely in a viable but non-cultivable state. There was a trend towards decreasing diversity in goldfish microbiota with the partial substitution by lupin, which seemed to inhibit some taxa.

Keywords : *Sparus aurata* ; *Carassius auratus* ; lupin meal ; rapeseed meal ; DGGE ; microbiota ; stomach ; intestine ; faeces

1. Introduction

The gastrointestinal microbiota in fish is constituted of facultative and obligate anaerobes, which may vary among fish species with different digestive apparatus (Cahill, 1990). Microbes are known to play crucial roles in maintaining gut integrity, in strengthening immunity and disease resistance, and in contributing to digestion in higher animals. Despite their potential importance, the role of gastrointestinal microbiota has been much less studied in fish than in human and homoeothermic animals (Gatesoupe, 2010). One of the most important issues in microbial ecology of the gastrointestinal tract is the understanding of how dietary factors influence digestive microbiota. Hosted microbes may bring exogenous enzymes and fermentation products to fish with various feeding habits, especially when their diet is rich in recalcitrant substrates such as fibre (Clements, 1997). The interest of the possible role of gut microbiota in fish digestion emerged with the increasing proportion of plant protein sources introduced in fish feed to compensate for the shortage of fish meal (Gatlin et al., 2007; Barrows et al., 2008), but there are yet few reports on the effect of plant protein sources on gastrointestinal microbiota in fish. Eighteen years ago, Patti et al. (1992) did not find any significant change in the bacterial counts on selective or non-selective agar media, when European sea bass were fed diets rich in soybean meal, but more recently, such influence on intestinal microbiota was demonstrated in salmonids by using molecular methods (Heikkinen et al., 2006; Ringø et al., 2006; Bakke-McKellep et al., 2007; Ringø et al., 2008; Merrifield et al., 2009). Dimitroglou et al. (2010) studied the effect on gut microbiota of feeding gilthead sea bream (*Sparus aurata*) with mannan oligosaccharide and soybean meal, and the authors demonstrated that microbial diversity and richness were affected by both of the feed components.

In a previous study, the effect of partial substitution of fish meal by either lupin or rapeseed meal on growth and enzymatic activities in intestinal brush border membrane of fishes was evaluated (Silva et al., 2010). This effect observed in the short digestive tract of a marine temperate fish was compared with those occurring in the relatively long intestine of the stomachless fresh warm water goldfish (*Carassius auratus*). Though there was no effect on growth in both species, the digestive enzymes were differently affected. The vegetable feedstuff decreased the activity of several enzymes in sea bream, whereas only maltase activity was decreased in goldfish fed with lupin. It was concluded that both species could adapt to partial substitution of fish meal with a possible interaction with microbiota, which remained to be investigated (Silva et al., 2010).

Therefore, the present study evaluated the effect of partial substitution of fish meal by either lupin or rapeseed meal on the diversity of microbial species associated to faeces and gastrointestinal contents in gilthead sea bream, and those associated to intestinal contents in goldfish using culture-based and molecular methods.

2. Materials and methods

2.1. Animals and experimental design

The rearing experiments were described elsewhere (Silva et al., 2010). In brief, gilthead sea bream (*Sparus aurata*) were reared in running seawater at 18-20°C (12 conical fibreglass tanks, 60 l capacity; 40 individuals per tank). The goldfish (*Carassius auratus*) were reared in running fresh water at 23-25°C (9 conical fibreglass tanks, 60 l capacity; 30 individuals per tank). Photoperiod was maintained at 12-12 light/dark in both experiments. The experiment lasted 30 days, during which the fish were fed a control diet F with fish meal as the sole protein source, or one of two mixes with fish meal and a plant protein source, which was introduced at the level of 20% of the diet - either lupin meal or rapeseed meal in diets L and R, respectively. The three diets were isoproteic (42% of crude protein, on dry matter basis, Table 1). The fish were fed twice a day, at 2% of daily ration on body weight basis. No mortality was observed during the experiment. The gilthead sea bream grew from 18.8 $\pm$ 2.1 g to 33.8 $\pm$ 5.5 g, and the goldfish grew from 21.5 $\pm$ 0.3 g to 29.2 $\pm$ 7.3 g. No significant difference was observed among the mean weights of the groups.

2.2. Microbial counts and isolation

A device for faeces collection (Choubert et al., 1983) was connected below the water outlet of each tank. Faecal samples were aseptically collected immediately after release for a period of 3 weeks, for quantification and characterization of microbiota. At the end of the experiments, in both gilthead sea bream and goldfish, six individuals were sampled in each tank. After euthanasia using excess anaesthetic, the stomach and the intestine of gilthead sea bream, or the anterior and posterior parts of the goldfish intestine were aseptically removed by dissection, and the contents were squeezed out and collected. The contents were weighed and homogenized for 3 min in a Stomacher 400 Laboratory Blender (AJ Steward Company LTD, London, England) in sterile half-salinity water (distilled water: seawater 1:1, v/v) for microbial marine fish and sterile 0.85% (w/v) saline for microbial fresh water fish.

Then, tenfold dilutions from faeces, stomach and intestine contents were prepared to obtain 10^{-1}-10^{-2}-10^{-3}-10^{-4}-10^{-5}, 10^{-1}-10^{-2}, and 10^{-1}-10^{-2}-10^{-3}-10^{-4}-10^{-5} dilutions, respectively. One millilitre of the dilutions 10^{-4}-10^{-5} (faeces), 10^{-1}-10^{-2} (stomach) and 10^{-4}-10^{-5} (intestine) was spread on Petrifilm (Aerobic Count Plates, 3M Microbiology Products, St Paul, MN, USA). Aliquots of 100 µl of the dilutions 10^{-1} and 10^{-2} from faeces, stomach and intestine contents were spread onto de Man, Rogosa and Sharpe agar (MRS; Oxoid) to counting lactic acid bacteria, and onto Sabouraud dextrose agar with antibiotics (Tovar-Ramirez et al., 2004) to counting yeast. The plates and Petrifilms were incubated at 20°C and inspected for up to five days. Approximately 24 colonies were isolated from each individual Petrifilm and five colonies were isolated per plate for phenotypic characterization of bacteria from gilthead sea bream and goldfish. The aerobes bacterias were cultured on Plate Count Agar (PCA, AES Laboratoires) dissolved in distilled water with pH adjusted to 7.8, and enriched with 18 g l^{-l} NaCl for the marine strains. The yeasts were cultured on Sabouraud dextrose agar.

Pure cultures were stored in liquid nitrogen in tryptone soya broth (TSB; Oxoid), or in Sabouraud dextrose broth, both supplemented with 15 % (v/v) glycerol as cryoprotectant.

2.3. Phenotypic characterization

The isolates were classified on the basis of cell morphology, motility, catalase and oxidase reactions. A randomly taken sample of bacteria and yeasts were characterized with API 20E and API 20 AUX strips, respectively (BioMérieux, Marcy l'Etoile, France). API 20E is one of the most commonly used kits for rapid diagnosis of bacteria from fish (Popovic et al., 2004). The strips were used according to the manufacturer's instructions, except for the suspension medium which was replaced by sterile half-salinity water for the marine isolates. The isolates were screened for similarity with their API 20E and 20 AUX profiles, before selection of representative strains for genotyping.

2.4. Amplified ribosomal DNA restriction analysis

After phenotypic characterization, the isolates were screened for similarity. One isolate corresponding to each phenotypical profile was compared by amplified ribosomal DNA restriction analysis (ARDRA) as described by Gatesoupe (2002). Briefly, DNA was extracted and prepared for PCR to amplify a fragment of the 16S rRNA gene with the universal primers for bacteria SA-dir (5'-AGAGTTTGATCATGGCTCAG-3') and S17-rev (5'-GTTACCTTGTTACGACTT-3'). A fragment of yeast DNA was amplified by using primers of internal transcribed segments ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). To characterize the amplicons by ARDRA, 4 µl aliquots of each PCR product were added to 5.5 µl of buffered premix with one of the two restriction enzymes, *Hae*III or *Cfo*I (Sigma, St Louis, USA). The incubation was carried out for 4 h at 37° C. The restriction products were deposited into wells of agarose gel (1.5 %, w/v) with ethidium bromide for staining, plus one well with DNA base-pair ladder, and the electrophoresis was run at 100 V. The resulting migration was visualized under UV light. One isolate corresponding to each restriction profile was selected, and purified with the GenElute^{TM} PCR Clean-up Kit according to the supplier's instructions (Sigma), before partial sequencing by MilleGen Biotechnologies (Labège, France). The nucleotide sequences obtained were checked with a sequence alignment editor (BioEdit), and the results were used for homology searches with BLAST (NCBI).

2.5. DNA isolation, and PCR- DGGE

In order to ensure the detection of non-cultivable bacteria, DNA was purified directly from the stomach contents, after the 4 weeks of feeding the respective diets. Total genomic DNA was extracted by glass beads beating (Sigma G4649) as described by Godon et al. (1997) with some modifications. The extracted genomic DNA was used as PCR template with the primers for the V6–V8 variable regions of 16S rRNA gene, F-968-GC (5′-AACGCGAAGAACCT TAC-3′ with GC clamp at the 5′ end) and R-1401 (5′-CGGTGTGTACAAGACCC-3′; Nübel et al., 1996). The PCR was performed in a volume final containing 50 ng μl^{-1} of genomic DNA, 0.025 U μl^{-1} Taq DNA polymerase (MP Biomedicals), 0.2 mM of each dNTP (Eurogentec premix), 0.4 µM of each primer, and 2 mM $MgCl_2$. The PCR conditions in thermocycler TC-512 Techne**)** were as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles including denaturation at 94°C for 1 min, annealing for 2 min - with 'touch down' from 65 to 52°C at the 10th cycle and followings - and 2 min elongation at 72°C. A final extension cycle was performed at 72 °C for 30 min. The amplification products were visualized by electrophoresis on 1.5% agarose gel containing ethidium bromide.

DGGE (Denaturing Gradient Gel Electrophoresis) was performed by using the Dcode Universal Mutation Detection System (Bio-Rad). A volume of 25 µl of PCR products

was loaded onto a 8% polyacrylamide gel (16 cm x 16 cm x 1.5 mm) prepared from 40% (w/v) acrylamide-N, N'-methylenebisacrylamide stock, 37.5:1 (Bio-Rad). The denaturing gradient was prepared by mixing 35 and 60% solution of 7 M urea and 40% formamide. The electrophoresis was run in 1X TAE buffer diluted from 50X TAE buffer stock solution (Bio-Rad). The migration was performed at 80 V (constant voltage) for 18 h, with a temperature gradient of 60°C. After electrophoresis, the gel was stained for 30 min by 200 ml of TAE solution with SYBR Green (0.1 µl ml^{-1}; Sigma). After rinsing, the gel was scanned on Typhoon 9400 (Amersham Biosciences), and the image was analysed with the GelCompar® software (version 6.1, Applied Maths).

Some well-separated bands with high peak intensity were excised from the gels, and left overnight soaking in 200-µl sterile milli-Q water at 4°C. The infusion was then used as template for a second PCR-DGGE similar to the first round, for purity control. The purified bands were again excised and soaked for PCR with primers F-968 without GC clamp and R-1401 (initial denaturation at 95°C for 3 min, followed by 25 cycles including denaturation at 95°C for 30 sec, annealing at 52°C for 30sec, and 1 min elongation at 72°C; a final extension cycle was performed at 72°C for 30 min). The products were purified with a GeneElute PCR clean-up kit (Sigma), and sent for sequencing.

2.6. Statistical analysis

Data were reported as mean ± standard error. The bacterial counts (colony forming units/CFU g^{-1} of contents) were $\log_{10}$ transformed. The DGGE profiles were analyzed with indices of similarity and diversity, computed with PAST (Harper, 1999; http://folk.uio.no/ohammer/past). The results were analyzed by ANOVA or by Kruskall-Wallis ANOVA on ranks, depending on normality and equality of variance, and compared by multiple comparison (Student-Newman-Keuls' method; SigmaStat®2.0). The differences were considered significant at $P < 0.05$.

3. Results

3.1. Characterization of cultivable microbes by culture dependent techniques and DNA sequences

Viable aerobic bacteria count ranged from 10^6 to 10^8 CFU g^{-1} in the samples of faeces, during the four weeks of experiment on gilthead sea bream (Fig. 1). A total of 1.152 bacterial strains from faeces and 576 bacterial strains from stomach and intestine were isolated from the gilthead sea bream. Lactic acid bacteria could not be cultivated in the samples. By the end of the feeding trial, the counts were significantly different between the three groups, and the faeces of group L contained approximately tenfold more cultivable bacteria than the control group F. At day 30, the counts of aerobes were ca. 10^1-10^2 CFU g^{-1} in the stomach, and 10^4-10^5 CFU g^{-1} in the intestine, without significant difference between treatments (Fig. 2).

The nucleotide sequences of the 16S rRNA gene of the isolates selected by ARDRA were submitted to GenBank, where they were assigned the accession numbers listed in Table 2. The composition of the bacterial population associated with gilthead sea bream faeces appeared generally dominated by *Vibrionaceae* - with variable proportions of *Vibrio* and *Photobacterium* - but the family was not detected in Group R by day 17 (Table 3). Two taxa of *Photobacterium* were found. The isolate DF123 had 760-bp nucleotide sequence with a 100% identity with *Photobacterium damselae*

subsp. *piscicida* strain PN510 (AY147860). The isolate DFC2.17 had a sequence of 1386 bp 99% identical with *Photobacterium* spp. JT-ISH-224 (AB293986), isolated from the stomach of Japanese barracuda (Tsukamoto et al., 2008). Two other taxa were found which were 99% identical for 1183 and 1163 bp with *Vibrio* spp. DAl 1-3-54 (AM159569) and *Vibrio ichthyoenteri* FK-1 (AM181657; Kim et al., 2004), respectively, and the two published sequences were 99% identical themselves. By day 24, genus *Photobacterium* was strongly dominant in the three groups. For the other sampling dates, *Alteromonadaceae* were also represented in Groups F and R, either by *Glaciecola* spp. DFC4.58 (99% identical on 729 bp with strain S577 # FJ457138; Gram et al., 2010) or by *Pseudoalteromonas* spp. DFH4.24 (99% identical on 775 bp with strain G23 # DQ677307).

At the end of the experiment, *Photobacterium damselae* subsp. *piscicida* was retrieved in the gastrointestinal microbiota of all groups of gilthead sea bream (Table 4; strain DFIL2.4 100% identity with PN510 on 1416 bp). Only *Vibrionaceae* of the genera *Vibrio* and *Photobacterium* were detected in the intestine, whereas *Bacillales* were also present in the stomach - *Bacillus* spp. DFEL3.4 in group L (100% identical on 736 bp with many *Bacillus* spp. sequences, e.g. strain CNE 9 # FR749864), and *Sporosarcina aquimarina* in the other groups (100% identical on 767 bp with strain SF237 # GQ141979; Khaneja et al., 2009). *Flavobacteriaceae* were also found in the stomach of groups F and R (100% identical on 781 bp with strain Rc6 # EU278338).

In goldfish by day 30, the aerobic bacteria count in intestinal contents were ca. 10^6-10^7 and 10^7-10^8 CFU g^{-1} in the anterior and posterior regions of the intestine, respectively. A total of 555 bacterial strains were isolated from the intestinal contents. These aerobes consisted of some members of the *Bacilli* and *γ-Proteobacteria* classes (Table 5). *Aeromonas* spp. CFC1P.24 was 99% identical on 1410 bp with many *Aeromonas* spp. sequences, e.g. GU566315 corresponding to strain 'JD3(2010)'. This taxon was retrieved in all the samples collected from goldfish stomach contents, but it was particularly dominant in group R (Table 6). Most samples also contained *Shewanella putrefaciens* CFC2P.24, 99% identical on 769 bp with strain PIC1 isolated from the intestinal content of yellow catfish (GQ359955; Wu et al., 2010). *Staphylococcus* spp. CFC2P.21, which was also present in most samples, was 100% identical on 720 bp with strain CTSP32 # EU855210. The strain was not detected in the anterior intestine of group R, while *Streptococcus parauberis* was retrieved instead (100% identical on 733 bp with strain DSM 6631 # AY584477; Picard et al., 2004). Cultivable microbes appeared more diverse in the anterior intestine compared with those from the posterior section. *Lactococcus* spp. CFL1A.6 was found in the anterior intestine of group L (99% identical on 529 bp with strain D124 # EF204363; Hantsis-Zacharov & Halpern, 2007). The highest diversity was observed in the anterior intestine of the control group F, where *Acinetobacter* spp. CFH2A.4 was 99% identical on 732 bp with strain LR20 (EU263286; Jung et al., 2009), and where yeasts were also retrieved (*Debaryomyces* spp. 100% identical on 583 bp with many sequences, e.g. NJ147 # HM032737; *Rhodotorula slooffiae* 99% identical on 539 bp with strain SY-84 # AB025994; Nagahama et al., 2003).

3.2. DGGE analyses of microbial diversity in fish gut

The culture-independent method with DGGE revealed further diversity in the gastrointestinal microbiota of gilthead sea bream (Fig. 3). The most important shift between gastric and intestinal contents was observed within group L, whose Dice similarity index dropped from 0.76 in the stomach to 0.26 in the intestine (Fig. 4). The replicate SF1 was also quite different from the other three samples collected in the stomach of fish fed the control diet F, but Dice similarity coefficients were generally

higher among the gastric replicates compared with the intestinal samples. This was confirmed by one-way ANOVA test on the mean Dice coefficients of the six groups of replicates (diet × digestive tract segment; Table 7). The trend was similar when abundance data were compared with the coefficient of Bray-Curtis. Considering diversity within each lane, the distribution of the taxonomical units were not significantly different among the six groups in terms of dominance and richness, based on presence/absence data, but the intestinal samples from group R had the lowest mean coefficients of equitability and Buzas-Gibson's evenness, both based on Shannon index which take into account peak intensities (Table 8).

Some PCR products were isolated from the polyacrylamide gel containing sea bream samples, re-amplified and partially sequenced. The prevalence of the genera *Photobacterium* and *Vibrio* was thus confirmed, with further insight into the diversity of their representatives. *Photobacterium* spp. 2A and 2B were retrieved in all the gastric samples, while another taxon *Photobacterium* spp. 2G appeared in some intestinal samples. The three taxonomical units were found 99% identical to *Photobacterium* spp. JT-ISH-224, while they were more distant from *Photobacterium damselae* subsp. *piscicida* strain PN510 (Fig. 5). *Vibrio* spp. 2F and 2H had 99% homology with partial sequence from *Vibrio* spp. DAl 1-3-54 and *Vibrio ichthyoenteri* FK-1 (Fig. 6). *Vibrio* spp. 2H was observed in most intestinal samples, whereas the other bands recognized as *Vibrio* appeared more variable among replicates. Though the bands corresponding to *Vibrio* spp. 2E and 2I had a low relative migration value (Rf; Fig. 3), their partial nucleotide sequences did not seem very different from those of the other *Vibrio* strains identified on the gel (98% identical).

The profiles obtained by DGGE with the samples collected from the intestinal content of goldfish appeared less variable than those from sea bream (Fig. 7). The samples from goldfish fed rapeseed did not produce suitable PCR amplicons, except one from the anterior section (AR3), and its DGGE profile did not seem particularly different from those of the other groups. The comparison was thus restricted to groups L and F, and the global similarity appeared relatively high compared with that obtained in sea bream. The lowest Dice similarity indice was 0.50, and the samples from the anterior intestine were generally more similar between them than the samples from the posterior section, except sample AF3 (Fig. 8). The two dietary groups have clearly distinct profiles in the posterior section, and contrary to what was observed in sea bream, the homogeneity remained relatively constant between the two sections of sampling. In a two-way ANOVA test comparing both sections and diets, there was no significant difference between the four mean similarity indices of Dice and Bray-Curtis. The diversity indices of each sample were also affected differently from those of sea bream. There was no significant difference in equitability and evenness, but there was an effect of the diet on the other diversity indices in a two-way ANOVA test taking into account the segment of sampling (Table 9). The diet L increased the dominance of few bands, while decreasing Shannon's entropy, Fisher's α, and indices of richness of Menhinick and Margalef were observed.

Five PCR products were isolated from the polyacrylamide gel with goldfish samples, re-amplified and partially sequenced. They were present in all experimental groups. The same taxon as *Aeromonas* spp. CFC1P.24 was retrieved in band 2O, 99% identical on 358 bp with strain 'JD3(2010)'. Though *Vibrionaceae* were not detected by culture methods, they were detected on the gel, with *Vibrio cholerae* 2L, 99% identical on 368 bp with strain PIM9 isolated from the intestinal mucus of yellow catfish (GQ359963; Wu et al., 2010). This strain was also 99% identical with *Vibrio* spp. 2K. *Vibrionaceae* bacterium 2Q was 97% identical on 369 bp with *Grimontia hollisae* LMG 17719 (AJ514909; Thompson et al., 2003). The presence of anaerobes was exemplified by

Cetobacterium somerae 2J, 100% identical on 362 bp with strain AG39, already isolated from the intestinal tract of goldfish by Tsuchiya et al. (2008; #AB353123).

4. Discussion

Intestinal microbiota has been seldom described in gilthead sea bream juveniles. Savas et al. (2005) followed the effect of weaning on the microbiota of early juveniles, and they stressed the importance of the food sequence on the composition of intestinal microbiota of larvae and juveniles. After weaning on to a compound diet, the dominant genera were of *Vibrio* and *Pseudomonas* in this study based on culture methods. In an epidemiological survey, Sitjà-Bobadilla et al. (2006) isolated mainly *Vibrio* spp., from the liver of farmed gilthead sea bream. *Pseudoalteromonas* spp. and *Photobacterium* spp. were also found, and less frequently, *Flavobacteriaceae*. These taxa were also present in our samples, suggesting that they are common in the stomach of healthy gilthead sea bream. There was no indication of virulence in the simple homology of a partial sequence of the 16S r-RNA gene with fish pathogens like *Photobacterium damselae* subsp. *piscicida* strain PN510. This subspecies, formerly *Pasteurella piscicida*, is known to have naturally avirulent strains (Magariños et al., 1996). Similarly, *Vibrio ichthyoenteri* was isolated from healthy fish (Gomez et al., 2001; Pujalte et al., 2003). Dimitroglou et al. (2010) did not study the taxonomy in intestinal microbiota, but they observed quite different profiles depending on whether or not the diet of gilthead sea bream included 31.3% of soybean meal. In the present experiment, the effect of the diet on luminal microbiota was much less marked, as well as the similarity within group replicates. A high variability was observed in all samples collected during this experiment on sea bream. This variability applied to bacterial counts, and to the composition of cultivable microbiota in faeces sampled at regular intervals, and finally to the gastric and intestinal contents, pooled from six fishes per replicate. There was no previous study about such variations in gilthead sea bream, but some information was available in goldfish.

Sugita et al. (1987a) examined the composition of faecal pellets collected from the anus of goldfish, and they showed that this was not markedly different from that recovered from intestinal contents. These authors used this non-invasive method to follow the daily fluctuations of faecal microbiota in goldfish, and they concluded that four individuals had distinct faecal microbiota, with different daily variations (Sugita et al., 1987b). Asfie et al. (2003) further studied these variations with non-cultural methods, which were in good agreement with the previous culture techniques, but *Bacteroides* type A seemed particularly fluctuant. This type was re-classified as *Cetobacterium somerae* (Tsuchiya et al., 2008), and we observed also quite variable relative band intensity for *C. somerae* 2J in the present experiment (Fig. 7). More generally, the bacterial composition of the luminal contents appeared variable in goldfish, though each sample was obtained from six individuals confined in the same tank. This variability was much more marked in the experiment with sea bream. Heikkinen et al. (2006) compared the microbiota in intestinal contents of rainbow trout fed with or without soybean meal, with five individuals pooled in five replicates per diet. These authors also found a great variability within the two groups clustered after Jaccard similarity of LH-PCR profiles, making the interpretation of the results difficult, especially after only 4 weeks of experiment.

The bacterial counts in goldfish were inferior to those recovered from whole intestinal content as reported by Sugita et al. (1987a, 1991) with other culture media for aerotolerant bacteria. For example, their estimates on Trypticase soy blood agar were higher than 10^9 CFU g^{-1}. However, the composition of the intestinal microbiota

appeared relatively similar to what was described in the literature. Besides *Cetobacterium somerae*, already mentioned, we retrieved the dominance of *Aeromonas* spp. described by Sugita et al. (1987a). The genera *Acinetobacter*, *Staphylococcus*, and *Streptococcus* were detected in faecal pellets of goldfish by Sugita et al. (1987ab). Lactic acid bacteria were isolated from closely related cyprinids by Hagi et al. (2004), such as *Lactococcus raffinolactis* from *Carassius cuvieri*. *Shewanella putrefaciens* was observed in the intestine of common carp (Al-Harbi & Uddin, 2008). Reddacliff et al. (1993) isolated *Vibrio cholerae* from septicaemic goldfish, but the species is a common inhabitant of aquatic environments, with many non-toxigenic strains (Rivera et al., 1995). *Vibrio* spp. 2K and 2L did not seemed pathogenic, and not even active in the digestive tract, since they were not detected by culture methods. They transited likely in a viable but non-cultivable state similar to that associated with zooplankton (Thomas et al., 2006; Locascio de Mitrovich et al., 2010). To our knowledge, there was no report of the presence of yeast in goldfish, but they were frequently isolated from fresh water fish (Gatesoupe, 2007). Merrifield et al. (2009) noted that the amount of yeast was promoted in the digestive tract of rainbow trout fed soybean meal.

Sugita et al. (1988a) compared the effect of a compound dry diet, *Tubifex* sp., and dried *Daphnia* sp. on the faecal microbiota of goldfish and found variability between individuals, without any clear influence of the diet. Since good correspondence was previously found between the microbial composition of faeces and the intestinal contents (Sugita et al., 1987a), these authors concluded that the diet exerted few influence on microbiota established in the intestine. Our results indicated that it was possible to show the influence of protein sources by sampling separately the contents from the anterior and posterior parts of the digestive tract. The diet containing about 20% plant protein sources tended to decrease the diversity among cultivable isolates in the anterior part of goldfish intestine. Interestingly, similar observations were made in the stomach of gilthead sea bream fed lupin but not rapeseed, though the composition of microbiota associated with marine fish are different from those of fresh water fish. The effect of dietary lupin meal was particularly clear in goldfish, when it was compared with that of the control diet by DGGE analysis. The two groups of samples from the posterior intestine were distinguished by Dice cluster analysis, and the bacterial diversity was reduced in the group fed lupin, independently of the sampling segment. In sea bream, the DGGE profiles indicated that there was a shift in the composition during digestive transit, with a trend to increasing dissimilarity between stomach and intestine. This shift was particularly obvious in the group of sea bream fed 20% lupin meal. The reason for such increased dissimilarity in the intestine of sea bream could not be elucidated, and that hindered from interpreting clearly the effects of the diets. However, there was a significant decrease of equitability and evenness of the bacterial community in the intestine of sea bream fed rapeseed. Dimitroglou et al. (2010) observed conversely a high Bray-Curtis similarity between duplicated intestinal samples of sea bream fed soybean meal. Future experiments should focus on the comparison of mucosal and luminal communities, with respect to the effect of the diet on similarity indices. In Atlantic cod and rainbow trout, the bacterial community adherent to mucosa was found different from that transiting with digesta, but the introduction of dietary soybean meal affected both compartments, as well (Ringø et al., 2006; Merrifield et al., 2009). In goldfish, Sugita et al. (1988b) distinguished three categories of bacteria: (1) the “transient type” coming from diet and water; (2) the “permanently indigenous type” which was not disturbed by the diet, and which included *Aeromonas* spp.; (3) the “adult type”, which was established during the juvenile stages, and included *Bacteroides* type A (re-classified as *Cetobacterium somerae)*. A fourth category could be distinguished as bacteria transiting in a non cultivable state, and likely inactive, as unaffected by the diet. That may be the case of *Vibrio* spp. 2K and 2L, whose abundance looked relatively uniform among all the DGGE profiles. Other

bacteria that seemed independent of the diet were *Aeromonas* spp. 2O, likely belonging to the "permanently indigenous type", and the so-called "adult type" *Cetobacterium somerae* 2J. The *Vibrionaceae* bacterium 2Q could belong to the "transient type", since it was mainly present in groups fed the control diet F, and much less abundant in group L. Lupin is generally rich in α-galactosides, which can influence faecal microbiota (Smith et al., 2006; Martínez-Villaluenga & Gómez, 2007). In healthy men fed lupin kernel fibre, colonic bifidobacteria were stimulated, whereas taxa of the clostridia group were repressed (Smith et al., 2006). A similar selection of competent strains seemed to occur at least in goldfish, but likely with other taxa than bifidobacteria, which were not commonly detected in fish (Wang et al., 1994). The genus *Clostridium* was sometimes isolated from goldfish faeces by Sugita et al. (1987b; 1988a), and it was possible that it was also present among the taxa that we did not succeed to sequence from our samples. The *Vibrionaceae* bacterium 2Q was likely repressed by dietary lupin.

5. Conclusion

The microbial content of the digestive tract was not affected in the same way by the substitution of protein sources in the diet of goldfish and gilthead sea bream. In the freshwater stomachless species, the variability within replicates was relatively low, and it was possible to observe the selection of a limited number of taxa that were likely promoted by dietary lupin. In sea bream, a high variability was observed within replicates, especially those from the intestinal contents of the group fed lupin, and it was not possible to conclude to a clear dietary influence. It seems particularly important to further study the individual fluctuations of faecal microbiota in fish having a stomach, and a relatively short intestine, since it appeared that the mix of five or six individual samples did not compensate for the variability observed in the present experiment, as well as in that of Heikkinen et al. (2006) on rainbow trout.

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Tables

Table 1. Ingredients and chemical composition of the experimental diets

6.	Fish meal	Lupin	Rapeseed
6.1. **Ingredients** *(g kg^{-1}, on a dry matter basis)*			
Fish meal [a]	570	451	478
Rapeseed [b]	0	0	200
Lupin [c]	0	200	0
Starch	276	193	168
Fish oil	124	124	124
Carob gum E410	5	5	5
Xanthan gum E415	5	5	5
Mineral mix [d]	10	10	10
Vitamin mix [d]	10	10	10
Amino acid mix [e]	0	2	0
Analysed composition			
Dry matter (DM, %)	97.8	94.8	97.6
Crude protein (% DM)	45.7	45.4	45.5
Crude fat (% DM)	19.0	20.5	18.6
Aminoacid composition (% crude protein)[f]			
Arginine	5.4	6.7	5.8
Histidine	2.0	2.2	2.3
Isoleucine	4.7	4.6	4.6
Leucine	7.5	7.0	7.5
Lysine	7.2	6.8	6.8
Methionine+cystine	4.1	3.6	4.4
Phenylalanine+tyrosine	6.8	6.9	6.9
Threonine	4.4	4.2	4.5
Tryptophane	1.1	1.1	1.1
Valine	4.8	4.6	5.0

[a] Norse-LT 94 supplied by La Lorientaise, Lorient, France. The amounts were adjusted to obtain isoproteic diets (42 %, dry matter basis).

[b] SAIPOL, Grand Couronne, France.

[c] Lup'Ingredients, Martigne Ferchaux, France.

[d] According to Cahu et al. (1999).

[e] leucine 36,8% ; lysine 31,6% , methionine 31,6%

[f] computed after data from raw feedstuffs

Table 2. Bacteria found in gilthead sea bream stomach, intestine and faeces, according to their homology to 16S rRNA gene partial sequences in GenBank.

Isolate	Origin	GenBank accession number	Taxonomical characterization
DFC4.58	Faeces	FR873778	*Glaciecola* sp.
DFH4.24	Faeces	FR873779	*Pseudoalteromonas* sp.
DFH1.25	Faeces	FR873780	*Vibrio* sp.
DFH2.13	Faeces	FR873781	*Vibrio ichthyoenteri*
DF123	Faeces	FR873782	*Photobacterium damselae*
DFIL2.4	Intestine	FR873782	*Photobacterium damselae*
DFC2.17	Faeces	FR873783	*Photobacterium* sp.
DFEL3.4	Stomach	FR873784	*Bacillus* sp.
DFC4.71	Faeces	FR873785	*Sporosarcina aquimarina*
DFEH2.1	Stomach	FR873785	*Sporosarcina aquimarina*
DFL1.44	Faeces	FR873786	*Flavobacteriaceae*

Table 3. Frequency (%) of the bacterial population associated with gilthead sea bream faeces by days 3, 10, 17 and 24.

Day	**3**			**10**			**17**			**24**		
Diet	**F**	**L**	**R**	**F**	**L**	**R**	**F**	**L**	**R**	**F**	**L**	**R**
Glaciecola sp.	ND	ND	ND	ND	ND	33%	25%	ND	40%	ND	ND	ND
Pseudoalteromonas sp.	30%	ND	25%	ND	ND	ND	ND	ND	ND	ND	ND	ND
Vibrio sp.	30%	33%	25%	75%	20%	ND	25%	75%	ND	ND	ND	ND
Photobacterium damselae	40%	33%	ND	ND	20%	ND	ND	ND	ND	25%	100%	100%
Photobacterium sp.	ND	33%	50%	25%	40%	67%	25%	25%	ND	75%	ND	ND
Sporosarcina aquimarina	ND	ND	ND	ND	ND	ND	25%	ND	60%	ND	ND	ND
Flavobacteriaceae	ND	ND	ND	ND	20%	ND	ND	ND	ND	ND	ND	ND
Total isolates numbers	10	6	8	4	8	9	4	8	10	8	10	10

The identification was based on alignment with nucleotide sequences available in NCBI database (Table 2); ND: not detected; F: Fish meal; L: Lupin; R: Rapeseed.

Table 4. Composition of the bacterial population detected in the stomach and in the intestine of gilthead sea bream at day 30.

Segment	**Stomach**			**Intestine**		
Diet	**F**	**L**	**R**	**F**	**L**	**R**
Vibrio sp.	25%	ND	ND	25%	20%	25%
Photobacterium damselae	25%	75%	25%	75%	40%	50%
Photobacterium sp.	ND	ND	25%	ND	40%	25%
Bacillus sp.	ND	25%	ND	ND	ND	ND
Sporosarcina aquimarina	25%	ND	25%	ND	ND	ND
Flavobacteriaceae	25%	ND	25%	ND	ND	ND
Total isolates numbers	12	8	4	8	6	12

The identification was based on alignment with nucleotide sequences available in NCBI database (Table 2); ND, not detected; F: Fish meal; L: Lupin; R: Rapeseed.

Table 5. Baterial found in *C. auratus* according their homology to nucleotide sequences in GenBank.

Isolate	Intestinal segment	GenBank accession number	Taxonomical characterization
Bacteria (16S rRNA gene, partial sequence)			
CFH2A.4	Anterior	FR873787	*Acinetobacter* sp.
CFC1P.20	Posterior	FR873788	*Shewanella putrefaciens*
CFC1P.24	Posterior	FR873789	*Aeromonas* sp.
CFC2P.21	Posterior	FR873790	*Staphylococcus* sp.
CFC1A.4	Anterior	FR873791	*Streptococcus parauberis*
CFL1A.6	Anterior	FR873792	*Lactococcus* sp.
Yeasts [a]			
CFH1.A	Anterior	FR873793	*Debaryomyces* sp.
CFH2.A	Anterior	FR873794	*Rhodotorula slooffiae*

[a] ITS1, 5.8S rRNA gene, ITS2 partial sequence

Table 6. Frequency (%) of the bacterial and yeast population found in goldfish intestine at day 30.

Intestinal segment	**Anterior**			**Posterior**		
Diet	**F**	**L**	**R**	**F**	**L**	**R**
Acinetobacter sp.	12,5%	ND	ND	ND	ND	ND
Shewanella putrefaciens	25%	29%	ND	33%	20%	25%
Aeromonas sp.	12,5%	43%	90%	33%	30%	62,5%
Staphylococcus sp.	25%	14%	ND	33%	50%	12,5%
Streptococcus parauberis	ND	ND	10%	ND	ND	ND
Lactococcus sp.	ND	14%	ND	ND	ND	ND
Debaromyces sp.	12,5%	ND	ND	ND	ND	ND
Rhodotorula slooffiae	12,5%	ND	ND	ND	ND	ND
Total isolates numbers	8	7	10	9	10	8

The identification was based on alignment with nucleotide sequences available in NCBI database (Table 5); ND, not detected. F: Fish meal; L: Lupin; R: Rapeseed.

Table 7. Comparison of bacterial similarity between the gastric and intestinal contents sampled in sea bream fed the three diets, and analysed by PCR-DGGE. The coefficient of Dice was computed to compare the presence or absence of the OTUs (Operational Taxonomic Units), whose peak intensity was taken into account with the index of abundance of Bray-Curtis (mean coefficients ± standard errors). For each index, the means were compared by ANOVA, and those without common superscript were significantly different after Tukey test.

Diet	**F**	**L**	**R**
OTUs			
Stomach	7.3 ± 1.9	12.3 ± 1.0	10.0 ± 1.0
Intestine	9.7 ± 2.0	7.0 ± 2.0	8.5 ± 0.9
Dice index			
Stomach	0.37^{c} ± 0.04	0.88^{a} ± 0.03	0.78^{ab} ± 0.11
Intestine	0.51^{bc} ± 0.01	0.24^{c} ± 0.06	0.57^{abc}
Bray-Curtis index			
Stomach	0.35^{bc} ± 0.10	0.83^{a} ± 0.06	0.59^{ab} ± 0.14
Intestine	0.37^{bc} ± 0.07	0.15^{c} ± 0.03	0.22^{bc}

Table 8. Comparison of bacterial diversity between the gastric and intestinal contents sampled in sea bream fed the three diets, and analysed by PCR-DGGE. The coefficients of Shannon's equitability, and those of Buzas and Gibson's evenness were found significantly different by ANOVA (mean coefficients ± standard errors). The means without common superscript were significantly different after Tukey test.

Segment	**Stomach**			**Intestine**			**ANOVA**
Diet	**F**	**L**	**R**	**F**	**L**	**R**	
Equitability	$0.95^{a} \pm 0.01$	$0.92^{a} \pm 0.01$	$0.97^{a} \pm 0.01$	$0.97^{a} \pm 0.01$	$0.90^{ab} \pm 0.03$	$0.83^{b} \pm 0.01$	P=0.001
Evenness	$0.91^{ab} \pm 0.02$	$0.82^{ab} \pm 0.02$	$0.94^{a} \pm 0.02$	$0.94^{ab} \pm 0.02$	$0.82^{bc} \pm 0.04$	$0.72^{c} \pm 0.02$	P<0.001

Table 9. Comparison of bacterial diversity between the anterior and posterior intestinal contents sampled in goldfish fed the three diets, and analysed by PCR-DGGE. The indices of dominance, Shannon's entropy, Menhinick's richness, Margalef's richness, and Fisher's α were found significantly different, depending on the diet by two-way ANOVA, while there was neither influence of the sampling segment, nor significant interaction. These indices were computed after the presence/absence and relative peak intensity of OTUs (Operational Taxonomic Units; mean coefficients ± standard errors).

Segment	**Anterior intestine**		**Posterior intestine**		**Two-way ANOVA**	
Diet	**F**	**L**	**F**	**L**	**Diet**	**Segment**
Dominance	0.11 ± 0.02	0.12 ± 0.02	0.08 ± 0.02	0.15 ± 0.02	*P*=0.047	n.s.
Shannon	2.78 ± 0.21	2.52 ± 0.21	3.08 ± 0.21	2.22 ± 0.21	*P*=0.026	n.s.
Menhinick	1.93 ± 0.29	1.55 ± 0.29	2.29 ± 0.29	1.06 ± 0.29	*P*=0.026	n.s.
Margalef	3.85 ± 0.61	3.09 ± 0.61	4.60 ± 0.61	2.05 ± 0.61	*P*=0.027	n.s.
Fisher's α	7.38 ± 1.47	5.28 ± 1.47	9.10 ± 1.47	3.00 ± 1.47	*P*=0.024	n.s.
OTUs	18.3 ± 2.7	15.0 ± 2.7	21.7 ± 2.7	10.3 ± 2.7	*P*=0.027	n.s.

Figure 1. Population levels of bacteria isolated from faecal samples of gilthead sea bream from day 3 to day 24 (log CFU g^{-1}). Letters a, b and c correspond to significant differences in the multiple comparison with Student-Newman-Keuls' method (P<0.05).

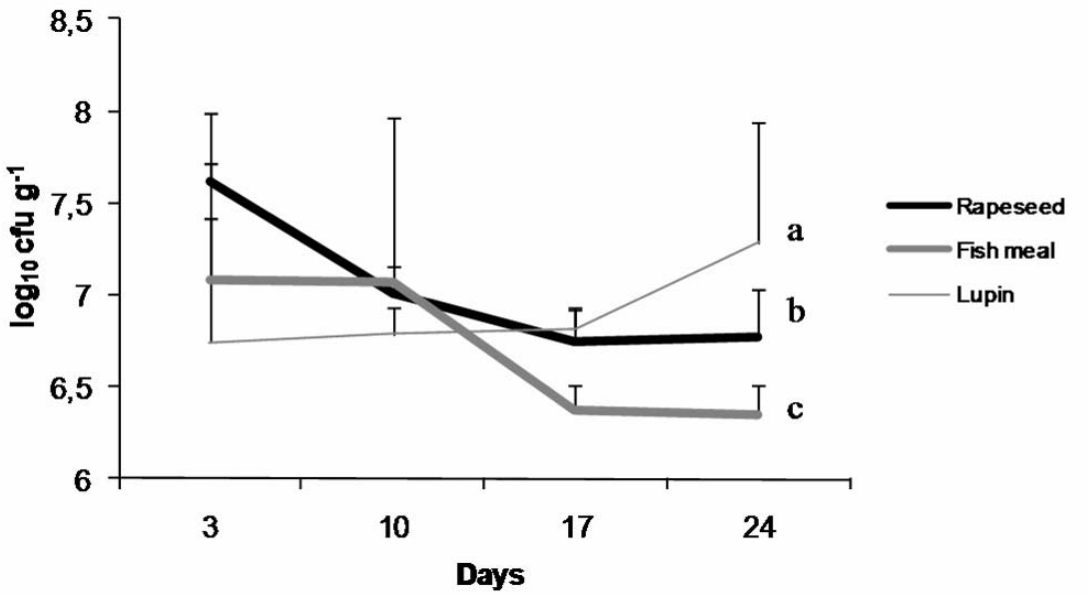


Figure 2. Bacterial count in the gastric and intestinal contents sampled in gilthead sea bream at day 30 (log CFU g^{-1}). Means ± S.E. with different superscripts are significantly different between sampling regions (Student-Newman-Keuls' method, $P<0.05$). The difference between diets were not significant.

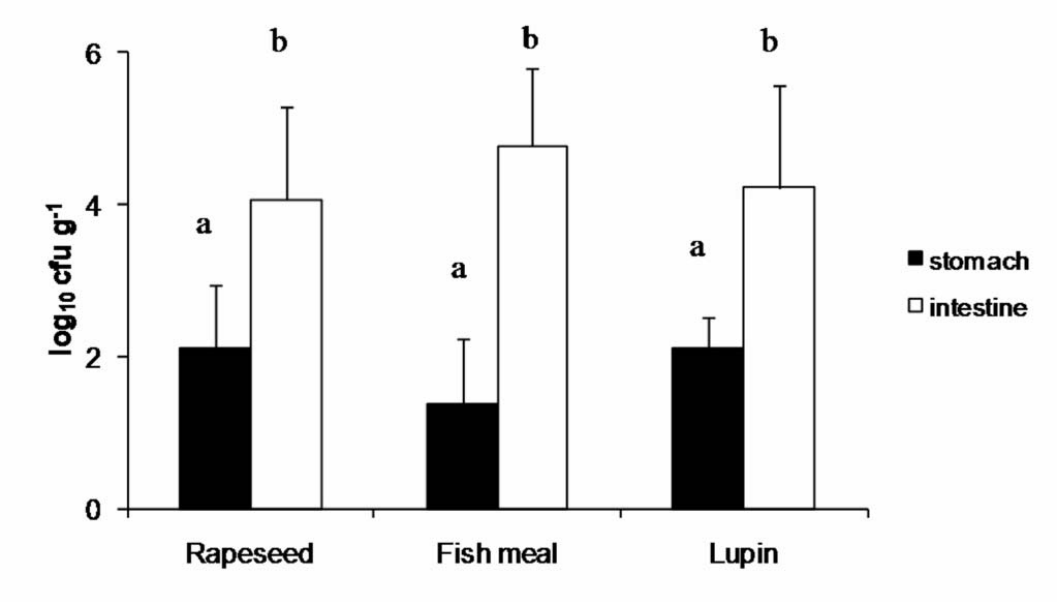


Figure 3. PCR-DGGE profiles from 16S rRNA gene V6-V8 variable regions, representing the biodiversity of bacteria in the gastric and intestinal contents of gilthead sea bream at day 30. Some taxa were identified by sequencing 2A, 2B, and 2G: *Photobacterium* sp.; 2F, 2H, 2E, and 2I: *Vibrio* sp.

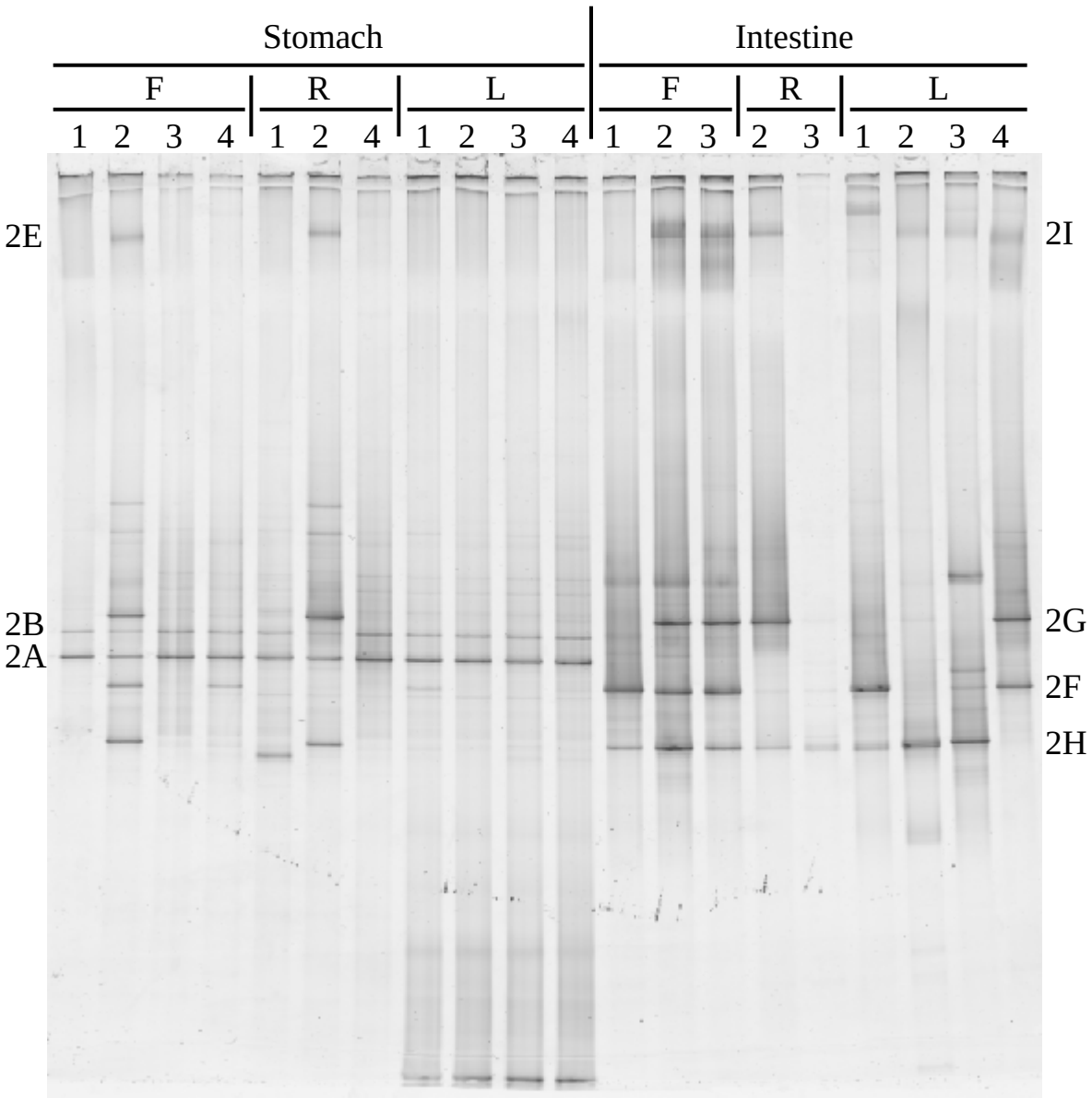


Figure 4. Dendrogram of Cluster analysis based on the DGGE profiles of Fig.3, computed using Dice similarity coefficients. The codes were constructed with first letter: S or I, depending on the sampling segment; second letter: F, L, or R, depending on the diet; the digit corresponded to the sampling tank. The bracket and the rectangle indicate the similarities within the gastric samples and within the intestinal samples of the group fed the control diet, respectively. The circle indicates an odd replicate of the gastric samples.

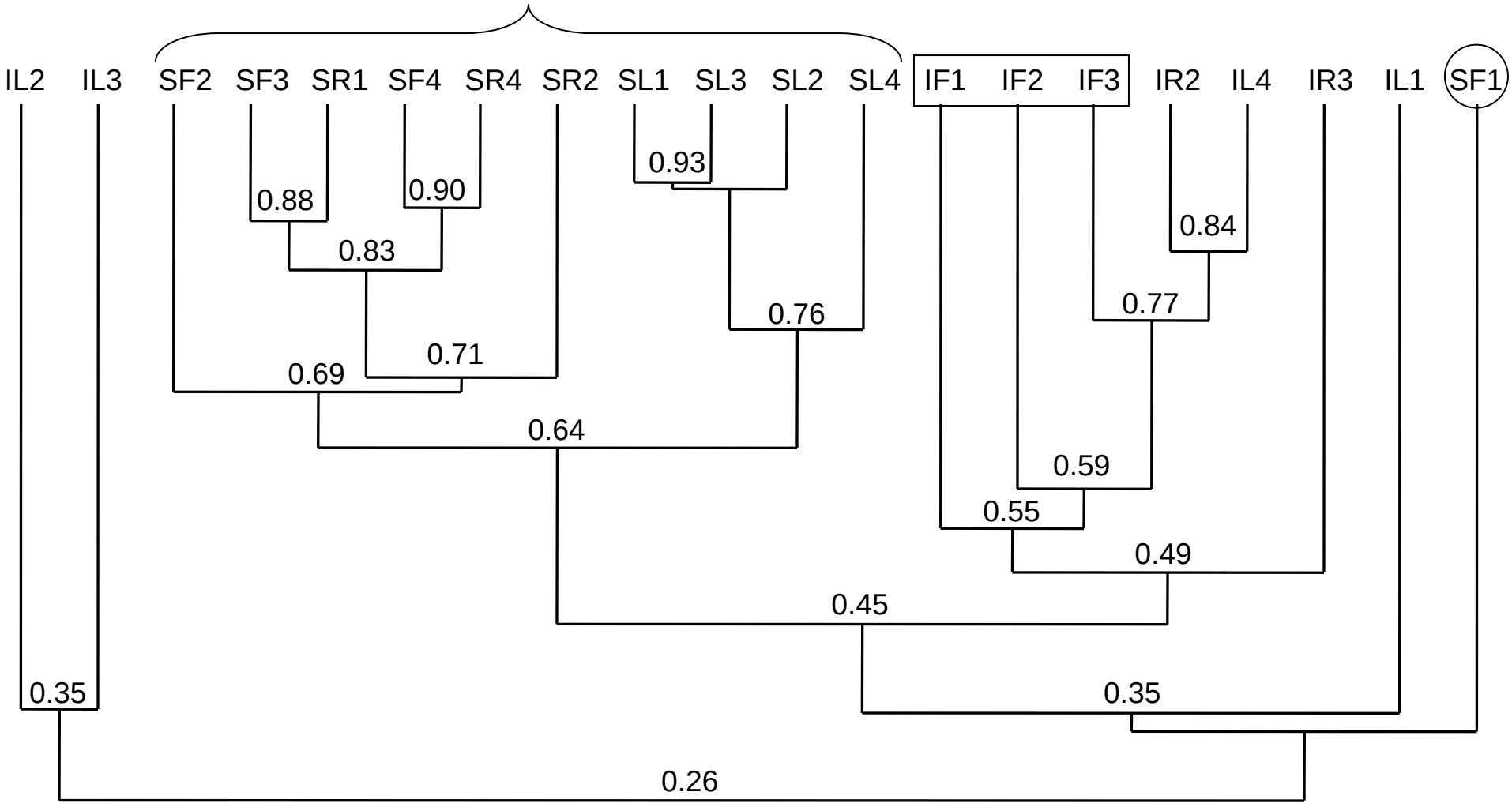


Figure 5. Dendrogram of Cluster analysis based on the similarity of nucleotide sequences of the 16S rRNA gene V6-V8 variable regions of *Photobacterium* sp. 2A, 2B and 2G, and the sequences found in GeneBank: *Photobacterium* sp. JT-ISH-224 (AB293986), and *Photobacterium damselae* subsp. *piscicida* strain PN510 (AY147860).

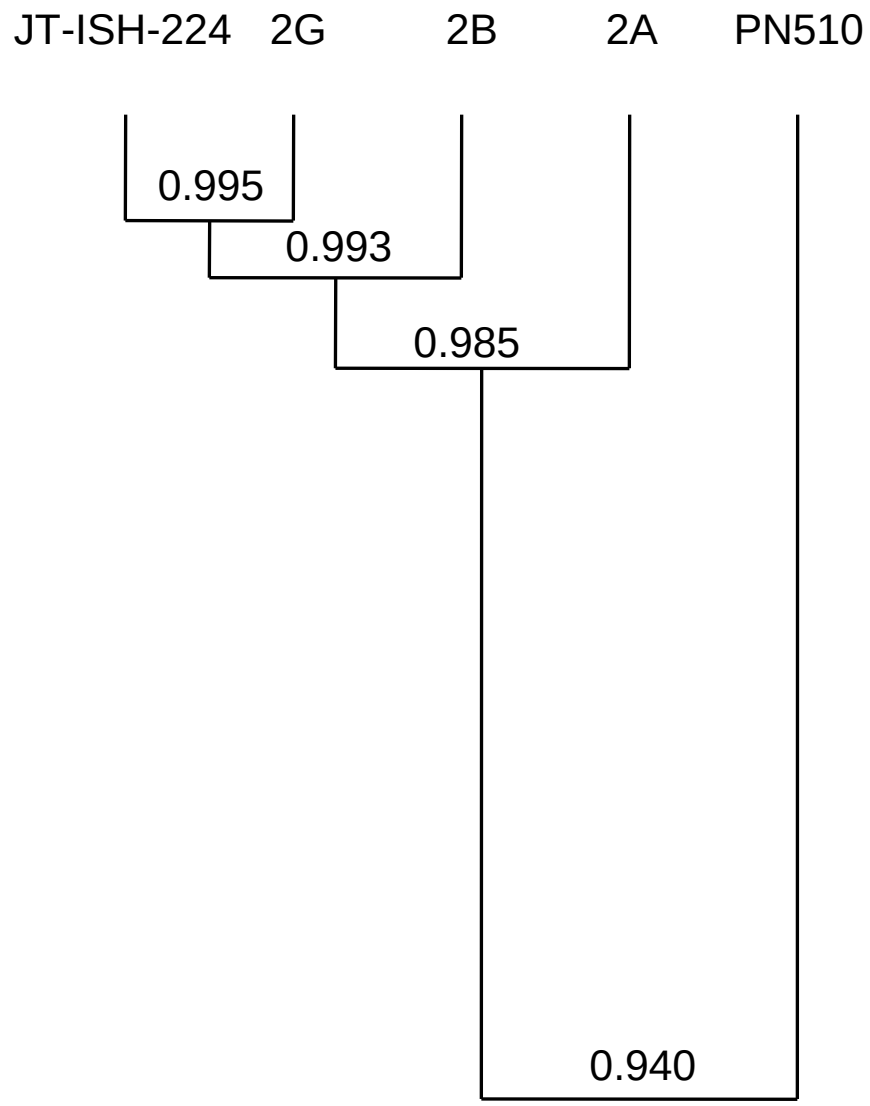


Figure 6. Dendrogram of Cluster analysis based on the similarity of nucleotide sequences of the 16S rRNA gene V6-V8 variable regions of *Vibrio* sp. 2E, 2F, 2H and 2I, and the sequences found in GeneBank with *Vibrio* sp. DAl 1-3-54 (AM159569) and *Vibrio ichthyoenteri* FK-1 (AM181657). 2I, and the sequences found in GeneBank with *Vibrio* sp. DAl 1-3-54 (AM159569) and *Vibrio ichthyoenteri* FK-1 (AM181657).

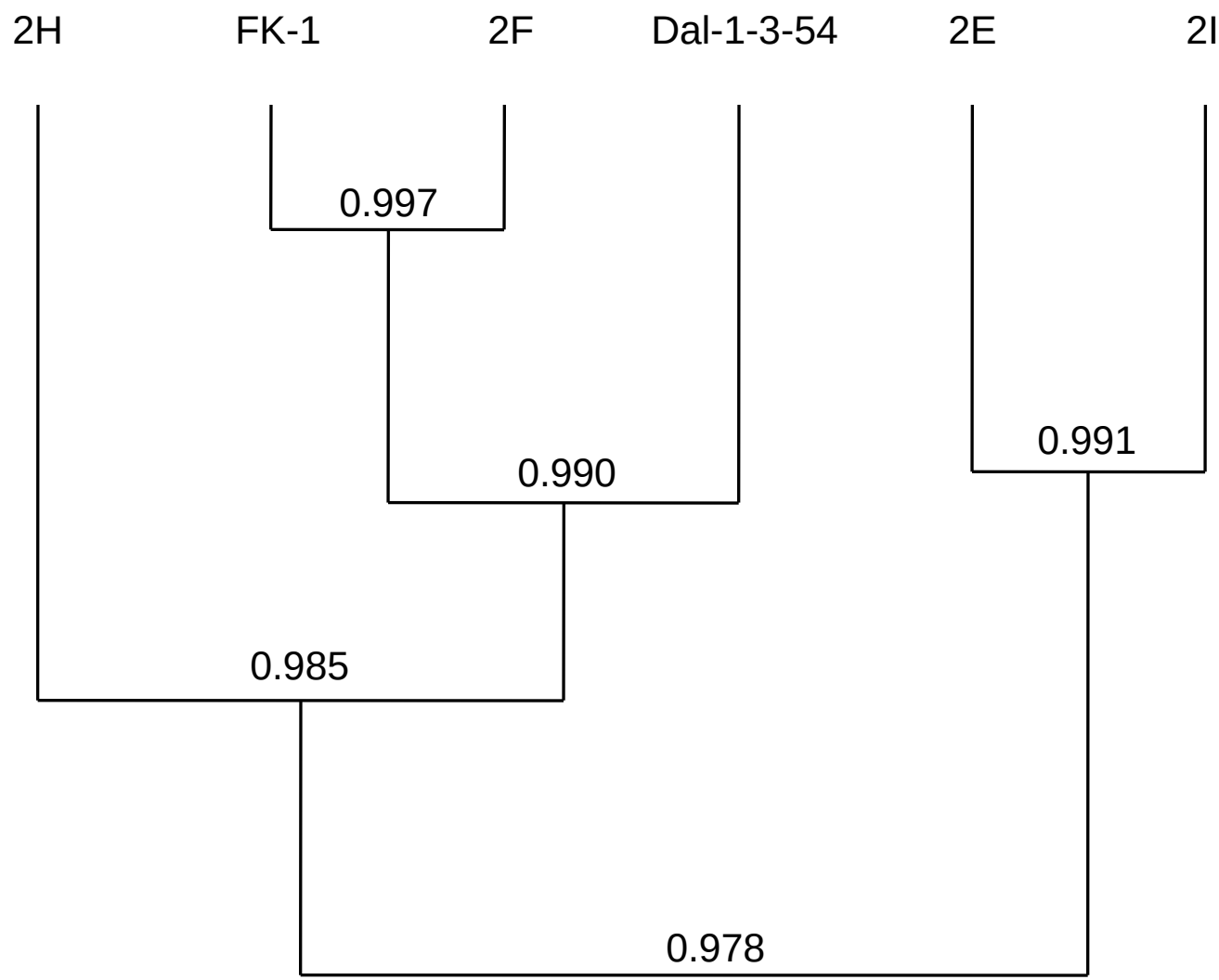


Figure 7. PCR-DGGE profiles from 16S rRNA gene V6-V8 variable regions, representing the biodiversity of bacteria in the anterior and posterior intestinal contents of goldfish. Some taxa were identified by sequencing, 2J: *Cetobacterium somerae*; 2K and 2L: *Vibrio cholerae* 2L; 2Q: *Vibrionaceae* bacterium; 2O: *Aeromonas* sp.

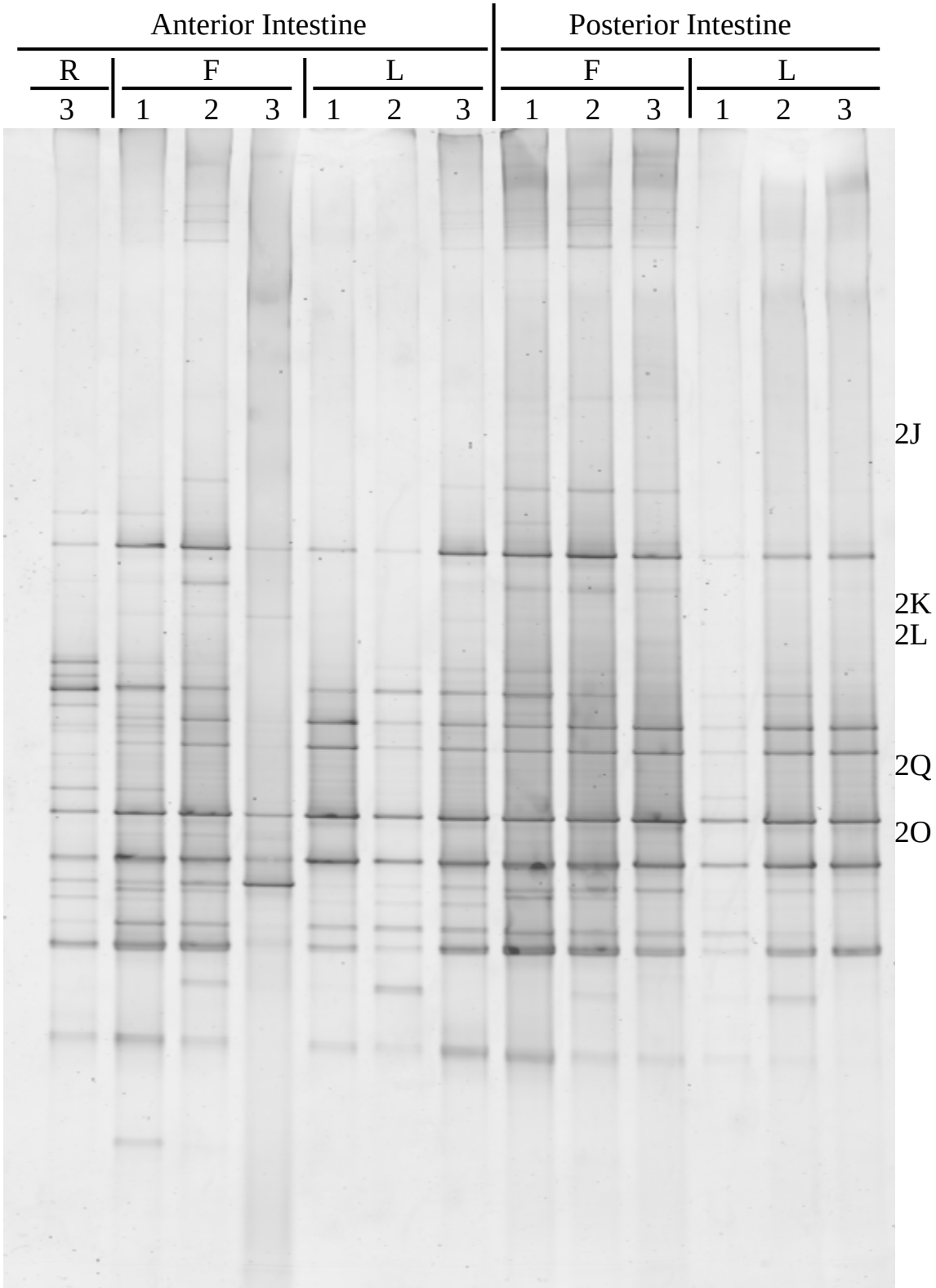
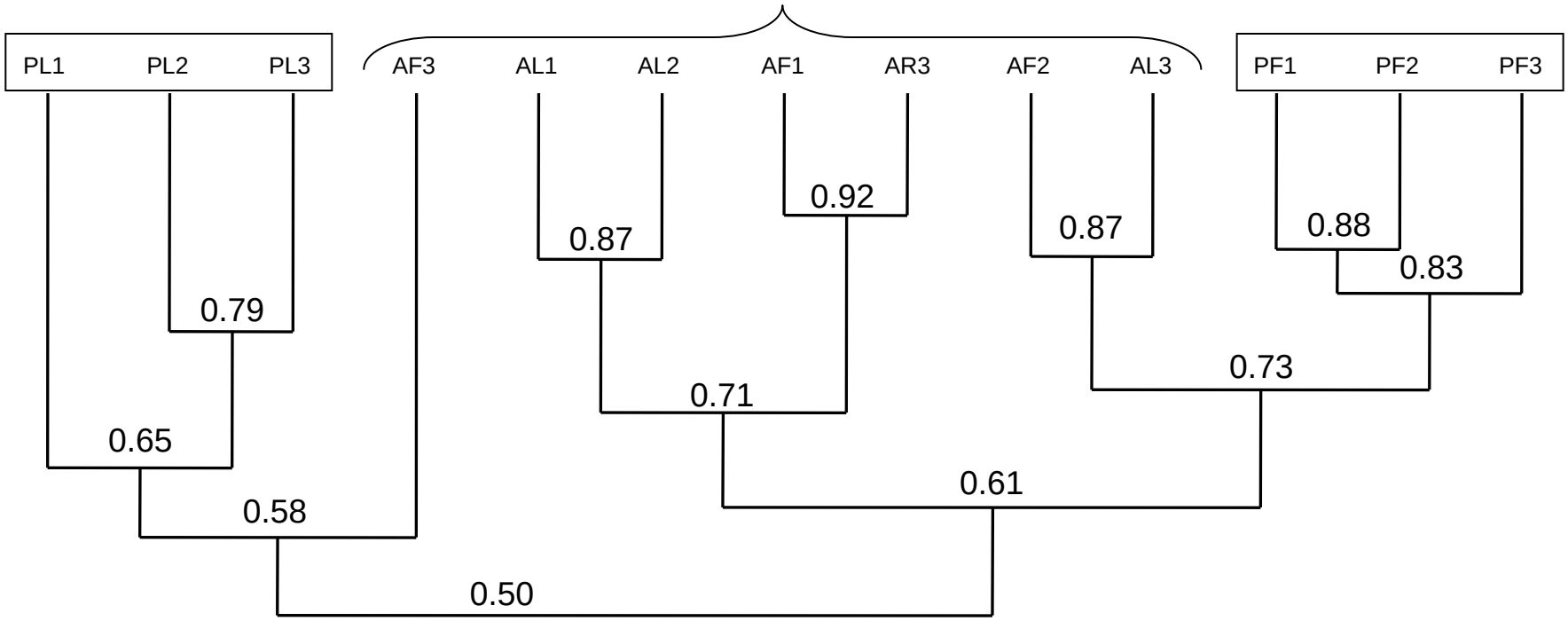
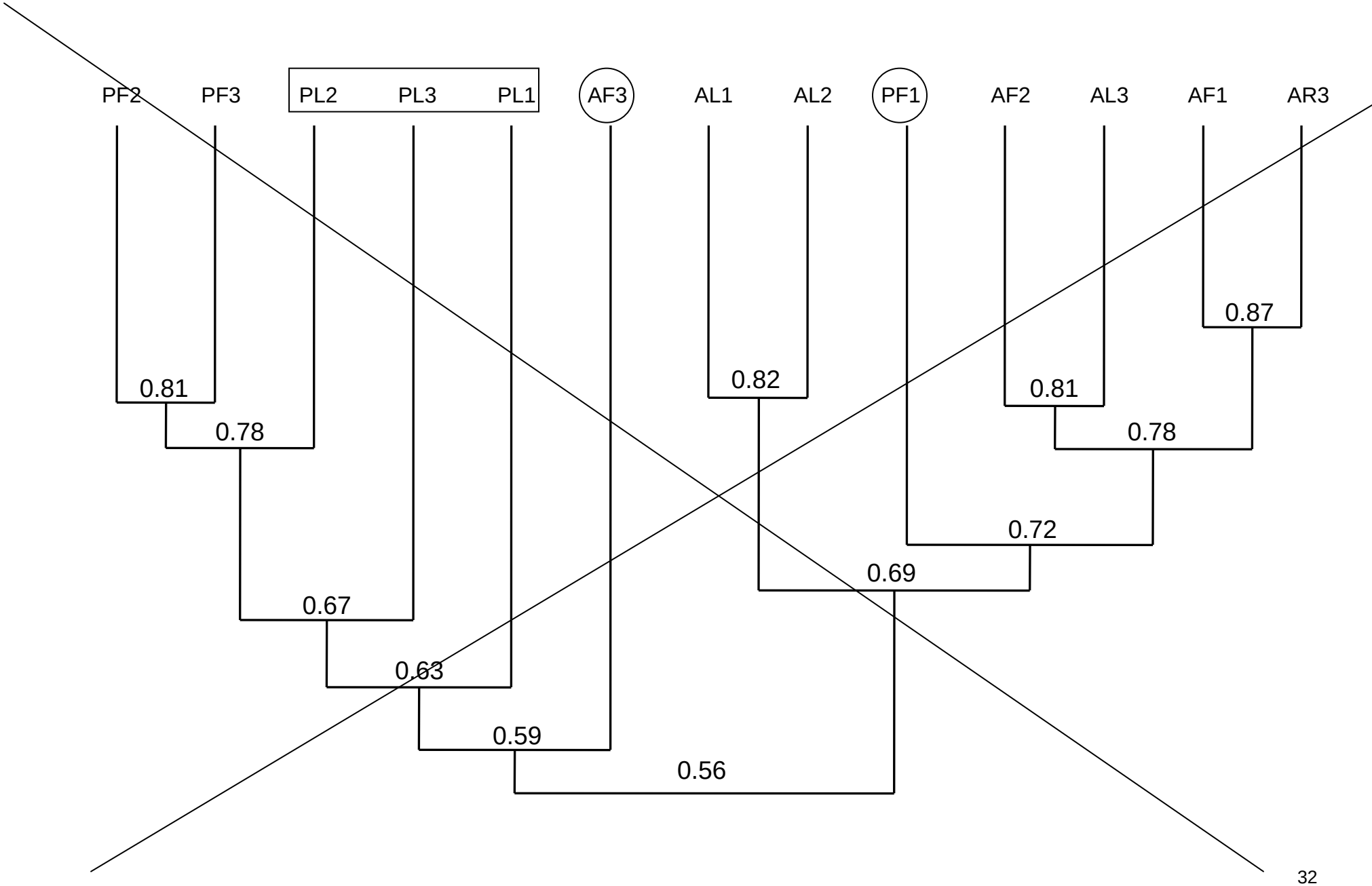


Figure 8. Dendrogram of Cluster analysis based on the DGGE profiles of Fig.7, computed using Dice similarity coefficients. The codes were constructed with first letter: A or P, depending on the sampling segment; second letter: F, L, or R, depending on the diet; the digit corresponded to the sampling tank. The bracket and the rectangles indicate the similarities within the anterior samples, and within the posterior samples.




PF2
PF3
PL2
PL3
PL1
AF3
AL1
AL2
PF1
AF2
AL3
AF1
AR3
0.87
0.81
0.82
0.81
0.78
0.78
0.72
0.69
0.67
0.63
0.59
0.56