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Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, Psetta maxima (Linnaeus, C. 1758)

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Abstract: Preliminary experiments were undertaken to investigate the effect of dietary inulin (Raftiline ST), oligofructose (Raftilose P95) and lactosucrose on the growth and intestinal bacteria of the marine carnivorous turbot, Psetta maxima. Turbot larvae were weaned on compound diets containing 2% Rafiline ST, 2% Raftilose P95 or 2% lactosucrose; 2% cellulose was the carbon source in the control group. The final mean weight of the group weaned with Raftilose P95 was significantly higher than those observed with the other diets. The bacterial load was highly variable in weaning turbot, especially with respect to the putative Vibrio spp. growing on TCBS agar which, in general, seemed to be dominant. Of the total load of bacterial isolates from turbot weaned on oligofructose, 14% consisted of a strain of Bacillus spp. This strain could use Raftilose P95 as a single source of carbon, and it might play a role in the beneficial effect of oligofructose on turbot growth, since Bacillus spp. have been documented as probiotics in fish.

Keywords: Fish nutrition - Fructo-oligosaccharides - Intestinal flora - Prebiotics - Turbot

Introduction

The main challenges in European turbot production is to improve feed formulation in order to optimize fish growth and fish resistance through the development of health promoting diets.

Prebiotics and probiotics were originally proposed for human health-enhancing food (Gibson and Roberfroid, 1995). Probiotics include viable lactic acid bacteria and other *Bacilli* that are able to survive in the intestine. Prebiotics include carbohydrates that are not digested in the upper part of the gastrointestinal tract, but selectively fermented by bacteria in the colon. This selective fermentation affects the composition of the intestinal microflora by stimulating *Bifidobacteria* and *Lactobacilli*, both in humans and in animals, where these bacteria have health promoting properties (Gibson and Roberfroid, 1995; Van Loo et al., 1999; Flamm et al., 2001). The importance of the intestinal microbiota in fish has been better understood during the last decade, while lactic acid bacteria were detected in fish microbiota (Ringø et al., 1995). To induce an artificial dominance, lactic acid bacteria were administrated to fish fry with encouraging results (Ringø and Gatesoupe, 1998). In fish larviculture, probiotics are inoculated in the rearing water, and they may be incorporated in the food chain by allowing the live food organisms, *Artemia* and rotifers, to graze on the probiotic strain. There were also some attempts to introduce probiotics into compound diets for fish (Gatesoupe, 1999; Ringø and Birkbeck, 1999; Verschuere at al., 2000). However, the probiotics strains can keep dominant in the gastrointestinal tract only during the dietary treatment. It is unlikely that such an exogenous addition of a single probiotic will result in long-term colonisation of the gut, especially when the strains used do not belong to the normal dominant intestinal flora. In those cases it is necessary to develop innovative strategies in order to establish stable and healthy gastrointestinal microbiota in fish.

The stimulation of specific indigenous microflora by supplementing fish feed with indigestible carbohydrates that act as prebiotic, could be an interesting approach to increase the proportion of health-promoting bacteria in the gut. There are few studies dealing with the influence of prebiotics on the intestinal microflora of fish. Lactosucrose was used as substrate for the intestinal microflora, and it increased the thickness of intestinal tunica muscularis in red sea bream, *Pagrus major* (Kihara et al., 1995). However, lactosucrose was poorly used by microbiota in carp (Kihara and Sakata, 2001a) and trout (Kihara and Sakata, 2001b). Recently, Olsen et al. (2001) observed a damaging effect of inulin on enterocytes of Arctic charr, *Salvelinus alpinus* L., but the concentration used was quite high (15% of the diet).

The present experiments were designed to study the effects of dietary inulin, oligofructose and lactosucrose, on the microbial ecology of the digestive tract, and on the growth of turbot at the weaning stage.

Material and methods

Animals

Turbot larvae (*Psetta maxima*) used in this study were obtained from France Turbot (Noirmoutier, France). The larval rearing was conducted at IFREMER, Brest, France, according to the method described by Gatesoupe (1990).

The newly hatched larvae were transferred to 150 litres conical fiber glass tanks (stocking density: 20 larvae l^{-1}). These tanks were supplied with running seawater passed through a sand filter, a heat exchanger, then a degassing column. The temperature was maintained at 18°C at a salinity of 35‰. The larvae were submitted to continuous illumination.

From 3 to 7 days post hatching (dph), turbot larvae were fed with enriched *Brachionus rotundiformis* with daily amounts increasing from 20 to 150 rotifers per larva. The rotifers were fed daily with backer's yeast (60 mg l^{-1}, on a dry matter basis), a self-emulsifying concentrate of essential fatty acids DHA Selco (INVE, Belgium, 18 mg l^{-1}) and T-*Isochrysis affinis galbana* (ca. 10^5 cells ml^{-1}). The rotifers were then transferred to clean seawater, enriched with DHA Selco (18 mg l^{-1}) and distributed continuously to the larvae with a peristaltic pump. *Artemia* nauplii (1 dph) were enriched with DHA Selco (150 mg l^{-1}) and dried brewer's yeast (50 mg l^{-1}). Then, they were distributed into the larval rearing tanks from 8 dph to 29 dph.

Prebiotics
Raftiline ST and Raftilose P95 are powdered food ingredients that contain mainly polydisperse β (2→1) linked fructans. Raftiline ST is a standard form of chicory inulin. Raftilose P95 contains mainly oligofructose produced by partial enzymatic hydrolysis of chicory inulin. The degree of polymerisation of the fructans ranged from 2 to 60 for Raftiline, and from 2 to 8 for Raftilose. The minimum amount of fructans guaranteed by the manufacturer was 90 and 93% for Raftline ST and Raftilose P95, respectively. The other components were mainly glucose, fructose, and sucrose. These products were supplied by Raffinerie Tirlemontoise (Tienen, Belgium).
Lactosucrose (4^G-β-D-galactosylsucrose) is a trisaccharide synthesized from lactose and sucrose by transfructosylation of β-fructofranosidase. Lactosucrose LS-55P, obtained from Ensuiko Sugar Refining Co. (Yokohama, Japan), is a powder containing more than 55 % lactosucrose, less than 25 % lactose, and less than 10 % sucrose.

Diet Preparation
Experimental diets were formulated to contain approximately 62 % protein and 12 % fat, in order to test the influence of oligosaccharides and inulin during the weaning of turbot (Table 1). A control diet (C) included 2% cellulose powder. In the test diets, cellulose (Arbocell B00, J. Rettenmaier & Söhne, Germany) was substituted by 2% Raftilin ST (diet S), 2% Raftilose P95 (diet P) or 2% lactosucrose (diet L). Extruded pellets were sieved to obtain particles with graded diameters (200-400 µm; 400-600 µm and 600-1000 µm), which were successively used according to fish size during the weaning experiment.

Analytical method
The diets were analysed with the following procedures: dry matter by drying at 105°C for 24 h, ash by combustion at 550°C for 12 h, crude protein by Dumas method, fat by dichloromethane extraction (Soxhlet), and energy in an oxygen bomb calorimeter. The proximate composition of the diets is given in Table 1.

Experimental design
At 29 dph, turbot larvae, previously fed on standard live food organisms (as explained above), were transferred to the experimental facilities. Their initial mean weight was 45.5 ± 1.9 mg. They were distributed randomly in 12 subsquare tanks (440 fish per tank). The tanks contained 50 l seawater and were subjected to 18 light/6 dark photoperiod. The temperature was maintained at 18°C. The larvae were weaned for 10 days by decreasing progressively the amount of *Artemia*, while increasing the amount of the experimental compound diets (Table 1).

The larvae were fed continuously in excess the diets during one month, at the daily rate of 20-30% of the biomass. Each diet was tested in triplicates. Fish were weighed at the start and at the end of the experiment. At 29 and 55 dph larvae were sampled for bacterial analysis as described below.

Sampling and bacterial examination

Two larvae were sampled in each tank at 29 and 56 dph. The fish were euthanized with an overdose of 2-phenoxyethanol, and the corporal surface was disinfected in a solution of 0.1% benzalkolium chloride (Sigma, USA). Larvae were then dissected under sterile conditions and the gut was removed, weighed and homogenized. After homogenisation, appropriate dilutions of the three samples for each treatment were spread on Petrifilm (Aerobic Count Plates, 3M Microbiology products, USA) and TCBS agar (thiosulfate-citrate-bile-salt agar, AES Laboratoire, France, dissolved in half-strength seawater). MRS and Sabouraud Agars were also used to detect lactic acid bacteria and yeast, respectively (Gatesoupe, 2002). All the plates were incubated at 20°C and inspected up to 5 days. Eight colonies were randomly isolated from each individual Petrifilm for phenotypic characterisation.

These selected isolates were cultivated on plate count agar (PCA, AES laboratoire, enriched with 18 g l^{-1} NaCl, pH adjusted to 7.8) and stored under liquid nitrogen before further genotypic characterisation.

Phenotypic characterisation

The Phenotypes of the isolates were characterised with API 20 E strips according to the manufacturer's instructions (BioMérieux, France). The API 20 E profiles were compared and classified with the Wagner parsimony method, by using Mix of Phylip (Felsenstein, 1996). The isolates representative of the different phenotypic profiles were cultured again for DNA extraction.

Genotypic characterisation

DNA was extracted and the fragments corresponding to 16S rDNA were amplified by PCR. The isolates were clustered according to their genotypic similarity, characterised by Amplified Ribosomal Dna-Restriction Analysis (ARDRA) of the PCR products by using Hae III and Cfo I (Gatesoupe, 2002). One isolate per each dominant cluster was selected for sequencing the 16S rDNA gene (partial sequences of 702 – 753 bp from primer SA-dir 5'-agagtttgatcatggctcag-3'). The phylogenic position of the cluster was then searched with BLAST (NCBI).

Ability of bacterial isolates to use inulin and oligofructose

Selected isolates were tested for Hugh and Leifson's fermentation test applied to Raftiline ST and to Raftilose P95 under anaerobic conditions (Hugh and Leifson, 1953), and for their ability to use these products as single carbon source in Baumann medium (Baumann et al., 1971). The test tubes were incubated at 20°C, and checked up to 5 days.

Statistical Analysis

To test differences between dietary treatments, all data were subjected to one-way analysis of variance (ANOVA, SigmaStat 2.0), after checking normality and homogeneity of variance. The bacterial counts were log-transformed. When the parametric analysis was not applicable, Kruskall-Wallis ANOVA on ranks was tested. When significant differences were observed, the effects of the individual treatments

were further compared with Tukey's or Dunn's method, after parametric or non-parametric ANOVA, respectively.

Results

Growth and survival

Feeds were well accepted during the experimental period. High weaning survival rates were obtained, without any significant difference (82-89 %, Table 2). The growth rate was significantly improved with diet P that contained oligofructose (final mean weight of 0.50 vs. 0.41-0.43 g).

Microbiota

The bacterial counts were highly variable, and it was not possible to observe any significant difference between the amounts of bacteria, the mean of which was generally ca. 10^3 CFU g^{-1} (Table 3). Before weaning at 29 dph, the intestinal microbiota was still dominated by *Vibrio* spp. which represented 96% of the isolates. After weaning at 55 dph, the dominance of *Vibrio* spp. was again observed in the control group, and in turbot fed with inulin. Two strains were dominant, *Vibrio* L2C55, closely related to *V. ordalii* 6.30 (Montes et al., 2003), and *Vibrio* sp. S12411, closely related to *Vibrio* sp. Csur-1 (Radjasa et al. 2001). Both strains were also observed in turbot weaned with oligofructose, but their dominance was less marked. Bacterial diversity was high in this latter group, with the emergence of *Bacillus* sp. L171P95, close to *B. subtilis* KL-077 and KL-073 (Venkateswaran et al., 2003). Microbiota could not be characterised in the group weaned with lactosucrose, because most of the isolates from Petrifilm did not grow on PCA plates. However, the high counts on TCBS plates suggested the dominance of *Vibrio* spp.
No colony was detected on MRS and Sabouraud agars along the experiment.

Ability of bacterial isolates to use inulin and oligofructose

Strains corresponding to the dominant *Vibrio* spp., and to *Bacillus* sp. were tested for their acid production, and for their use of Raftilose P95 and Raftilin ST as single carbon source. The growth of *Bacillus* sp. with Raftilose P95 as carbon source was faster than that obtained with glucose (Table 4). It is noteworthy that these strains did not produce organic acids, and that they did not grow with Raftilin ST, indicating that the nutritive value of Raftilose P95 was due to oligofructose, rather than due to the small proportion of mono- and disaccharadides contained in the commercial products.

Discussion

Raftilose P95 had a positive effect on the growth of weaning turbot. Further study is needed to interpret this effect, but the increased diversity in gut microbiota, and the specific emergence of *Bacillus* sp. may have played a role in this effect. *Bacillus* sp. have been seldom isolated from turbot (Austin, 1982, 1983). This genus has been evaluated as source of probiotics in aquaculture systems, by trying either to improve water quality or to reduce the incidence of pathogens in farmed species (Irianto and Austin, 2002). Autochthonous isolates have been used as biocontrol agents to reduce vibriosis in fish (Kennedy et al., 1998; Sugita et al., 1998), and in shrimp (Moriarty, 1998; Rengipat et al., 1998, 2000; Vaseeharan and Ramasamy, 2003). Commercial probiotic preparations containing *Bacillus* spp. have been also tested in fish (Gatesoupe, 1989, 1993; Queiroz and Boyd, 1998; Raida et al., 2003). In our study, the *Bacillus* strains were isolated only from turbot fed with Raftilose P95, while the proportion of *Vibrio* spp. was lower than in the other groups. The isolates of *Bacillus*

sp. were able to use Raftilose P95 as single carbon source at a faster rate than *Vibrio* spp. These isolates inhibited also the growth of *Vibrio anguillarum* strains isolated from turbot (data not shown). Their potential as probiotics should be further investigated.

The high variability of the bacterial counts in the weaning experiment may be an indication that the prebiotic treatments were not sufficient alone to regulate microbiota. The combination of the probiotic and prebiotic treatments may be particularly important for fish where the gastrointestinal microbiota are variable (Ringø and Birkbeck, 1999), and much more dependent on the environment than those of terrestrial species. The strain of *Bacillus* sp. isolated in the present experiment could be particularly interesting in this context.

The treatment with lactosucrose did not significantly improved the growth of turbot. However, it is not possible to draw any conclusion about the interest of this compound as prebiotic for turbot. The relatively high amounts of lactose and sucrose contained in the commercial product might have affected microbiota, which seemed different from those of the other groups, though it was not efficiently characterised.

Conclusion and perspective

This preliminary study should encourage to further investigate the potential of prebiotics in fish compound diets. It should be necessary to improve the methods to survey the effect of prebiotics on microbiota, while considering also their possible effects on uncultivable and anaerobic bacteria. Preferably combined with probiotics, some prebiotics like oligofructose could be useful for the establishment of a stable and healthy gastrointestinal flora, especially in intensive larval culture where high mortalities are frequently encountered.

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Table 1
Composition of the diets

Ingredients (%)				
Fish meal	59.9			
Fish concentrate protein	14			
Pre-cooked starch	12			
Cod liver oil	4			
Soybean lecithin	2			
Mineral mixture (INRA 736)[§]	2			
Vitamin mixture (INRA CV762)[§]	2			
Guar	2			
Inosin	0.1			
Cellulose or Prebiotic[£]	2			
Proximate analyses (% dry matter)	**C**	**S**	**P**	**L**
Dry matter	93.5	95.7	93.0	92.8
Moisture (% wet weight)	6.5	4.3	7.0	7.2
Protein	61.7	62.2	62.0	61.5
Fat	11.5	11.8	11.8	12.5
Ash	10.4	10.3	10.3	10.3
Energy	22.6	22.6	22.9	22.8

[§] Mineral and vitamin mixes detailed by Fournier et al. (2002)
[£] Cellulose powder, Raftilin ST, Raftilose P95 and lactosucrose were added in diets C, S, P and L, respectively

Table 2
Effect of prebiotics on turbot growth at the weaning stage

Treatment	Final mean weight[£] (g)	SGR[§]	Survival rate[#] (%)
C	0.41 ± 0.02[b]	8.4 ± 0.4	88.6 ± 1.7
S	0.41 ± 0.02[b]	8.4 ± 0.2	82.1 ± 1.8
P	0.50 ± 0.02[a]	9.2 ± 0.2	87.4 ± 1.3
L	0.43 ± 0.02[b]	8.6 ± 0.2	85.7 ± 0.9

[§] Specific Growth Rate = [(LnFW – LnIW)/t] × 100; FW: final mean weight for each replicate; IW: initial mean weight (46 ± 2 mg); t = time (26 days)
[£] Means ± S.E. with different superscripts for significantly different means in Tukey's test; ***P*<0.01, n = 90
[#] Survival rate (not significantly different, n = 3)

Table 3
Effect of prebiotics on intestinal microbiota in weaning turbot

	29 dph	55 dph			
Group	Initial	C	S	P	L
Petrifilm counts[@] (log CFU g^{-1})	3.2 ± 1.4	3.6 ± 0.3	2.8 ± 0.3	3.0 ± 0.4	2.3 ± 0.5
TCBS counts[@] (log CFU g^{-1})	3.1 ± 1.4	2.7 ± 0.9	3.8 ± 0.9	1.8 ± 1.2	3.7 ± 1.3
Vibrio sp. S12411[§] (%)	nd	28	22	16	5
Vibrio sp. L2C55[£] (%)	nd	36	44	25	5
Bacillus sp. L171P95[#] (%)	nd	nd	nd	14	nd
Other bacteria (%)	100	36	34	45	90

[@] Bacterial counts: means ± S.E., Kruskall-Wallis ANOVA on ranks not significant, n = 6
The identification was based on alignment with nucleotide sequences currently available in NCBI database; [§] Accesion number of an homologous fragment: AF293974, position of the first and last nucleotides corresponding to the fragment in the referenced sequence: 43-605; [£] Accession number: AJ634477, sequence deposited for this study; [#] accession numbers of an homologous fragment: AY030331, position of the first and the last nucleotides corresponding to the fragments in the referenced sequence: 53-785.
nd: not detected

Table 4
Carbohydrate utilization of selected strains isolated from turbot weaned with Raftilose P95

Strains	Acid production (Hugh and Leifson)			Utilization as single source of carbon		
	Glucose	Raftilose P95	Raftiline ST	Glucose	Raftilose P95	Raftiline ST
Vibrio sp. S12411[£]						
L144P95	(+)	+	(+)	+	(+)	-
L145P95	(+)	+	-	+	-	-
L173P95	-	-	-	nt	nt	nt
Vibrio sp. L2C55[#]						
L183P95	(+)	+	(+)	+	-	-
L136P95	(+)	+	(+)	+	(+)	-
L133P95	(+)	+	(+)	+	-	-
L134P95	(+)	+	(+)	+	(+)	-
L131P95	(+)	+	(+)	+	-	-
Vibrio sp. L162P95[@]	(+)	+	(+)	+	+	-
Bacillus sp.[#]						
L161P95	-	-	-	+	++	-
L171P95	-	-	-	+	++	-

The identification was based on alignment with nucleotide sequences currently available in NCBI database; [£] see Table 3; [#] see Table 3; [@] accession numbers of an homologous fragment: AJ437193, position of the first and the last nucleotides corresponding to the fragments in the referenced sequence: 37-755.
nt: not tested; (+):weak or delayed reaction, +: positive reaction; ++: faster growth; -: negative reaction