Published December 5, 2014

Dietary mannan oligosaccharide supplementation modulates intestinal microbial ecology and improves gut morphology of rainbow trout, *Oncorhynchus mykiss* (Walbaum)[1]

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ABSTRACT: A study was conducted to investigate the effect of mannan oligosaccharide (MOS) on the gut microbiota and intestinal morphology of rainbow trout under commercial farming conditions. Juvenile (mean initial BW 38.2 ± 1.7 g) and subadult (111.7 ± 11.6 g) trout were fed 2 dietary treatments for 111 and 58 d, respectively. The control treatment consisted of a standard commercial diet, and the MOS treatment consisted of the control diet supplemented with 0.2% MOS. Morphology of the anterior and the posterior intestine was examined with light and electron microscopy. Light microscopy demonstrated increased gut absorptive surface area in the subadult MOS group. Additionally, electron microscopy revealed an increase in microvilli length and density in the subadult MOS group compared with the control ($P < 0.05$). However, no significant improvements were detected in the juvenile group. Culture-based evaluation of the intestinal microbiota showed that MOS significantly reduced ($P < 0.05$) the viable intestinal bacterial populations (by approximately 2 log scales in all cases). Levels of *Aeromonas/Vibrio* spp. were significantly decreased ($P < 0.05$) in the juvenile MOS group (9% of the total microbiota) compared with the juvenile control group (37%). Additionally, analysis of microbial communities was conducted using denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA. The denaturing gradient gel electrophoresis fingerprinting revealed an alteration of bacterial populations; analysis of similarity, similarity percentages, and nonmetric multidimensional scaling analysis showed that MOS reduced species richness and increased similarity of bacterial populations found within the subadult and juvenile groups. The current study shows that MOS modulates intestinal microbial communities, which subsequently improve gut morphology and epithelial brush border.

Key words: histology, mannan oligosaccharide, microbiota, prebiotic, rainbow trout

J. Anim. Sci. 2009. 87:3226–3234
doi:10.2527/jas.2008-1428

INTRODUCTION

Improving and protecting fish health in commercial production practices is a major factor in the aquaculture industry. This has prompted new initiatives toward the development/appraisal of novel agents or functional dietary supplements in commercial feeds for fish and crustacean species. Such products include immunostimulants (Huttenhuis et al., 2006), probiotics (Burr et al., 2005; Balcázar et al., 2006), and prebiotics (Li and Gatlin, 2004). Many are yeast-based products, either whole cells incorporated into the diet as a probiotic (Waché et al., 2006) or cellular derivatives included as a prebiotic (Li and Gatlin, 2004; Li et al., 2005). Many of these products were developed for poultry, swine, and cattle production. One such example is mannan oligosaccharide (**MOS**), which has been shown to improve gut function and health by increasing villi height, uniformity, and integrity (Iji et al., 2001; Hooge, 2004; Castillo et al., 2008). As a result, the digestive tract has potential for greater absorptive efficiency (Spais et al., 2003; Sims et al., 2004). The documented benefits of MOS in terrestrial animals have led to initiatives to evaluate the potential of MOS with regards to finfish aquaculture. The use of functional feed additives such

[1]The authors thank Christopher G. Saunders-Davies (Test Valley Trout farm, Romsey, UK) and his technical staff for providing the samples and all the relative information regarding the trial. The authors also thank Michele Kiernan, Paul Waines, and Mike Hockings for their technical assistance at the laboratories of the University of Plymouth, Plymouth, UK.

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Received August 23, 2008.
Accepted July 6, 2009.

as MOS to improve growth and health performance in the aquaculture industry is increasingly important as consumers demand eco-friendly production practices. There is a clear lack of information regarding the effect of MOS on the economically important species, particularly rainbow trout (*Oncorhynchus mykiss* Walbaum). The aim of the present study is to evaluate the prebiotic effects of MOS on gut structure, morphology, and the intestinal microbial ecology of rainbow trout (*Oncorhynchus mykiss* Walbaum).

MATERIALS AND METHODS

No work was conducted that required ethical approval, and no acts were conducted that fall under legislation related to scientific procedures in the United Kingdom.

Fish

The present study was conducted under commercial rearing conditions at Test Valley Trout farm, Romsey, UK. During the experimental period 2 separate batches of fish were monitored: batch 1, initial mean BW of 111.7 ± 11.6 g (referred to as subadult group hereafter) and batch 2, mean BW of 38.2 ± 1.7 g (referred to as juvenile group hereafter). Subadult and juvenile trout were subjected to 2 dietary treatments: (i) control group, fed a standard Skretting commercial feed (Wincham, Northwich, Cheshire, UK) and (ii) MOS group, fed a standard Skretting feed supplemented with a commercial MOS product (Bio-Mos, Alltech Inc., Lexington, KY) at 2 $g \cdot kg^{-1}$ (0.2%). Bio-Mos is derived from the outer cell wall of *Saccharomyces cerevisiae* strain 1026. Supplementation level of 0.2% was chosen based on previous studies (Salze et al., 2008; Staykov et al., 2007). Excel 30 diet (Skettring; 48% protein, 19% lipid, 9% ash, 16% nitrogen-free extract, 8% moisture) was fed until fish reached approximately 100 g, and Royale Crystal 45 + astaxanthin (43% protein, 26% lipid, 8% ash, 16% nitrogen-free extract, 7% moisture) was fed thereafter (Skretting). Main dietary components were fishmeal, fish oil, corn gluten meal, soybean meal, wheat, vitamins, minerals, and lysine. Batches of approximately 20,000 fish were randomly distributed into 4×450 m^3 outdoor earth ponds. Juvenile groups were fed for 111 d on the experimental diets (81 d on Excel 30 and 30 d on Royale Crystal 45 + astaxanthin), and subadult groups were fed experimental diets for 58 d. Fish were fed approximately 1.5% of $BW \cdot d^{-1}$. The juvenile and subadult trials were conducted simultaneously to prevent seasonal factors from affecting the groups. The farm was fed by a local river, so temperature (11 to 16°C) and pH (~7 to 8) fluctuated naturally with seasonality.

Sampling

At the end of the experiment 12 fish from each experimental group were randomly selected. Mean final BW of fish sampled were 232.2 ± 18.5 g for subadult control group, 267.9 ± 30.9 g for subadult MOS group, 132.8 ± 23.4 g for the juvenile control group, and 153.5 ± 32.1 g for juvenile MOS group.

Histological Examination

Intestinal samples from 6 fish per experimental group were retained for histological examination by electron and light microscopy. Sections from the intestine distal to the pyloric cecae (anterior region) and the intestinal section before the anus (posterior region) were taken. Material for light microscopy was fixed in 4% saline formalin. The tissue samples were dehydrated in graded ethanol before equilibration in xylene and embedding in paraffin wax (Refstie et al., 2006). Eight-micron transverse sections were cut and stained using Alcian blue periodic acid-Schiff staining technique (Kiernan, 1981).

Samples for scanning and transmission electron microscopy (**SCEM** and **TEM**; respectively) were fixed in 2.5% glutaraldehyde with 0.1 *M* cacodylate acid sodium salt solution (1:1 vol/vol), pH 7.2. Before fixation, SCEM samples were rinsed in 1% S-carboxymethyl-L-cysteine (Sigma) for 30 s to remove epithelial mucus. The SCEM samples were dried using a K850 critical point drier (Emithech, Kent, UK) with ethanol as the intermediate fluid and CO_2 as the transition fluid. All samples were coated with gold using K550 sputter coater (Emithech) and screened with a Jeol JSM 5600 LV electron microscope at 15 kV (Jeol, Tokyo, Japan). Samples for TEM were postfixed in OsO_4 for 1 h and embedded with the standard resin procedure. Resin blocks were sectioned using a diamond knife (~90 nm). Ultrathin sections from each sample were placed in copper grids and stained with uranyl acetate, post stained with lead citrate, and screened with a Jeol JSM 1200 transmission electron microscope at 120kV (Jeol).

All digital images were analyzed using Image J version 1.36 (National Institutes of Health). Images from light microscopy were analyzed to determine the perimeter ratio (**PR**) between the internal perimeter (***IP***) of the gut lumen (villi and mucosal folding length) and the external perimeter (***EP***) of the gut [$PR = IP/EP$, arbitrary units (**AU**)]. A high PR value indicates high villi length, increased mucosal folding, or both. High magnification (× 20,000) SCEM images were analyzed to measure the density of the microvilli (AU) after Merrifield et al. (2009b). The TEM images (magnification × 20,000) were analyzed to measure the microvilli length as described by Hu et al. (2007).

Culture-Based Microbial Analysis

Time between termination and dissection did not exceed 2.5 h. After aseptic dissection, the entire digestive tract was removed and the digesta from 6 fish per treatment were pooled into 2 samples to avoid interfish variation (Hovda et al., 2007; Merrifield et al., 2009a,b).

Water samples from the main supply of the farm were also taken. All samples were then serially diluted to 10^{-7} with PBS (Oxoid, Basingstoke, UK), and 100 μL was spread onto duplicate tryptone soy agar plates (**TSA**; Oxoid) after Huber et al. (2004). Colony-forming units per gram for aerobic heterotrophic populations were then calculated after 7 d aerobic incubation at 20°C. Random colonies (n = 25) from plates containing 30 to 300 cfu were taken and subcultured on TSA until pure cultures were achieved. A total of 450 isolates were then tentatively placed into groups or genera based on the colony morphology, cell morphology, gram stain, production of catalase, oxidase, glucose fermentation, motility, and endospore formation after Merrifield et al. (2009a).

Bacterial DNA Extraction and 16S rRNA Amplification

The DNA was extracted from 3 intestinal samples (pooled from 9 of the 12 fish sampled) per treatment using QIAamp Stool Mini Kit (Qiagen, Crawley, West Sussex, UK) with a lysozyme pretreatment (50 mg·mL^{-1} of TE buffer for 30 min at 37°C). Concentration of DNA was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop 1000, Wilmington, DE), and standardized. PCR amplification of the 16S rRNA genes was undertaken using the forward primer P3 with a GC clamp (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and the reverse primer P2 (5′-ATT ACC GCG GCT GCT GG-3′) after Muyzer et al. (1993). The following reagents were included in each PCR tube: 1 μL of primer P2 and P3 (50 pmol·μL^{-1}; MWG-Biotech AG, Ebersberg, Germany), 3 μL of DNA template, 25 μL of ReadyMix Taq PCR Reaction Mix with $MgCl_2$ (Sigma-Aldrich, Poole, Dorset, UK), and PCR-grade water to a final volume of 50 μL, giving a final concentration of 1.5 units of Taq DNA polymerase, 10 m*M* Tris-HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatin, 0.2 m*M* dNTP. The touchdown thermal cycling was conducted using a GeneAmp PCR System 9700 (Perkin-Elmer, Waltham, MA), under the following conditions: 94°C for 10 min, then 30 cycles starting at 94°C for 1 min, 65°C for 2 min, 72°C for 3 min. The annealing temperature decreased by 1°C every second cycle until 55°C and then remained at 55°C for the remaining cycles. The PCR products were stored at 4°C until used.

Denaturing Gradient Gel Electrophoresis

The denaturing gradient gel electrophoresis (**DGGE**) was performed using a DGGE-2001 system (CBS Scientific, Del Mar, CA). Ten microliters of standardized PCR products was run on 8% acrylamide gels with a denaturing gradient of 40 to 60% (where 100% denaturant is 7 *M* urea and 40% formamide). All samples were run on the same gel to prevent issues of reproducibility, and outside lanes were not used. The gel was run at 65 V for 17 h at 60°C in 1 × TAE buffer (66 m*M* Tris, 5 m*M* Na acetate, 1 m*M* EDTA). Visualization of the DGGE bands was achieved by the optimized silver staining method of Benbouza et al. (2006). The gel was scanned in a Bio-Rad universal hood II (Bio-Rad Laboratories, Segrate, Italy) and optimized for analyses by enhancing contrast and grayscale.

Statistical Analysis

Independent samples 2-tailed *t*-test was applied to evaluate the effect of MOS on intestinal histology and culture-based microbiota on each of the 2 batches of fish. Analyses were carried out using SPSS (SPSS Inc., Chicago, IL), and significance was accepted at the $P < 0.05$ level. The results are presented as mean values followed by the SD. The DGGE banding patterns were transformed into presence/absence matrices for similarity assessment between treatments using Quantity one version 4.6.3 analyses software (Bio-Rad Laboratories) after Schauer et al. (2000). Band intensities were measured and analyzed using Primer v6 (Clarke and Gorley, 2006). Nonmetric multidimensional scaling analysis (**nMDS**) was used to represent the relative similarities between treatments (Powell et al., 2003). Stress level was minimal (0.04), and cluster analysis was used to check the groupings by the nMDS procedure. Furthermore, similarity percentages (**SIMPER**) were performed on observed clusters shown on the MDS plots. A 1-way analysis of similarity (**ANOSIM**) was used for pairwise comparison to determine differences between DGGE banding profiles (Abell and Bowman, 2005).

RESULTS

Histological Examination

The results of the histological examination of the absorptive surface area, and microvilli morphology are presented in Table 1. The light microscopy assessment of the rainbow trout intestinal tract demonstrated that MOS had a beneficial effect on the absorptive area in the subadult fish groups. The anterior region of the subadult MOS group (4.50 ± 0.30 AU) displayed a greater absorptive surface area ($P = 0.032$) than the control group (3.46 ± 0.48 AU). The absorptive surface area of the posterior region of the subadult MOS group (5.29 ± 0.39 AU) was also greater ($P = 0.041$) than the control group (3.03 ± 0.19 AU). However, there were no significant differences between the juvenile fish groups in either intestinal region. The analysis of SCEM images showed that the microvilli density of the posterior intestine in the subadult MOS-treated fish (6.54 ± 1.08 AU) was increased ($P = 0.015$) compared with the control-fed fish (2.53 ± 0.75 AU). However, there were no other significant differences in microvilli density. The TEM revealed that MOS increased the microvilli length in subadult fish for anterior and poste-

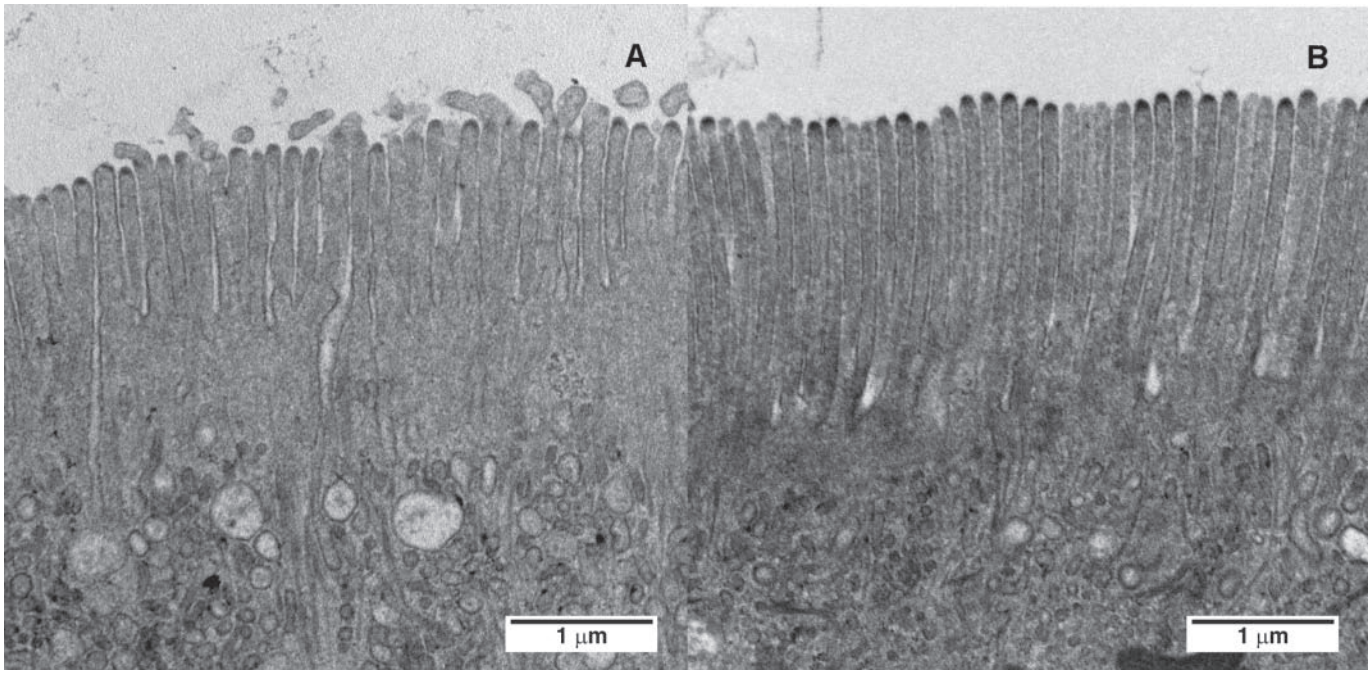

Figure 1. Comparative transmission electron microscopy (TEM) micrographs of posterior region of subadult rainbow trout fed fish meal (A) or mannan oligosaccharide (MOS; B). Although microvilli appear healthy in both treatments, microvilli are more regular and significantly longer in MOS-treated fish ($P = 0.007$).

rior intestinal regions. Microvilli length from the anterior region increased from 1.33 ± 0.72 μm in the control fish to 1.90 ± 0.15 μm in the MOS fish ($P = 0.002$). Similarly, microvilli length in the posterior intestinal region increased from 1.22 ± 0.01 μm in the control group to 1.53 ± 0.11 μm in the MOS-fed fish ($P = 0.007$). A comparative example is illustrated in Figure 1. However, no significant differences were found in the juvenile fish groups.

Culture-Based Analysis

Table 2 presents the aerobic heterotrophic intestinal microbial composition of each group and the rearing water. Viable counts in the rearing water were determined to be 10.8×10^3 cfu mL^{-1}. Dominant groups in the rearing water were identified as *Aeromonas/Vibrio* spp., *Pseudomonas* spp. and members of the Enterobacteriaceae, which accounted for approximately 80% of the total microbial population.

Viable gastrointestinal populations ranged between 10^6 and 10^8 cfu·g^{-1}. Viable populations in MOS-supplemented groups were significantly less than the control groups; approximately 2 log scales less (down from 10^8 to 10^6 cfu·g^{-1}; $P < 0.05$). Changes in the relative abundance of the microbiota identified were also observed. Compared with the juvenile control, juvenile MOS-fed fish displayed a significant reduction of the relative abundance of *Micrococcus* spp. (from 22 to 7% of the total microbiota; $P = 0.027$), *Aeromonas/Vibrio* spp. (from 37 to 9%; $P = 0.021$), and unidentified gram-positive rods (from 25 to 6%; $P = 0.037$). Coinciding with these changes a significant increase of *Enterococcus* spp. (from 3 to 19%; $P = 0.024$) and Enterobacteriaceae (from 5 to 39%; $P = 0.049$) were observed. Compared with the subadult control group, subadult

Table 1. Anterior and posterior gut morphology of subadult and juvenile rainbow trout fed a control diet or mannan oligosaccharide (MOS)-supplemented diet

		Juvenile trout		Subadult trout	
Variable	Intestinal region	Control	MOS	Control	MOS
Absorptive surface (perimeter ratio)[1]	Anterior	3.68 ± 0.22	3.75 ± 0.21	3.46 ± 0.48[a]	4.50 ± 0.30[b]
	Posterior	4.25 ± 0.65	3.00 ± 1.09	3.03 ± 0.19[a]	5.29 ± 0.39[b]
Microvilli density[1]	Anterior	4.54 ± 1.43	4.74 ± 0.86	3.51 ± 0.23	3.13 ± 0.52
	Posterior	2.45 ± 0.12	4.53 ± 1.15	2.53 ± 0.75[a]	6.54 ± 1.08[b]
Microvilli length, μm	Anterior	1.29 ± 0.17	1.65 ± 0.21	1.33 ± 0.72[a]	1.90 ± 0.15[b]
	Posterior	1.21 ± 0.37	1.37 ± 0.05	1.22 ± 0.01[a]	1.53 ± 0.11[b]

[a,b]Values (mean ± SE) within the same age group, in the same row, with different superscripts are different ($P < 0.05$); n = 6/treatment.
[1]Arbitrary units.

MOS-fed fish also displayed a significant reduction of *Micrococcus* spp. (27 to 6%; $P = 0.006$). A reduction of Enterobacteriaceae (from 22 to 5%; $P = 0.01$) and an increase of *Pseudomonas* spp. (from 7 to 26%; $P = 0.012$) were also observed.

DGGE Analysis

The similarity half matrix of presence/absence banding patterns from the 12 samples run on the DGGE is shown in Table 3. Similarity between dietary treatments within the same age groups were high, with 77.22 ± 11.06% similarity between the subadult control/MOS groups and 79.22 ± 14.92% similarity between the juvenile control/MOS groups. The Primer analysis is shown in Table 4. Species richness is greater in the subadult fish than the juvenile fish. Mannan oligosaccharide supplementation resulted in the removal of certain species; species richness reduced from 11.7 in the juvenile control to 8.7 in the juvenile MOS group and from 14.0 in the subadult control to 12.3 in the subadult MOS group. The ANOSIM R statistic (values closer to 1 indicate replicates within a group are more similar than those from the groups being compared) and its significance should be used with caution due to the small number of replicates. However, the subadult control group appears different to both juvenile groups. The nMDS analysis (Figure 2) shows that the juvenile control group is clustered with SIMPER average similarity of 94.5%. The MOS treatment of the juvenile group shows a distinct spatial shift (with some overlap remaining with the control) and a SIMPER of 72.2%. The subadult control group is again distinct from the juveniles and much less clustered (SIMPER = 72.5%). Mannan oligosaccharide treatment shows less similarity (SIMPER = 54.7%); however, spatial movement toward the juvenile groups is evident (resulting in moderate spatial overlapping). Average dissimilarity between groups also showed that the microbiota of juvenile groups were the least dissimilar (25.1%). The subadult control group was the most dissimilar from both juvenile groups (44.4 and 42.8% for juvenile MOS and control, respectively). Dissimilarity was considerably less between the MOS groups (33.7%).

DISCUSSION

The light and electron microscopy showed that MOS caused significant differences of gastrointestinal morphology in the subadult trout. Dietary MOS improved intestinal morphology in the anterior and posterior intestinal regions. Mannan oligosaccharide supplementation increased absorptive surface area by promoting longer mucosal foldings. Furthermore, increased absorptive surface area was confirmed at the electron microscopy level, where micrographs showed that MOS was able to increase microvilli density and length. Histological examination of juvenile trout showed that MOS was unable to confer significant improvements despite the

Table 2. Composition of subadult and juvenile rainbow trout culturable gut microbiota fed a control or mannan oligosaccharide (MOS)-supplemented diet[1]

			Juvenile group				Subadult group			
	Rearing water		Control trout		MOS trout		Control trout		MOS trout	
Item	cfu·mL^{-1}	%	cfu·g^{-1}	%	cfu·g^{-1}	%	cfu·g^{-1}	%	cfu·g^{-1}	%
Viable population	10.8×10^3		2.50×10^{8a}		2.35×10^{6b}		5.87×10^{8a}		4.83×10^{6b}	
Micrococcus spp.	—	—	5.49×10^7	22[a]	1.65×10^5	7[b]	1.58×10^8	27[a]	2.9×10^5	6[b]
Acinetobacter spp.	1.13×10^2	2	—	—	7.05×10^4	3	5.87×10^6	1	2.42×10^5	5
Enterococcus spp.	—	—	7.48×10^6	3[a]	4.47×10^5	19[b]	1.06×10^8	18	1.64×10^6	34
Staphylococcus spp.	—	—	—	—	—	—	2.93×10^7	5	—	—
Aeromonads/Vibrio spp.	1.13×10^2	20	9.23×10^7	37[a]	2.12×10^5	9[b]	9.97×10^7	17	7.73×10^5	16
Pseudomonas spp.	1.81×10^3	32	9.98×10^6	4	2.35×10^5	10	4.11×10^7	7[a]	1.26×10^6	26[b]
Enterobacteriaceae	1.58×10^3	28	1.25×10^7	5[a]	9.17×10^5	39[b]	1.29×10^8	22[a]	2.42×10^5	5[b]
Bacillus spp.	—	—	4.99×10^6	2	—	—	—	—	—	—
Carnobacteria/Lactobacillus spp.	1.13×10^2	2	2.49×10^6	1	—	—	—	—	1.93×10^5	4
Kurthia spp.	3.39×10^2	6	2.49×10^6	1	1.18×10^5	5	—	—	4.83×10^4	1
Unidentified gram-negative spp.	4.52×10^2	8	—	—	—	—	5.87×10^6	1	4.83×10^4	1
Unidentified gram-positive spp.	1.13×10^2	2	6.24×10^7	25[a]	1.41×10^5	6[b]	1.17×10^7	2	9.67×10^4	2
Yeast	—	—	—	—	4.7×10^4	2	—	—	—	—

[a,b]Values within the same row, in the same age group, with different superscripts are different ($P < 0.05$); n = 2/treatment (pooled from 6 fish).

[1]Expressed as log cfu·g^{-1} or mL^{-1} and percentages (calculated from percentage of total viable load).

Table 3. Similarity half matrix between presence/absence of denaturing gradient gel electrophoresis (DGGE) bands of rainbow trout gut microbial populations subjected to the experimental diets (values expressed as %)[1]

			Subadult						Juvenile					
			Control diet			MOS[2] diet			Control diet			MOS diet		
Fish group	Diet	Replicate	1	2	3	1	2	3	1	2	3	1	2	3
Subadult	Control	1	100	77.9	74	74	80	100	45.6	66.7	63.6	46.9	53.8	45.6
		2		100	80	68.7	73.1	71.4	47.4	61.5	57.1	54.5	60.9	66.7
		3			100	65.8	72	90	45.6	66.7	63.6	70.6	77.8	63.6
	MOS	1				100	71.4	90	63.6	73.3	72.7	70.6	77.8	63.6
		2					100	71.4	57.1	69	66.7	56	61.5	73.3
		3						100	63.6	73.3	72.7	82.4	88.9	72.7
Juvenile	Control	1							100	95.7	100	73.7	70	100
		2								100	95.7	66.7	63.2	95.7
		3									100	73.7	70	100
	MOS	1										100	93.3	73.7
		2											100	70
		3												100

[1]n = 3/treatment (pooled from 9 fish).
[2]MOS = mannan oligosaccharide.

observation that juveniles received MOS supplementation for a longer period of time. The reason for this is not understood. Literature regarding the effect of MOS on rainbow trout gut histology is scarce; however, 2 recent investigations in other fish species have provided valuable information. Torrecillas et al. (2007) demonstrated that dietary administration of MOS (at 0.2 or 0.4%) to European sea bass (*Dicentrarchus labrax)* for 67 d did not affect villi length. However, similar to the findings of the present study, MOS supplementation significantly increased microvilli length in cobia (*Rachycentron canadum*) larvae (Salze et al., 2008). Similarly, improvements of gut morphology have been reported in poultry and swine fed dietary MOS (Iji et al., 2001; Hooge, 2004; Peet-Schwering et al., 2007; Castillo et al., 2008). An improvement in gut morphology is not only likely to benefit feed utilization, but the maintenance of an intact, healthy mucosal epithelium may help to prevent opportunistic indigenous bacterial infections. The findings of the current study may help to explain the improved growth performance, feed utilization, and survival of rainbow trout observed by Staykov et al. (2007).

In the current study, culturable populations from the gastrointestinal tract ranged between 10^6 and 10^8 cfu·g^{-1}, which is within the range of values reported in previous rainbow trout investigations (Spanggaard et al., 2000; Heikkinen et al., 2006; Kim et al., 2007; Merrifield et al., 2009a,b). The intestinal microbial communities of rainbow trout are predominantly culturable, with reported culturability estimates of 50 to 90% (Spanggaard et al., 2000; Huber et al., 2004). Dominant groups in the present study were identified as belonging to the γ subclass of Proteobacteria, in particular *Aeromonas/ Vibrio* spp. and Enterobacteriaceae, which have been commonly isolated from trout (Spanggaard et al., 2000, 2001; Huber et al., 2004; Heikkinen et al., 2006; Pond et al., 2006; Kim et al., 2007; Merrifield et al., 2009a,b). The gut microbiota appeared to reflect the microbiota of the rearing water, which was dominated by *Aeromonads/ Vibrio* spp., *Pseudomonas* spp., and members of the Enterobacteriaceae. Culturable populations in MOS-supplemented groups were significantly less than the control groups (10^6 and 10^8, respectively). Mannan oligosaccharide supplementation is able to bind to certain gram-negative bacteria, preventing intestinal colonization and thus resulting in a removal mechanism of bacteria from the gut (Spring et al., 2000). Binding and removal of selected gram-negative bacteria (i.e., from the *Aeromonas/ Vibrio* spp., *Pseudomonas* spp., Enterobacteriaceae, or the unidentified gram-negative rod group) may explain the changes of viable populations observed in the present study. Large reductions of the number of these organisms were observed (i.e., several log scales less in the MOS group than the control group), but this was not always reflected in the relative proportion of total microbiota.

Differences in the relative abundance of the microbiota identified were also observed. Compared with the

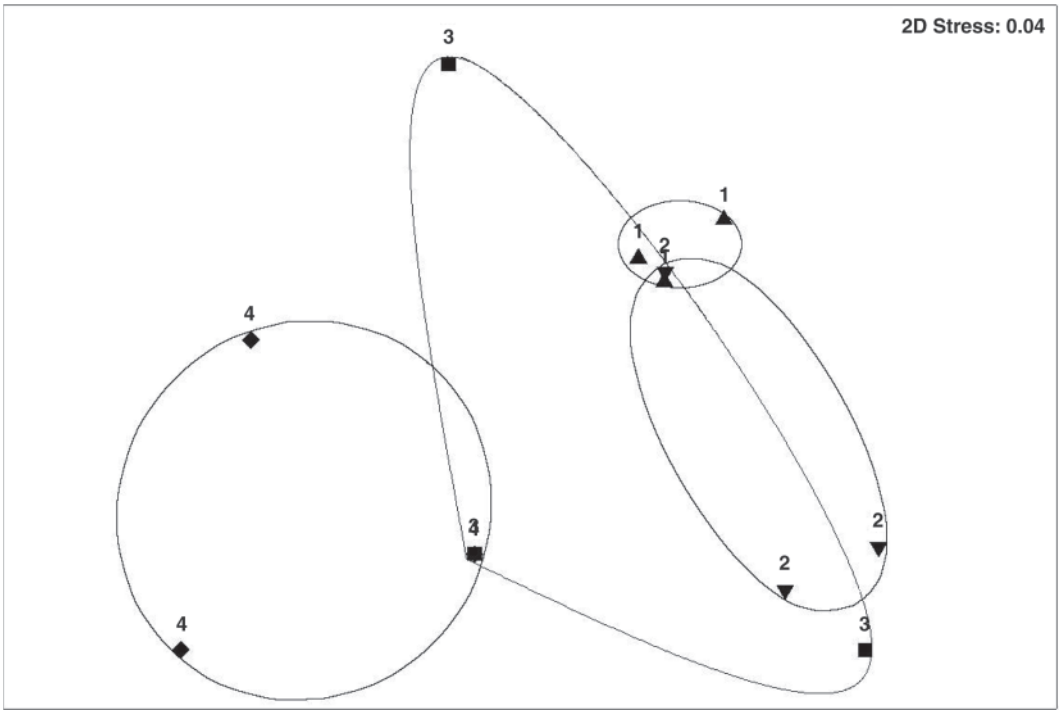

Figure 2. Nonmetric multidimensional scaling analysis plot of denaturing gradient gel electrophoresis fingerprints showing similarities between treatments. 1. Juvenile control; 2. juvenile mannan oligosaccharide (MOS); 3. subadult MOS; 4. subadult control. Clustering indicates that MOS supplementation of juvenile groups causes a shift in the bacterial community structure. Subadult plots are not closely clustered, but MOS supplementation appears to cause a shift toward the juvenile groups.

juvenile control, juvenile MOS-supplemented fish displayed a significant reduction of *Aeromonas/ Vibrio* spp. This may be of particular interest for rainbow trout aquaculture where some of the most common diseases, such as Vibriosis and furunculosis, are caused by *Vibrio* and *Aeromonas* spp. Such species include *V. anguillarum*, *V. ordalii*, *V. alginolyticus*, and *A. salmonicida,* which are often indigenous to fish and are thought to infect via the gastrointestinal tract (Austin and Austin, 1999; Ringo et al., 2007). A recent study showed that dietary MOS can provide protection against *Vibrio alginolyticus* infection in European sea bass (Torrecillas et al., 2007). The exact mechanism of protection was not clear; however, enhanced immune responses were observed, but the findings from the present study suggest that a potential direct role of MOS against *V. alginolyticus* could have been a factor.

The elevation in the relative population of *Enterococcus* spp. in the juvenile MOS group is also interesting because lactic acid bacteria such as *Enterococcus* spp. may be considered as beneficial bacteria. For example, probiotic administration of *Enterococcus faecium* has demonstrated an alteration of the gut microbiota of sheet fish (*Silurus glanis*) by reducing numbers of En-

Table 4. Summary of species diversity, richness, and pairwise comparisons obtained from denaturing gradient gel electrophoresis (DGGE) fingerprints (Primer analysis) of rainbow trout gut microbiota from each group[1]

			1-way analysis of similarity		
Group	Species richness	SIMPER[2] similarity %	R-value	*P*-value	Dissimilarity, %
Juvenile control	11.7	94.5			
Juvenile MOS	8.7	72.2			
Subadult MOS	12.3	54.7			
Subadult control	14	72.5			
Pairwise comparison					
A, B			0.26	0.30	25.1
A, C			0.22	0.20	38.2
A, D			1	0.10	42.8
B, C			−0.19	0.90	33.7
B, D			0.89	0.10	44.4
C, D			0.26	0.10	38.1

[1]n = 3/treatment (pooled from 9 fish). Feeding groups: A. juvenile control; B. juvenile mannan oligosaccharide (MOS); C. subadult MOS; D. subadult control.

[2]SIMPER = similarity percentages.

terobacteriaceae (including *Escherichia coli*), *Staphylococcus aureus*, and *Clostridium* spp. (Bogut et al., 2000). Subsequently growth performance was increased. The benefits of probiotic administration of *E. faecium* has also been demonstrated in European eel, *Anguilla anguilla* L. (Chang and Liu, 2002), and rainbow trout (Panigrahi et al., 2004). The reason for this relative increase is not clear; it may be a direct result of the MOS supplementation or a secondary effect produced by altering other microbial populations, thus reducing competition and antagonism, creating a more favorable environment for *Enterococcus* spp. If such microbial modulation occurs in other fish species, it may have been a contributory factor toward the enhanced intestinal epithelial morphology of cobia observed by Salze et al. (2008) and the improved soybean-meal digestibility by red drum (*Sciaenops ocellatus*) observed by Burr et al. (2008).

Diversity indices calculated from gradient gel electrophoresis analysis are an effective quantitative means of comparing microbial community profiles from different environmental samples (Eichner et al., 1999). The DGGE statistical analysis from the present study shows intestinal microbiota of the subadult and juvenile fish to be distinctly different. Presence/absence half matrix of the DGGE banding patterns showed similarity between the different feeding regimes within the same age group were relatively high (~80%). The SIMPER analysis showed that the subadult control group has the greatest species richness. Mannan oligosaccharide supplementation feeding appears to decrease the species richness in the subadult and juvenile fish. Results from nMDS confirm that dietary MOS has a considerable effect on the gut microbiota; however, this is not always seen in all fish. Plots indicate that MOS supplementation of juvenile groups causes a shift in the bacterial community structure. Subadult plots are not as closely clustered, but MOS supplementation appears to cause a shift toward the juvenile groups. Thus, MOS treatment of subadult and juvenile fish appears to have similar effects. Subadult fish were fed MOS-supplemented diets for 58 d (until approximately market BW); however, juvenile fish were fed for 111 d. The accumulated effect of continual supplementation of a subadult group may reduce the variability of the microbiota, as was evident in the juveniles. Despite the relative similarity between groups, DGGE analysis showed some bands present in the control groups that were not present in the MOS groups; therefore, we must consider the gut microbiota of the MOS groups distinctly different from the control groups. Future studies should incorporate 16S rRNA sequence analysis to identify these unique species. Despite the evident changes in populations, the spatial overlapping of each treatment demonstrates that the result of MOS addition is a subtle alteration of the microbiota as opposed to a dynamic shift.

General observations from nMDS plots, ANOSIM, and SIMPER comparing the subadult trout with the juvenile trout indicate that microbial communities in subadult trout are more diverse with reduced similarity within replicates. This would suggest that the complexity of the intestinal microbial community structure may be related to duration of exposure to environmental conditions and microbiota.

In conclusion, dietary MOS alters the characterization of rainbow trout gut microbiota and enhances gut morphology by increasing absorptive surface area and improving microvilli structure. Continuing from Torrecillas et al. (2007), further work should be undertaken to assess the possible potential of MOS against other *Aeromonas* and *Vibrio* infections in challenge studies. It would also be interesting to investigate if a synergistic effect could be achieved between applying a synbiotic of MOS and *E. faecium*. A longer duration trial may reveal if the shift in microbial populations would continue indefinitely or to some specific end point.

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