RESEARCH ARTICLE

Prebiotic effects of arabinoxylan oligosaccharides on juvenile Siberian sturgeon (*Acipenser baerii*) with emphasis on the modulation of the gut microbiota using 454 pyrosequencing

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Abstract

The potential of a novel class of prebiotics, arabinoxylan oligosaccharides (AXOS), was investigated on growth performance and gut microbiota of juvenile *Acipenser baerii*. Two independent feeding trials of 10 or 12 weeks were performed with basal diets supplemented with 2% or 4% AXOS-32-0.30 (trial 1) and 2% AXOS-32-0.30 or AXOS-3-0.25 (trial 2), respectively. Growth performance was improved by feeding 2% AXOS-32-0.30 in both trials, although not significantly. Microbial community profiles were determined using 454-pyrosequencing with barcoded primers targeting the V3 region of the 16S rRNA gene. AXOS significantly affected the relative abundance of bacteria at the phylum, family, genus and species level. The consumption of 2% AXOS-32-0.30 increased the relative abundance of *Eubacteriaceae*, *Clostridiaceae*, *Streptococcaceae* and *Lactobacillaceae*, while the abundance of *Bacillaceae* was greater in response to 4% AXOS-32-0.30 and 2% AXOS-3-0.25. The abundance of *Lactobacillus* spp. and *Lactococcus lactis* was greater after 2% AXOS-32-0.30 intake. Redundancy analysis showed a distinct and significant clustering of the gut microbiota of individuals consuming an AXOS diet. In both trials, concentration of acetate, butyrate and total short-chain fatty acids (SCFAs) increased in fish fed 2% AXOS-32-0.30. Our data demonstrate a shift in the hindgut microbiome of fish consuming different preparation of AXOS, with potential application as prebiotics.

Introduction

Gut microbiota play an important role in the health of aquatic vertebrates (Gomez & Balcazar, 2008). There is increasing evidence that the microbial community of the intestinal tract provides both nutritional benefits and protection against pathogens in fish (Ringø *et al.*, 2010). Manipulation of the gut flora is therefore an important mechanism to achieve an increased feed efficiency, growth and health of fish in aquaculture. Certain substrates such as dietary fibres are able to shift the intestinal bacterial composition towards health-promoting bacteria and are now a central issue in nutritional applications (Gibson & Roberfroid, 1995). They are commonly referred to as prebiotics. Prebiotics are not digested by enzymes of the upper gastrointestinal tract, but instead selectively fermented by some types of intestinal bacteria in the large intestine, as shown in humans (Gibson & Roberfroid, 1995) and animals (Flickinger & Fahey, 2002; Patterson & Burkholder, 2003), and they alter the gut bacterial composition (Gibson & Roberfroid, 1995). This shift in intestinal microbiota is associated with a higher production of short-chain fatty acids (SCFAs) in the gastrointestinal tract, promotes a better gut development and possibly improves overall host health (Van Loo, 2004; Kolida & Gibson, 2011). Feed additives such as prebiotics are

considered to be a promising alternative for antibiotics in aquaculture, as the traditional use of antibiotics in aquaculture has been criticized because of the potential development of antibiotic-resistant bacteria, the destruction of natural microbial communities in the aquatic environment and the suppression of the immune system of cultured species (Ringø *et al.*, 2010). Enhancement of fish growth and survival, abundance of beneficial gut bacteria, immune responses and reduction in susceptibility to pathogenic bacteria have been reported for some fish species upon ingestion of prebiotics (Ringø *et al.*, 2010).

Despite the importance of sturgeon in aquaculture, data on the impact of prebiotics are limited. Positive impacts of prebiotics such as inulin, oligofructose, Immunoster, Immunowall and Immunogen have been reported for some sturgeon species on growth performance, innate immunity, haematological and serum biochemical parameters, microbial fermentation and autochthonous intestinal microbiota (Jeney & Jeney, 2002; Mahious *et al.*, 2006; Mohajer Esterabadi *et al.*, 2010; Hoseinifar *et al.*, 2011a, b; Ta'ati *et al.*, 2011). In this study, we investigated the prebiotic effects of arabinoxylan oligosaccharides (AXOS), a new class of candidate prebiotics, on juvenile *A. baerii*. AXOS are fragmentation products of arabinoxylans (AX), which occur in the cell wall of many cereal grains, consisting of a main chain of ß-1,4-linked D-xylopyranosyl units to which O-2 and/or O-3-L-arabinofuranosyl units are linked (Swennen *et al.*, 2005, 2006; Grootaert *et al.*, 2007). AXOS preparations are obtained by chemical extraction of AX from cereal grains or bran followed by enzymatic hydrolysis of the extracted AX by specific AX-degrading enzymes (Swennen *et al.*, 2005; Grootaert *et al.*, 2007). Based on the fragmentation processes, different preparations of AXOS with varying degrees of polymerization (DP) and arabinose-to-xylose ratio (avDS) are produced. AXOS compounds are characterized by both the average DP (x) and DS (y), reflected in their denomination as AXOS-x-y. In recent years, the prebiotic potential of AXOS has been proved in some terrestrial animals and in humans (Broekaert *et al.*, 2011). Studies on rat and chicken showed that AXOS consumption was associated with multiple effects often related to health promotion, such as an increase in SCFAs and a higher concentration of *Bifidobacteria* in the gut (Courtin *et al.*, 2008a; Van Craeyveld *et al.*, 2008). The structural properties of AXOS determine to a large extent their physicochemical effects and fermentation processes (Van Laere *et al.*, 2000; Monobe *et al.*, 2008) as well as their immunological modulation (Monobe *et al.*, 2008; Geraylou *et al.*, 2012) in different organisms.

This study investigated the effects of supplementation of *i*) different concentrations (2% and 4%) and ii) different structures [high DP (AXOS-32-0.30) compared with low DP (AXOS-3-0.25)] of AXOS on *A. baerii* growth performance, gut microbiota diversity and composition, and fermentative products through two independent *in vivo* experiments. To analyse the microbiota modulation by AXOS diets, 454 high-throughput pyrosequencing was applied. Pyrosequencing allows high resolution determination of the eubacterial phylogenetic spectrum and taxonomic characterization with flexibility to analyse the microbiota at different taxonomic levels.

Materials and methods

Preparation of AXOS and experimental feed

AXOS-32-0.30 [average degree of polymerization (avDP) = 32; average degree of substitution (avDS) = 0.30] and AXOS-3-0.25 [avDP = 3; avDS = 0.25] were prepared as described earlier (Swennen *et al.*, 2006) and kindly provided by Laboratory of Food Chemistry and Biochemistry (KU Leuven, Belgium). The composition of the two AXOS preparations is summarized in Supporting Information, Table S1. Preliminary evaluation in our laboratory indicated a better performance and survival of *A. baerii* with increasing amounts of AXOS in the basal diet from 0.0 to 0.25, 0.50 up to 1.0% (Rurangwa, unpublished results, Table S2). Therefore, higher dosages of AXOS (2% and 4%) were supplemented to the fish diet in the present study. In the first trial, fish basal diet was supplemented with 3.2% or 6.4% of AXOS-32-0.30, corresponding to 2% or 4% AXOS after correction for purity. In the second trial, the different preparations of AXOS were added at the same final (corrected) concentration of 2% to the basal diet (2.5% of AXOS-3-0.25 and 3.2% of AXOS-32-0.30). In both trials, cellulose (2%) was used in the control diets as inert fibre. The basal diet for growing sturgeon (powder) was purchased from Joosen & Luyckx Aquabio (Turnhout, Belgium; Table 1). AXOS and binder (carboxymethyl cellulose, 2%) were added to the commercial feed powder, blended thoroughly in a mixer, minced and pelletized with a laboratory scale kitchen meat grinder (Kenwood) to obtain 3-mm-sized pellets. Feeds were dried overnight at 50 °C in a Heraeus oven with ventilation.

Experimental design

Two independent *in vivo* trials were conducted at the Laboratory of Aquatic Ecology, Evolution and Conservation (KU Leuven, Belgium). For both trials, tanks of 60 L supplied with water flow-through (400 mL min^{-1}) and aeration were used. Water quality parameters including dissolved oxygen (DO) and pH were measured daily, whereas nitrate, nitrite and ammonia concentrations were monitored biweekly. Nitrate, nitrite and ammonia concentrations were

Table 1. Composition of the commercial basic sturgeon grower feed and experimental diets in trials 1 and 2

	Diet type			
Ingredient	Control	AXOS-3-0.25 (2%)	AXOS-32-0.30 (2%)	AXOS-32-0.30 (4%)
Fish meal (%)	36.8	36.8	36.8	36.8
Soya (%)	16.3	16.3	16.3	16.3
Corn gluten (%)	12.7	12.7	12.7	12.7
Soybean meal (%)	8.0	8.0	8.0	8.0
Wheat flour (%)	9.6	9.6	9.6	9.6
Fish oil (%)	11.1	11.1	11.1	11.1
Vitamin/mineral premix (%)	1.5	1.5	1.5	1.5
Antioxidant (%)	0.05	0.05	0.05	0.05
Binder (carboxymethyl cellulose, %)	2.0	2.0	2.0	2.0
AXOS-3-0.25 (%)	0.0	2.5*	0.0	0.0
AXOS-32-0.30 (%)	0.0	0.0	3.2*	6.4*
Cellulose (%)	2.0	0.0	0.0	0.0
Proximate composition (dry matter)				
Crude protein (%)	45.0	–	–	–
Crude lipid (%)	20.0	–	–	–
Crude fibre (%)	1.4	–	–	–
Ash (%)	9.3	–	–	–
Starch (%)	9.5	–	–	–

*Corresponding to 2% or 4% AXOS after correction for purity.

determined using a portable Hach DR/2400 spectrophotometer. Water temperature, DO, pH, ammonia and nitrate ranged from 20.8 to 21.4 °C, 6.6 to 6.9 mg L^{-1}, 6.8 to 7.1, 0.34 to 0.39 mg L^{-1} and 9.12 to 10.53 mg L^{-1}, respectively. The light–dark cycle was fixed at 12-h light and 12-h dark. Fish were acclimatized to tank conditions for 2 weeks. Fish health status was verified by physical examination (excess of mucous secretion, normal colouration, erosion of scales or fins, skin, bulging of eyes and presence of cysts, spots or patches over the body and gills) and behavioural signs (swimming and feeding reflexes). In the first trial, juvenile sturgeons (Joosen & Luyckx, Turnhout, Belgium) of ca. 30 g were randomly distributed over nine tanks in triplicate (25 fish per tank, three tanks per treatment). The fish were fed a diet supplemented with 2% or 4% AXOS-32-0.30 at a rate of 3.0% body weight for 10 weeks using automatic feeders. For the second trial, 288 juvenile sturgeons (ca. 25 g) were distributed over 24 tanks (12 fish per tank, eight replicates per treatment), and two different preparations of AXOS, namely AXOS-32-0.30 and AXOS-3-0.25, were incorporated to the basal diet at a level of 2% for 12 weeks. The fish of this second trial were also used for an immunology study published in Geraylou *et al.* (2012). Total fish biomass in each aquarium was weighted biweekly, and the daily ration was adjusted accordingly.

Growth performance and survival

At the end of both trials, growth performance was assessed in terms of weight gain (WG), specific growth ratio (SGR) and feed conversion ratio (FCR). The calculations were performed using the following formulae: WG = 100 $(W_t - W_0)$/W0; SGR = 100 (ln W_t − ln W_0)/t; FCR = FO/$(W_t - W_0)$, where W_t is the weight of the fish at day t, W_0 is the initial weight of the fish, t is the duration of feeding (in days) and FO is amount of feed offered (g). The survival rate (%) = 100 × survival number/total number was calculated at the end of both trials.

Hindgut sampling procedure

A preliminary investigation of different sections of Siberian sturgeon's gastrointestinal tract using plating on brain heart infusion agar (BHIA, Difco) revealed that the density and diversity of cultivable bacteria in the hindgut (spiral vesicle) were significantly higher in comparison with the midgut and caecum. Therefore, the sampling was only performed from the hindgut of the fish. Twelve (four fish per replicate, trial 1) or 16 (two fish per replicate, trial 2) fish per treatment were randomly caught. Fish were killed by destructing the brain using a dissecting needle. Thereafter, fish were disinfected with 0.1% benzalkonium chloride solution and dissected with a scalpel to remove the hindgut. The hindgut was then aseptically removed and the content collected. The content of each fish hindgut was frozen at −80 °C until further analysis.

Hindgut SCFAs quantification using gas chromatography

For the analysis of SCFAs, the hindgut content of four (first trial) or five (second trial) fish were pooled, resulting in three samples per treatment for both trials. Quantitative analysis of SCFAs of hindgut samples was carried out by capillary gas chromatography. Of each pooled sample, 500 mg was brought in a centrifuge tube, and the following products were added: 0.2 mL 9.2 M sulphuric acid, 0.1 mL of 0.75% (v:v) 2-methylhexanoic acid (internal standard), 0.2 mL of 25% NaCl solution and 0.8 mL diethyl ether samples. Samples were rotated for 2 min and centrifuged at 3000 ***g*** for 3 min (Van Craeyveld *et al.*, 2008). The etheric layer containing SCFA extracts was injected to a Shimadzu GC-15A gas chromatograph equipped with a flame ionization detector and a Shimadzu C-R3A integrator. Helium was used as carrier gas. Temperatures during the process ranged from 115 to 245 °C with a linear temperature increase of 5 °C min^{-1}.

Hindgut DNA extraction and pyrosequencing

Total DNA was extracted from hindgut content using the NucleoSpin *1* Tissue kit (Macherey-Nagel) according to the manufacturer's protocol. For the 454 pyrosequencing, an amplicon library was prepared using eubacterial universal primers. Given the mean sequencing length of 400–450 bp for the 454 GS FLX Titanium platform, we composed different primer combinations targeting the variable 16S rDNA (V3) region, based on Wang & Qian (2009). The optimal primer combination should comprise a region with high genetic variability, cover *c.* 450 bp and have a high taxonomic coverage for the eubacterial community. The final primer set was chosen based on a series of *in silico* simulations investigating these characteristics and trial PCRs on test samples. The 16S rRNA gene was amplified from extracted DNA using the composite forward primer 5′-CGTATCGCCTCCCTCGCGCCATCAG MID *ACTCCTACGGGAGGCAGCAGT*-3′, where the underlined sequence is the 454 Life Sciences® primer A and in italics is the broad range bacterial primer E338F. Every amplicon library contained a multiplex identifier (MID), which is a unique 10-base code to identify the sample (18 tags in total). The reverse primer was 5′-CTATGCGCCTTGCCAGCCCGCTCAG *GGGTATCTAATCCTG*-′3, where the underlined sequence is the 454 Life Sciences® primer B and in italics is the broad range bacterial primer E797R. As sequencing was performed only in the forward direction, no MIDs were necessary within the reverse primer. Using these primers, the amplicon size was 459 bp long, had a coverage of 96.3% for the *Eubacteria* and contained the complete variable V3 region.

PCR mixture conditions were as follows: 2.5 μL 10 × PCR buffer II (Eurogentec), 1 μL $MgCl_2$ (50 mM, Eurogentec), 2.5 μL deoxyribonucleoside triphosphates (2 mM), 1.0 μL forward primer and 1.0 μL reverse primer (20 pmol μL^{-1} each), 0.2 μL Silverstar Taq DNA polymerase (2.5 U; Eurogentec) and 50 ng template DNA in a total reaction volume of 25 μL. The PCR conditions were as follows: 5 min denaturing at 95 °C followed by 30 cycles of 1 min at 95 °C (denaturation), 1 min at 55 °C (annealing) and 1 min at 72 °C (elongation), with a final extension at 72 °C for 5 min. For each sample, an independent PCR was performed. PCR products of four (first trial) or five (second trial) samples of the same treatment were pooled, resulting in three replicate samples per treatment for each trial and purified using NucleoFast (Macherey-Nagel, Germany). The 18 amplicon libraries (nine for trial 1 and nine for trial 2) were checked by agarose (1.5%) gel electrophoresis, their DNA concentration was measured using Quant-IT PicoGreen (Invitrogen), and they were then pooled in equimolar concentrations. Pyrosequencing was performed with a Roche GS FLX Titanium sequencer by Biogenomics at the Genomics Core Facility of the KU Leuven.

Sequence processing

The sequences generated by pyrosequencing were mainly analysed with the software PANGEA for the identification of operational taxonomic units (OTUs), taxonomic assignment and community comparison (Giongo *et al.*, 2010). Raw 454 FLX data were parsed using the *trim2.pl* script from the PANGEA pipeline to only keep the reads that are longer than 100 bp, without ambiguous base calls (minimum phred score 20), multiple barcode or primer motifs and without erroneous nucleotides in the barcodes.

OTU assignment, identification and classification

The reads that passed the quality check were subjected to a trimming round of primers and barcode sequences using the *barcode.pl* script from the PANGEA pipeline, resulting in 13 274 and 16 044 high-quality pyrosequencing reads for trials 1 and 2, respectively. Sequences were analysed by Megablast (part of the BLAST package at NCBI; Zhang *et al.*, 2000) using a standalone BLAST to attach taxonomic information of the closest bacterial relative to each sequence. Therefore, we used the best match in a modified bacterial RDP-II database prepared using TaxCollector (http://www.microgator.org) containing 164 517 almost full-length 16S rRNA gene sequences. Classified sequences by Megablast were clustered into OTUs based on the relatedness of classification at similarity levels of 0.80 for domain/phylum, 0.90 for class/order/family, 0.95 for genus, and 0.97 and 0.99 for species level. Sequences that remained unclassified by Megablast were clustered into OTUs by CD-HIT (Li & Godzik, 2006) based on the relatedness of the sequences as mentioned above.

For the diversity analyses, the number of sequences per barcode (and thus per sample) was normalized to a same value using the script *selector.pl* of the PANGEA pipeline, which selects at random a same number of sequences of each barcode file. This number is based on the sample with the smallest number of reads. This resulted in subsets of 614 (first trial) or 998 (second trial) randomly selected sequences for each sample.

Community analysis

To determine whether the type and/or concentration of AXOS as an explanatory variable could significantly explain the variation in hindgut bacterial community of Siberian sturgeon, a canonical redundancy analysis (RDA) was performed based on the relative abundances of OTUs

(normalized data set) detected by 454 pyrosequencing using Canoco 4.5 (Biometris – Plant Research International, the Netherlands), and its significancy was assessed by 999 Monte Carlo permutation tests. All analyses had samples as scaling focus, and species data were Hellinger-transformed (Legendre & Gallagher, 2001). Detrended correspondence analysis of the transformed OTU abundance data showed a length of gradient < 3.0, suggesting a linear species response (Ramette, 2007). Because the patterns were identical using the 97% and 99% sequence similarity level for species level, we only show the results based on the 99% sequence similarity level.

Calculation of diversity indices and rarefaction analysis

Taxonomic richness was calculated by summing the number of OTUs per replicate, including singlet OTUs (OTUs with only a single observation in a single replicate), based on the normalized data set. Shannon diversity was calculated following $H' = -\sum p_i \ln(p_i)$, where p_i is the proportion of taxon i using the script *Shannon.pl* of the PANGEA pipeline. A 99% similarity level value was used for assigning an OTU. Rarefaction curves were computed for the separate replicates using the rarefaction command (source: http://www.jennajacobs.org/R/rarefaction.txt) in the statistical package R (version 2.14.1).

Statistical analyses

The overall effect of treatment on the relative abundance of OTUs was assessed at phylum, family, genus and species level. To limit multiple testing at the species level, only OTUs that were likely positively or negatively related to one of the specific treatments were selected for statistical analysis. This was based on the RDA sample–species diagram as visualized by the length and angle of the species arrow compared to the position of the treatment variable in the RDA diagram. For each selected OTU (99% sequence identity level), it was first determined whether the relative abundances were significantly different from zero using a *t*-test, after which the effect of treatment on relative abundance was assessed by analysis of variance (ANOVA) in (version 11) for nonzero relative abundances. Relative abundances were $\log_2$-transformed, and a threshold of $P < 0.05$ was used to determine statistical significance.

Results

Fish performance

The effects of the different concentrations and structures of AXOS on WG, SGR, FCR and survival of sturgeon juveniles are summarized in Table 2. In the first trial, the group of fish fed for 10 weeks with 2% AXOS-32-0.30 had the highest WG and SGR, although not significantly different from the control and 4% AXOS treatments ($P > 0.05$). No significant change was observed for WG and SGR in fish fed 4% AXOS-32-0.30 compared with the control treatment. The FCR for sturgeons fed 2% AXOS-32-0.30 was significantly different from that of fish fed the control diet. The survival rate of fish fed 2% AXOS-32-0.3 was significantly higher (95%) compared with fish fed 4% AXOS-32-0.30 ($P < 0.05$). In the second trial, the fish fed AXOS-32-0.30 obtained a 464 % increase in weight in comparison with 416 % for the control. However, no significant effect on final weight, weight gain, SGR or FCR was observed. Survival was high in all treatments of this trial (98%, $P > 0.05$).

SCFAs production in the hindgut

The experimental diets supplemented with AXOS resulted in modulated fermentation in both trials (Fig. 1A and B) compared with the control diet. Acetate, propionate and butyrate were the main SCFAs produced in the hindgut of Siberian sturgeon. Acetate and propionate were the

Table 2. Zootechnical performances of *Acipenser baerii* fed a control diet or diets supplemented with different preparation of AXOS

	Trial 1			Trial 2*		
Parameter	Control	AXOS-32-0.30 (2%)	AXOS-32-0.30 (4%)	Control	AXOS-3-0.25 (2%)	AXOS-32-0.30(2%)
Initial body weight (g)	29.7 ± 1.8	29.80 ± 1.0	28.9 ± 1.11	25.8 ± 0.8	25.6 ± 0.7	26.2 ± 1.0
Final body weight (g)	65.1 ± 14.0	73.3 ± 5.5	58.9 ± 2.4	133.8 ± 5.8	134.9 ± 6.7	145.1 ± 7.5
Weight gain rate (%)	119.8 ± 15.2	146 ± 19.6	103 ± 3.0	416 ± 24.0	426 ± 25.9	464 ± 29.0
Specific growth ratio	1.08 ± 0.26	1.31 ± 0	1.05 ± 0.19	1.90 ± 0.02	1.99 ± 0.06	2.07 ± 0.05
Feed conversion ratio	2.40 ± 0.70^{a}	1.97 ± 0.34^{b}	2.59 ± 0.40^{ab}	1.48 ± 0.05	1.51 ± 0.02	1.47 ± 0.05
Survival (%)	90.0 ± 3.4^{ab}	95.0 ± 2.4^{b}	88.0 ± 4.5^{a}	100 ± 0.0	98.0 ± 2.2	98.0 ± 1.4

Values are means ± SE (3 or 8 replicates (tank) in first and second trial, respectively). Values with a different superscript in the same line are significantly different ($P < 0.05$).

*Growth parameters of trial 2 were taken from Geraylou *et al.* (2012).

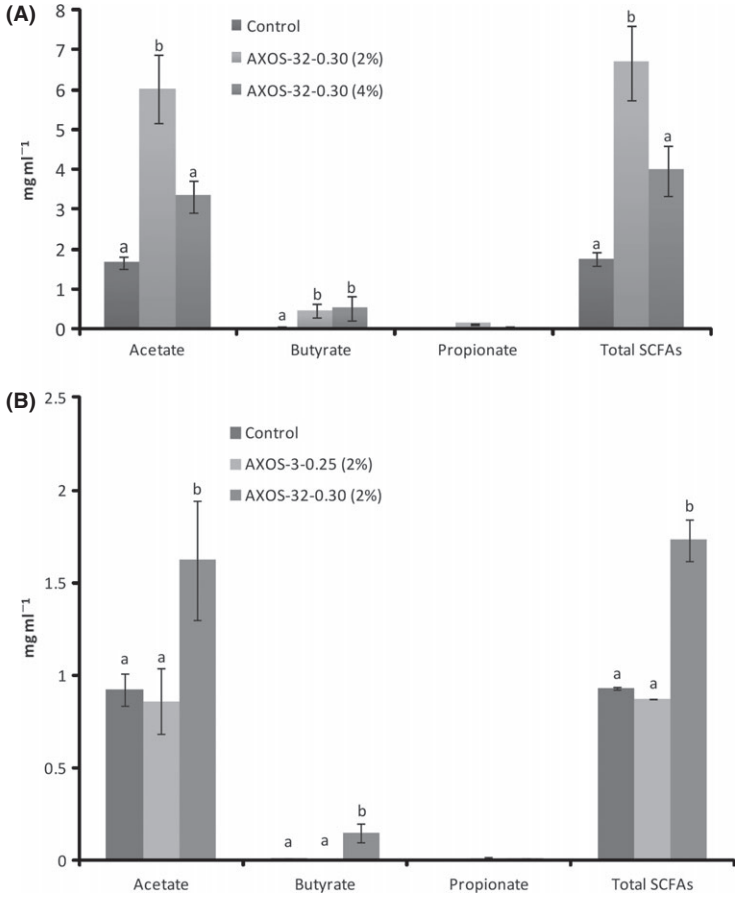

Fig. 1. Short-chain fatty acid production of hindgut microbiota in *Acipenser baerii* fed (A) a control diet or diet supplemented with 2% or 4% AXOS-32-0.30 (trial 1); (B) a control diet or diet supplemented with 2% AXOS-32-0.30 or AXOS-3-0.25 (trial 2). Values are means ± SE (trial 1, $n = 12$, trial 2 $n = 16$). Data of trial 2 are taken from Geraylou *et al.* (2012).

and least produced SCFAs, respectively. In the first trial, supplementation of 2% AXOS-32-0.30 increased the concentration of acetate, butyrate, propionate and total amount of SCFA in hindgut of fish significantly ($P < 0.05$) compared with the control diet. Although the concentration of these organic acids also increased in the hindgut of fish fed a diet supplemented with 4% AXOS-32-0.30, this increase was not significant in comparison with the control (Fig. 1A). Similarly, AXOS-32-0.30 (2%) resulted in significantly higher concentrations of acetate, butyrate and total amount of SCFA in the hindgut of Siberian sturgeon ($P < 0.05$) in the second trial compared with the control treatment, whereas no significant difference was found in the concentration of propionate between treatments (Fig. 1B). No significant effect of supplementation of 2% AXOS-3-0.25 on hindgut SCFAs production was observed in the second trial.

Phylotype coverage and bacterial diversity in the *A. baerii* hindgut

A total of 13 274 and 16 044 high-quality pyrosequencing reads were obtained after quality control processes, covering 1279 and 1593 OTUs (99% similarity level) for the first and second trial, respectively. The number of sequences per sample varied between 614 and 3322 in the first trial and between 998 and 3475 in the second trial (Table S3). At phylum level (80%), the number of OTUs in the different treatments averaged between 8 and 29 in trial 1 and 8 and 65 in trial 2, while being between 102 and 291 (trial 1) and 124 and 462 (trial 2) at species level (99%; Table S3). In an effort to determine whether all the OTUs present in the hindgut microbiota had been recovered in the pyrosequencing data set, a rarefaction analysis per sample was performed at the 99% similarity level. As can be seen in Fig. 2A and B, the number of OTUs is still increasing with increasing number of reads, even at the highest numbers of reads.

To assess the bacterial richness and Shannon index of the hindgut community of fish treated by AXOS, the normalized number of reads was used. In the first trial, the Shannon index and richness did not change significantly in fish fed 2% or 4% AXOS-32-0.30 in comparison with the control group, while in trial 2, Shannon index and bacterial richness significantly increased in hindgut of fish fed 2% AXOS-3-0.25 (Table 3, $P < 0.05$).

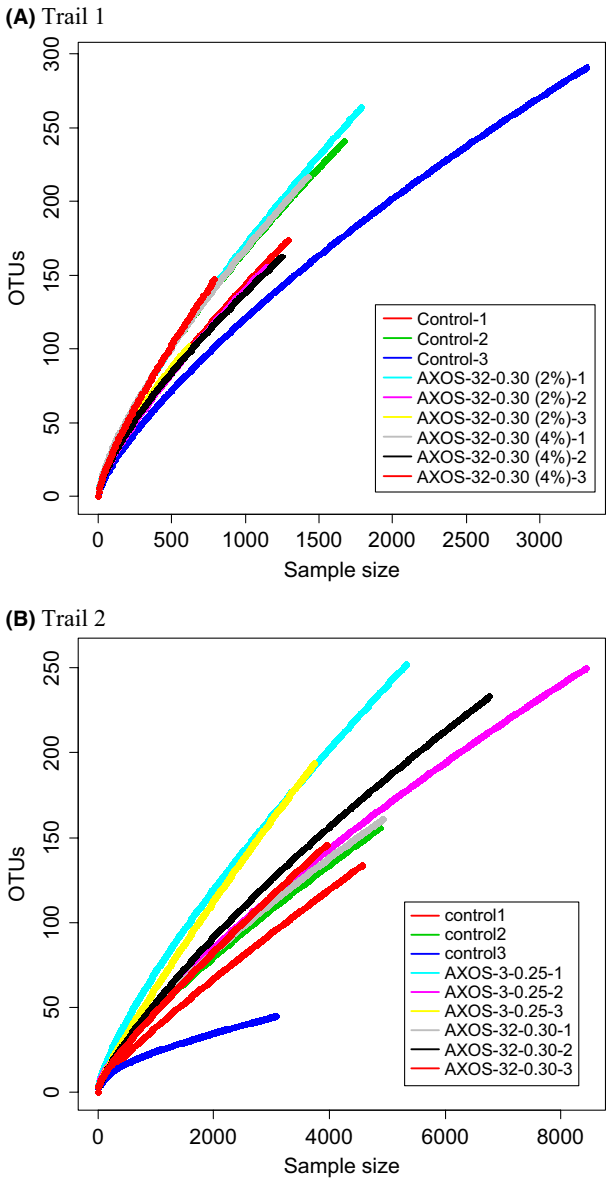

Fig. 2. Collectors curves analysis of the completeness of sampling. Repeated samples of OTU subsets were used to evaluate whether further sampling would likely yield additional taxa (rarefaction analysis), as indicated by whether the curve has reached a plateau value. The *y*-axis indicates the number of OTUs detected, and the *x*-axis indicates the number of taxa in the sequence subset analysed. Rarefaction analysis of OTUs clustered at % 99 ID across different feeding diet for *Acipenser baerii*. trial 1 (A) and trial 2 (B).

Effect of AXOS on Siberian sturgeon gut microbial community

RDA based on the relative abundance of the OTUs revealed that the intestinal community composition clustered significantly according to feed type in both trials (Fig. 3). In trial 1, the first and second constrained axes corresponded to 37.2% and 9.2% of the total variance in the bacterial community, respectively, and explained all the cumulative percentage variance of the species–environment relationship (Fig. 3A). Monte Carlo significance tests (999 unrestricted random permutations) indicated that both axes explained a significant proportion of the variation in the data ($P = 0.05$). Similarly, distinct clustering by dietary treatments was found for the second trial by RDA (Fig. 3B).The first two constrained axes explained 35% and 11.8% of the total variance in the bacterial community, respectively. Assessment of the significance of the RDA analysis by Monte Carlo permutation test indicated that the bacterial community composition was significantly related to the AXOS treatment [$P = 0.016$ (first canonical axis), $P = 0.010$ (all canonical axes)].

Table 3. Shannon index of diversity and OTU richness for the hindgut microbial communities from juvenile sturgeon fed different preparations of AXOS

Treatment		Shannon index	Richness
Trial 1	Control	2.58 ± 0.08	93.0 ± 4.2
	AXOS-32-0.30 (2%)	2.26 ± 0.29	84.6 ± 9.9
	AXOS-32-0.30 (4%)	2.78 ± 0.09	91.3 ± 4.3
Trial 2	Control	1.63 ± 0.24[a]	110.5 ± 20.3[a]
	AXOS-3-0.25 (2%)	2.12 ± 0.13[b]	154.6 ± 4.8[b]
	AXOS-32-0.30 (2%)	1.61 ± 0.16[a]	95.3 ± 9.1[a]

Values are means ± SE (12 and 16 replicates fish in the first and second trial, respectively). Values with a different superscript in the same column are significantly different ($P < 0.05$).

Phylum, class and family level

Phylogenetic analysis of the sequences using the RDP classifier identified 7 (trial 1) and 6 (trial 2) phyla in the hindgut microbial community. Approximately 2.3% and 2.5% of the 16S rRNA gene sequence reads in trial 1 and trial 2, respectively, have yet to be assigned to a phylum. Fusobacteria, Firmicutes and Proteobacteria were the most abundant phyla detected in both studies. Fusobacteria was detected as the most dominant phylum in the hindgut of Siberian sturgeon fed the control diet (57.6% and 89.9% in trial 1 and trial 2, respectively). Fusobacteriaceae was the unique family in this phylum and their relative abundances decreased in fish fed AXOS diets; however, this decrease was not statistically significant (Table S4A and B). In the first trial, the Firmicutes phylum covered *c.* 24% of the sequences in the control fish and increased up to 31% or 60% in fish fed diet supplemented with 4% or 2% AXOS-32-0.30, respectively. Clostridia and Bacilli were the most abundant classes and dominated by the Clostridiaceae, Eubacteriaceae, Streptococcaceae, Bacillaceae and Lactobacillaceae. Eubacteriaceae was the most abundant family in control fish and

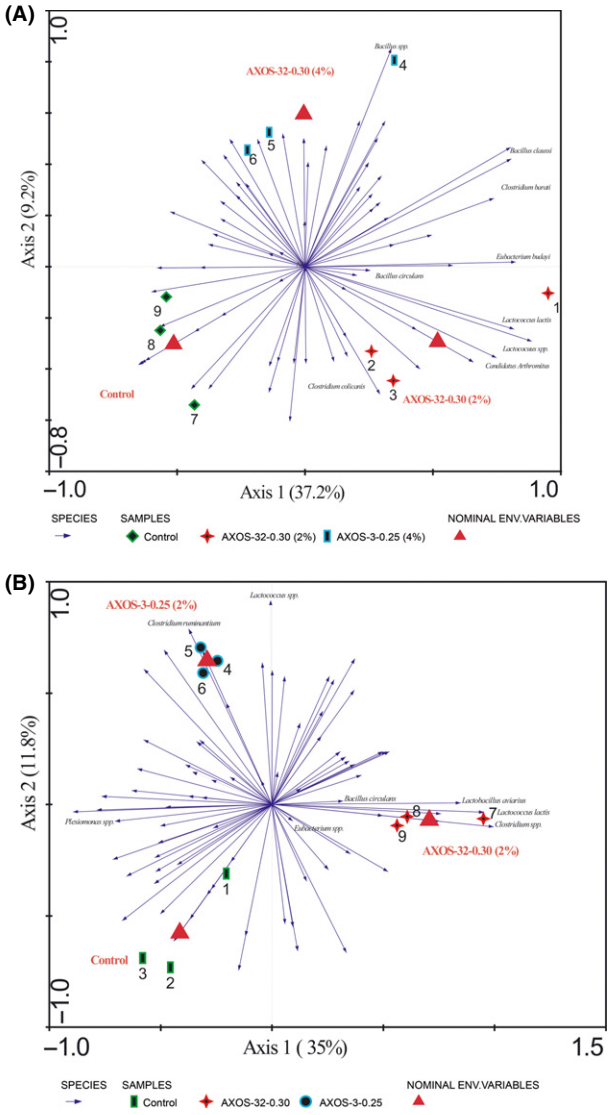

Fig. 3. Distance triplot of the RDA of Siberian sturgeon's hindgut microbiota. Nominal environmental variables: (A) control, AXOS-32-0.30 (2%) and AXOS-32-0.30 (4%) (trial 1); (B) control, AXOS-3-0.25 (2%) and AXOS-32-0.30 (2%) (trial 2). The species having significant changes in their relative abundance are labelled.

significantly increased in the group of fish fed 2% AXOS-32-0.30. The relative abundance of Clostridiaceae was higher in fish fed a diet supplemented with 2% AXOS-32-0.30 in comparison with the control and fish fed 4% AXOS-32-0.30. The Streptococcaceae and Lactobacillaceae showed a significant increase in hindgut of fish fed 2% AXOS-32-0.30. The relative abundance of Bacillaceae increased by 4% AXOS-32-0.30 (Table S4A).

In the second trial, *c.* 4% of sequences belonged to the Firmicutes phylum in the control fish. Firmicutes bacteria significantly increased in fish fed 2% of AXOS-32-0.30. The Firmicutes phylum included four main families: Clostridiaceae, Streptococcaceae, Lactobacillaceae and Bacillaceae. The relative abundance of Clostridiaceae increased in fish fed 2% AXOS-32-0.30. Interestingly, the relative abundance of Lactobacillaceae (containing a well-known probiotic species) was significantly higher ($P < 0.05$) in hindgut of fish fed 2% AXOS-32-0.30 than that in the control or fish treated with AXOS-3-0.25 (Table S4B). Similarly, the relative abundance of Streptococcaceae increased significantly in fish fed 2% AXOS-32-0.30 in second trial ($P < 0.05$). No significant change in abundance of Bacillacea was observed.

Proteobacteria was another predominant phylum in the hindgut of Siberian sturgeon. The most abundant family within the Proteobacteria was Rhodobacteraceae, but its relative abundances were not affected by the AXOS treatments. The phyla Chlamydiae, Actinobacteria and Bacteroidetes were represented at low relative abundances in the hindgut of Siberian sturgeon and were not significantly affected by the treatments (Table S4A and B).

Genus level

In the first trial, among the well-characterized genera within the phylum Firmicutes, *Eubacterium*, *Bacillus*, *Clostridium*, *Lactococcus* and *Lactobacillus* were predominant (Table S5A). The relative abundances of *Lactococcus* and *Lactobacillus* were significantly higher in the hindgut of fish fed a diet supplemented with 2% AXOS-32-0.30 ($P < 0.05$) compared with the fish fed control or 4% AXOS-32-0.30. Relative abundances of *Eubacterium* increased in the group of fish fed 2 or 4% AXOS-32-0.30. Moreover, *Bacillus* significantly increased in fish receiving 4% AXOS-32-0.30. *Bacillus*, *Candidatus Arthromitus*, *Clostridium*, *Lactobacillus* and *Lactococcus* were the dominate genera within the Firmicutes in the hindgut of Siberian sturgeon in the second trial (Table S5B). Feeding the fish by a diet supplemented with 2% AXOS-32-0.30 resulted in an increase in relative abundances of *Bacillus*, *Lactobacillus, Clostridium* and *Lactococcus*.

In both trials, the relative abundance of *Cetobacterium*, the dominant genus within the phylum Fusobacteria, decreased in fish fed AXOS diets, but these differences were not statistically significant. Similarly, the relative abundances of the genera within the phyla Proteobacteria, Chlamydiae, ctinobacteria and Bacteroidetes did not significantly respond to the AXOS treatments in both trials (Table S5A and B).

Species level

In the first trial, eight taxa differed significantly between sturgeons fed the AXOS-supplemented diets or the control diet (Table 4A). The relative abundance of *Bacillus*

clausii and *Clostridium baratii* increased significantly in fish fed AXOS diets. *Bacillus circulans* and *Bacillus clausii* had a higher relative abundance in fish fed 4% AXOS-32-0.30 than control fish. *Lactococcus lactis* and *Lactobacillus* spp. were the third and fourth most abundant taxa and had significantly greater relative abundances in fish fed 2% AXOS-32-0.30 than the control and 4% AXOS-32-0.30 treatments. Moreover, *Eubacterium budayi* and *Clostridium colicanis* had significantly higher relative abundances in the hindgut of fish fed a diet supplemented with 2% of AXOS-32-0.30 in comparison with the control (Table 4A). In the second trial, the relative

Table 4. Relative abundances (% of sequences per treatment) ± standard deviation of the most abundant bacterial OTUs in the hindgut of juvenile *Acipenser baerii* fed with diet supplemented with (A) 2% or 4 % AXOS-32-0.30 (trial 1) and (B) 2% AXOS-3-0.25 or 2% AXOS-32-0.30 (trial 2) as determined by 16S rRNA gene pyrosequencing

(A)

	% sequences per treatment		
Taxon	Control	AXOS-32-0.30 (2%)	AXOS-32-0.30 (4%)
Anoxybacillus flavithermus	0.16 ± 0.13	0.27 ± 0.16	0.92 ± 0.56
Bacillus circulans	0.76 ± 0.20^{a}	3.09 ± 0.19^{ab}	5.53 ± 0.74^{b}
Bacillus clausii	0.00 ± 0.00^{a}	0.70 ± 0.18^{b}	0.70 ± 0.33^{b}
Bacillus cereus	0.30 ± 0.05	0.05 ± 0.03	0.06 ± 0.04
Bacillus sp.	0.21 ± 0.09	0.81 ± 0.43	0.92 ± 0.63
Bacillus sp.	0.16 ± 0.07	0.65 ± 0.23	0.70 ± 0.25
Clostridium baratii	0.46 ± 0.02^{a}	1.00 ± 0.08	0.96 ± 0.10^{ab}
Clostridium beijerinckii	0.03 ± 0.02	0.10 ± 0.04	0.06 ± 0.02
Clostridium chartatabidum	0.00 ± 0.00	0.10 ± 0.00	0.10 ± 0.04
Clostridium colicanis	4.19 ± 0.53^{a}	8.04 ± 1.04^{b}	5.91 ± 0.19^{ab}
Eubacterium budayi	6.80 ± 1.45^{a}	11.2 ± 2.59^{b}	9.09 ± 0.51^{ab}
Lactococcus lactis	0.70 ± 0.45^{a}	14.6 ± 4.55^{b}	2.17 ± 1.04^{a}
Lactobacillus aviarius	0.10 ± 0.04	0.10 ± 0.09	0.00 ± 0.00
Lactobacillus sp.	0.10 ± 0.04^{a}	2.87 ± 0.70^{b}	0.32 ± 0.07^{a}
Streptococcus sp.	0.16 ± 0.00	0.10 ± 0.04	0.10 ± 0.04
Cetobacterium somerae	56.1 ± 3.11	25.0 ± 8.69	30.1 ± 10.16
Plesiomonas shigelloides	0.76 ± 0.50	0.00 ± 0.0	0.00 ± 0.00
Candidatus arthromitus	4.39 ± 1.61^{a}	9.39 ± 2.39^{ab}	3.36 ± 0.57^{b}
Mycoplana sp.	0.00 ± 0.00	0.05 ± 0.04	0.21 ± 0.11
Catellibacterium sp.	0.10 ± 0.04	0.00 ± 0.00	0.06 ± 0.02
Alpha proteobacterium	0.21 ± 0.18	0.10 ± 0.04	0.21 ± 0.09
Rhodobacter sp.	6.56 ± 2.43	3.96 ± 1.21	14.82 ± 7.08

(B)

	% sequences per treatment		
Taxon	Control	AXOS-3-0.25 (2%)	AXOS-32-0.30 (2%)
Bacillus circulans	0.10 ± 0.07^{a}	0.23 ± 0.15^{a}	0.93 ± 0.16^{b}
Bacillus sp.	0.10 ± 0.06	0.16 ± 0.10	0.10 ± 0.07
Clostridium chartatabidum	0.05 ± 0.03	0.05 ± 0.03	0.03 ± 0.0
Clostridium ruminantium	0.10 ± 0.00^{a}	1.40 ± 0.14^{b}	0.13 ± 0.09^{a}
Clostridium colicanis	3.25 ± 0.39	2.53 ± 0.26	3.10 ± 0.04
Clostridium sp.	0.45 ± 0.25^{a}	0.66 ± 0.29^{a}	11.59 ± 3.3^{b}
Lactobacillus aviarius	0.15 ± 0.03^{a}	0.23 ± 0.07^{ab}	0.90 ± 0.20^{b}
Lactococcus sp.	0.00 ± 0.00^{a}	0.66 ± 0.07^{ab}	0.16 ± 0.07^{b}
Lactococcus lactis	0.03 ± 0.00^{a}	0.16 ± 0.13^{a}	11.69 ± 3.09^{b}
Candidatus Arthromitus sp.	0.65 ± 0.46	3.60 ± 2.91	0.10 ± 0.08
Cetobacterium somerae	76.05 ± 4.22	62.09 ± 4.18	57.51 ± 7.05
Plesiomonas shigelloides	0.65 ± 0.25	0.80 ± 0.19	0.10 ± 0.04
Plesiomonas sp.	5.21 ± 1.07^{a}	7.98 ± 2.76^{a}	1.23 ± 0.11^{b}
Plesiomonas sp.	0.70 ± 0.28	0.43 ± 0.19	0.10 ± 0.00
Catellibacterium sp.	0.50 ± 0.21	0.86 ± 0.29	1.30 ± 0.45
Fusobacterium sp.	0.10 ± 0.07	0.03 ± 0.02	0.03 ± 0.00
Unidentified Eubacterium clone	0.05 ± 0.03^{a}	0.43 ± 0.10^{b}	1.03 ± 0.02^{b}

abundance of *Bacillus circulans, Clostridium* spp., *Lactococcus lactis*, *Lactobacillus aviaries* and an unidentified *Eubacterium* increased significantly in the gut of fish fed a diet supplemented with 2% AXOS-32-0.30 ($P < 0.05$), whereas *Clostridium ruminantium* and *Lactococcus* sp. had higher abundance in the gut of fish fed 2% AXOS-3-0.25 (Table 4B). Besides, the relative abundance of *Plesiomonas* spp. showed a significant decrease in fish fed 2% AXOS-32-0.30.

Discussion

This study was conducted to determine the impact of two preparations and concentrations of AXOS on the growth performance and gut microbial community composition of Siberian sturgeon. Based on our results, growth performance of fish in terms of WG and SGR survival were not significantly affected in both trials. In the first trial, incorporation of 4% AXOS-32-0.30 resulted in a significantly lower survival than the 2% AXOS-32-0.30 diet ($P < 0.05$). Harmful effects of high levels of inulin have been documented by Olsen *et al.* (2001). They reported that inclusion of 15 % inulin in diet of Arctic charr (*Salvelinus alpines*) resulted in the damaging of enterocytes. The authors speculated that inulin that cannot be degraded by the cells would accumulate to an extent at which cell function became impaired.

The effects of prebiotics on the growth performance of sturgeon species reported in previous studies are ambiguous. Negative and positive impacts of dietary addition of prebiotics have been reported. Similar to our results, no significant differences in growth performance were observed for the Gulf sturgeon (*Acipenser oxyrinchus desotoi*) fed mannanoligosaccharides (Pryor *et al.*, 2003), Beluga sturgeon (*Huso huso*) fed oligofructose (Hoseinifar *et al.*, 2011a) and Siberian sturgeon fed poly-α-hydroxybutyrate (Najdegerami *et al.*, 2011). Furthermore, Akrami & Hajimoradloo (2009) observed a negative relationship between the supplementation level of inulin on growth performance and feed utilization in Beluga sturgeon. In contrast, Ta'ati *et al.* (2011) and Mohajer Esterabadi *et al.* (2010) observed that the prebiotics of Immunoster, Immunowall and Immunogen (0.5% or 1.0%) significantly increased the growth performance of juvenile Beluga sturgeon (*Huso huso*).

The growth performance parameters varied largely within treatments in the present study. Increasing the number of replicates from three (trial 1) to eight aquaria (second trial) did not reduce the interindividual variation in the growth parameters in the present study. The higher growth rate observed in the second trial compared with the first trial could be linked to the lower densities of juvenile sturgeon in the second trial. Density-dependent growth has been documented for several fish (including sturgeon species) (Bohlin *et al.*, 1994; Sullivan *et al.*, 2003; Yang *et al.*, 2011). Growth suppression caused by higher stocking density might be related to both crowding stress and a decline in peripheral circulating levels of thyroid hormones and be associated with the reduction in food consumption and decrease in food conversion efficiency (Li *et al.*, 2012).

Changes induced by specific AXOS preparations on the hindgut microbiota community were analysed using a high-throughput pyrosequencing approach to allow the comparison of communities at various taxonomic levels and the identification of the taxa. The use of pyrosequencing methods for the identification of fish gut microbiota is still very limited (Roeselers *et al.*, 2011; Wu *et al.*, 2012; Van Kessel *et al.*, 2012; Desai *et al.*, 2012). Our results indicate a higher bacterial richness compared with studies using the conventional DNA fingerprinting (Pryor *et al.*, 2003). The microbial diversity in the Siberian sturgeon samples even at 99% similarity (102–291 or 124–462 OTUs in the first or second trial, respectively) is considerably below that that in Grass Carp samples (259–2773, 97% similarity) (Wu *et al.*, 2012), whereas it is close to the microbial diversity found for zebrafish (178–259) (Roeselers *et al.*, 2011). These differences may be attributed to many variables, including host genotype (McKnite *et al.*, 2012), geography (Ley *et al.*, 2005) and diet (Tannock *et al.*, 2004). At the technical level, differences among experiments in sampling method, DNA extraction and PCR amplification method, the variable regions of the 16S rRNA gene analysed and sequence processing techniques can also play a role (Wu *et al.*, 2010). In our study, the rarefaction analyses indicated a steep increase in OTUs, showing that the full extent of the bacterial diversity of the *A. baerii* hindgut has not yet been surveyed. This suggests that a large number of OTUs remain undetected, and a higher sequencing effort may be required to detect the additional phylotypes and the full diversity. Although diversity estimates based on observed OTU may differ among host species, a general observation is that even pyrosequencing-based data sets with high read counts do not yet fully cover the complete diversity in gut environments. This is demonstrated in our study and elsewhere (Eckburg *et al.*, 2005; Middelbos *et al.*, 2010).

The bacterial species richness and Shannon diversity index did not differ in hindgut of fish fed AXOS-32-0.30 and the control group in trials 1 and 2 (2% or 4%). This indicates that incorporation of AXOS-32-0.30 in the *A. baerii* diet does not alter overall hindgut bacterial diversity. In contrast, feeding Siberian sturgeon with 2% AXOS-3-0.25 resulted in an increase in bacterial diversity (bacterial species richness and Shannon diversity index)

in hindgut. From a health perspective, an increase in bacterial diversity in the gut may have positive effects at various levels. Several studies suggest that higher species richness in gut microbiota is associated with a decreased ability of pathogens to colonize the gut (Dillon *et al.*, 2005). Bacterial species may facilitate each other's growth, perhaps due to more effective resource use when more species are present, leaving less niche space for invaders, including pathogens. Ringø and Olsen (Ringø & Olsen, 1999) reported that the amount of carbohydrates included in the diet of Arctic charr (*Salvelinus alpinus*) affected the diversity of the microbial population, but not the total numbers of bacteria isolated. In another study, Najdegerami *et al.* (2011) indicated that a well-balanced diet with poly-β-hydroxybutyrate (PHB) increased the bacterial species richness in juvenile Siberian sturgeon and that there was a relationship between high growth rate, low mortality and bacterial diversity in the GI tract of fish fed 2% PHB. In contrast, Bakke-McKellep *et al.* (2007) showed that inclusion of inulin in the diet reduced the diversity of gut microbiota in the teleost Atlantic salmon (*Salmo salar* L.). In the present study, the higher hindgut bacterial diversity in fish fed 2% AXOS-3-0.25 did not affect the growth performance of Siberian sturgeon juveniles. Moreover, although incorporation of 2% AXOS-32-0.30 in the fish diet did not significantly affect the overall diversity of the hindgut microbiota, it resulted in higher relative abundances of beneficial bacteria including *Clostridium* and LAB, potentially leaving less niche space for other bacteria. These data thus support the idea that prebiotics can have a positive influence on growth or metabolism of bacteria without significant changes in bacterial diversity of the gut, but simply by a shift in the community composition itself, towards health-promoting bacteria.

The community composition of the gut microbiota was significantly affected by diet type. The RDA analyses support the hypothesis that the overall composition of the gut microbial communities in Siberian sturgeon fed diets including the AXOS candidate prebiotic is distinctly different from that of the control diet and interestingly also from each other. Fusobacteria showed the highest relative abundance among the phyla, most notably in the control treatment in both trials. This phylum was almost exclusively represented by *Cetobacterium* at the genus level with a high similarity to *Cetobacterium somerae* at species level. *Cetobacterium somerae* has been previously reported in the intestinal tract of freshwater fish (Tsuchiya *et al.*, 2008) and was also detected in Siberian sturgeon hindgut at relatively high abundances using denaturing gradient gel electrophoresis (Geraylou *et al.*, 2012). AXOS diets significantly shifted the Firmicutes/Fusobacteria ratio in favour of Firmicutes. The dynamics within the Firmicutes over the different treatments were very complex, with changes in composition at the family level (Table S4A and B) but also on lower taxon levels (Table S5A and B; Table 4A and B). The relative abundance of *Eubacterium*, *Clostridium*, *Lactobacillus*, *Bacillus* and *Lactococcus* increased significantly in the hindgut of fish fed different preparations of AXOS. Incorporation of higher avDP of AXOS in fish diet resulted in the highest significant increase in relative abundance of certain species of LAB, *Clostridium* and *Bacillus*. Different studies have shown that incorporation of different preparations of AXOS resulted in an increase in relative abundance or metabolic rate of beneficial bacteria such as *Bifidobacterium* in chicken (Courtin *et al.*, 2008a, b), humans (Cloetens *et al.*, 2010) and porcine faeces (*in vitro*) (Van Laere *et al.*, 2000) and *Lactobacillus* in humans (*in vitro*) (Moura *et al.*, 2007; Grootaert *et al.*, 2009), Siberian sturgeon hindgut (*in vitro*) (Rurangwa *et al.*, 2009), as well as *Bacteroides* and *Clostridium* sp. (nonpathogenic) in humans (*in vitro*) (Sanchez *et al.*, 2009). The presence of *Bifidobacterium* has been reported in some fish species (Vlková *et al.*, 2012), but to our knowledge, there is no evidence yet for the presence of *Bifidobacterium* in the gut of *A. baerii* and other species of sturgeon. Our data confirm this, as no reads related to *Bifidobacterium* were detected in the hindgut of Siberian sturgeon in all treatments despite the reports that supplementation of AXOS resulted in an increase in the growth of *Bifidobacterium* in other organisms (Van Laere *et al.*, 2000; Courtin *et al.*, 2008a, b; Cloetens *et al.*, 2010). *Lactobacilli*, *Bifidobacteria*, *Enterococci*, *Bacteroides*, *Prevotella*, *Clostridia*, *Ruminococcus*, *Eubacterium* and *Lactococcus lactis* are all able to break down the AX molecule by different enzymes including endo-1, 4-β-xylanases, α-L-arabinofuranosidases, β-xylosidases, α-glucuronidases and ferulic acid esterases (Grootaert *et al.*, 2007). The increase in relative abundance of some of these bacteria (*Lactobacillus*, *Clostridium*, *Eubacterium* and *Lactococcus lactis*) following the AXOS diets in the present study is thus very likely directly related to the presence of AX molecules in the hindgut.

Both the average degree of avDS and the avDP influence the growth of intestinal microbiota and the fermentability of AXOS (Grootaert *et al.*, 2007; Moura *et al.*, 2007; Van Craeyveld *et al.*, 2008). In our study, the two AXOS preparations had a similar avDS but different avDP. The observed differences in impact of AXOS treatment may therefore be associated with differences in the avDP. Van Craeyveld *et al.* (2008) reported that mainly the avDP, but not or only to a lesser extent the avDS of AXOS preparations determines the effects they produce in the intestine of rats. While we did not evaluate the influence of differences in avDS, our results confirm that differences in avDP lead to a large influence on the prebiotic effects.

Functional foods such as prebiotics that target the colon and affect the internal environment and bacterial community composition enhance the concentration of SCFAs such as acetate, propionate and butyrate. SCFAs, the main acidic products of bacterial fermentation, contribute towards a low colonic pH, have a direct inhibitory activity towards important gastrointestinal pathogens (Gibson, 2004) and provide a major source of useful energy and nutrients for the host and energy for the colonocytes (McNeil, 1984). Studies on the prebiotic properties of different preparations of AXOS have indicated that acetate, propionate and butyrate are the main fermentation products (Kabel *et al.*, 2002; Hughes *et al.*, 2007). This has been confirmed in the present study, as AXOS administration significantly increased SCFA concentration in the hindgut of Siberian sturgeon. The concentrations of acetate, butyrate and total SCFAs in fish fed AXOS-32-0.30 (2%) were significantly higher than those in the AXOS-3-0.25 (2%, trial 2), AXOS-32-0.30 (4%, trial 1) and control treatments. Acetate was the dominant SCFA in all treatments. This high proportion of acetate was also reported for the intestinal microbiota of various fish species (Clements & Choat, 1995; Mahious *et al.*, 2006). The higher production of acetate in fish fed AXOS-32-0.30 (2%) could be explained by the increasing relative abundance of acetate producers such as *Clostridium* spp. and LAB in the hindgut of sturgeon species fed this AXOS diet. These results are in agreement with data obtained *in vitro* by Rurangwa *et al.* (2009). These authors found that LAB and *Bacillus* spp. inoculated with AXOS (avDP = 30) produced mainly acetate as SCFA, while propionate, butyrate and branched acid production remained very low.

Butyrate is another major SCFA produced by the intestinal microbiota. Butyrate-producing bacteria are limited to some genera: sugar fermenters, such as *Clostridium* spp., *Eubacterium* spp., *Fusobacterium* spp. and *Butyrivibrio* spp., and acid utilizers (Holdeman *et al.*, 1977). Butyrate production can also be stimulated by the administration of LAB (Sakata *et al.*, 1999). Lactic acid bacteria do not directly enhance butyrate production because they produce lactate and acetate rather than butyrate (Cummings & Macfarlane, 1991). Lactate is a typical intermediate product that will be further metabolized by bacteria to acetate, propionate and/or n-butyrate in the large intestine (Ushida *et al.*, 2002). To our knowledge, lactic acid utilizers have not been found yet in the gut of fish. In the present study, butyrate production increased significantly by diets containing 2% AXOS-32-0.30. In the first trial, *Eubacterium* spp. and *Clostridium* spp. could have been the butyrate-producing bacteria, eventually stimulated by the increase in LAB. Similarly in the second trial, the abundance of *Clostridium* spp. and LAB increased by dietary AXOS-32-0.30. Lower amount of *Eubacterium* was detected in the hindgut of *A. baerii* fed an AXOS or control diet in the second trial in comparison to the first trial. This difference between initial gut microbiota composition of both trials could be due to various factors. Several parameters including genetic, nutritional and environmental factors could affect the gut microbiota composition of the fish. The juvenile Siberian sturgeons for the two trials were obtained from the same farm and cultivated in the same conditions. However, they belonged to two different batches, and differences in gut microbiota composition could be related to genetic differences. Nevertheless, the results of both trials show that AXOS supplementation increased the relative abundance of SCFA-producing bacteria, this way very likely promoting hindgut fermentation and SCFA production, as observed.

Health-promoting effects of AX have already been proven in humans (Garcia *et al.*, 2007), rat and mice (Lu *et al.*, 2000; Ogawa *et al.*, 2011). It has also been shown that AX with a long chain has the strongest impact (Monobe *et al.*, 2008). Similarly, the immunological results of our previous study (sampled from trial 2) indicated that the effects of AXOS on the innate immune response were more pronounced with the AXOS compound presenting a longer avDP (Geraylou *et al.*, 2012). These immunomodulatory effects of AXOS could potentially be attributed to a higher production of SCFAs s in the hindgut, which could then be taken up by the fish. Alternatively, the positive effects of AXOS could be due to the stimulation of LAB bacterial growth in the hindgut, which results in a higher availability of the cell wall components of these LAB that have immunostimulatory properties (Bricknell & Dalmo, 2005).

In conclusion, analysis of the bacterial 16S rRNA gene clearly demonstrated a significant impact of AXOS on the composition of the gut bacterial microbiome. The consumption of 2% AXOS-32-0.30 led to beneficial shifts in gut microbiota, primarily in the phylum Firmicutes, and higher concentrations of SCFAs. The impact of AXOS on Siberian sturgeon is dose dependent, as supplementation with 4% of AXOS-32-0.30 did not lead to the same responses. Dietary supplementation of AXOS with a lower degree of polymerization (AXOS-3-0.25, trial 2) increased the species richness in hindgut microbiota, but had lower impacts on SCFA concentration and fish growth. Therefore, the prebiotic potential of AXOS strongly depended on the avDP. The health-promoting effects of AXOS consumption, resulting from the changes in relative abundances of LAB and Clostridium, could be used to prevent certain diseases and thus support the potential application of these oligosaccharides as prebiotics in sturgeon nutrition. However, as AXOS is a new class of candidate prebiotics, further research is needed to better understand the pathways and mechanisms of its immunostimulatory impacts.

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Supporting Information

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

Table S1. Characteristics of AXOS preparations.
Table S2. Zootechnical performances of Siberian sturgeon fed a control diet or diets supplemented with AXOS.
Table S3. Number of obtained sequences (# reads) per replicate for each treatment, and number of OTUs per taxonomic level detected in the bacterial hindgut community of *Acipenser baerii* fed with a specific feed preparation for the two trials (non-normalized dataset).
Table S4A. Relative abundances (% of sequences per treatment) ± standard deviation of the most abundant bacterial phyla and families in the hindgut of juvenile *Acipenser baerii* fed with diet supplemented with 2% or 4 % AXOS-32-0.30 (trial 1) as determined by 16S rRNA gene pyrosequencing.
Table S4B. Relative abundances (% of sequences per treatment) ± standard deviation of the most abundant bacterial phyla and families in the hindgut of juvenile *Acipenser baerii* fed with diet supplemented with 2% AXOS-3-0.25 or 2% AXOS-32-0.30 (trial 2) as determined by 16S rRNA gene pyrosequencing.
Table S5A. Relative abundances (% of sequences per treatment) ± standard deviation of the most abundant bacterial genera in the hindgut of juvenile *Acipenser baerii* fed with diet supplemented with 2% or 4 % AXOS-32-0.30 (trial 1) as determined by 16S rRNA gene pyrosequencing.
Table S5B. Relative abundances (% of sequences per sample) ± standard deviation of the most abundant bacterial genera in the hindgut of juvenile *Acipenser baerii* fed with diet supplemented with 2% AXOS-32-0.30 or 2% AXOS-32-0.30 (trial 2) as determined by 16S rRNA gene pyrosequencing.