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Cell wall fractions from *Methylococcus capsulatus* prevent soybean meal-induced enteritis in Atlantic salmon (*Salmo salar*)

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ABSTRACT

Plant based feed ingredients pose a risk for developing intestinal inflammatory diseases in fish. The salmon is in particular sensitive to solvent extracted soybean meal (SBM). Recent studies have shown that dietary inclusion of a bacterial meal (BM) grown on natural gas containing mainly *Methylococcus capsulatus* can prevent SBM-induced inflammation. The present study aimed to identify BM fractions and products that have this beneficial effect. A fish meal (FM) based diet, a diet with 200 g kg^{-1} SBM, and six diets with 200 g kg^{-1} SBM in combination with basic BM, autolyzed BM (AUT), permeate (PER) or retentate (RET) from filtration of AUT, nucleic acid reduced *M. capsulatus* (MCap), and *M. capsulatus* grown on methanol (MeOH) were made for this study. The inclusion of the BM products were equivalent to 150 g kg^{-1} basic BM. The diets were fed for 4 weeks to triplicate tanks of juvenile Atlantic salmon (*Salmo salar*) reared in salt water. No significant ($P < 0.05$) differences were found for feed intake and growth among fish fed the experimental diets. Morphometric measurements of the length of proliferating cell nuclear antigen (PCNA) stained cells in distal intestinal tissue revealed that all BM products except the PER provided significant protection against SBM-induced enteritis. The effective component in the BM products was present in the retentate that mainly consisted of large molecules and water insoluble components. Nucleic acids and small water soluble molecules did not seem to provide any protective effect.

1. Introduction

Intestinal inflammatory disease is a major concern when plant based ingredients are used in formulated diets for fish. Salmonids are especially sensitive towards solvent extracted soybean meal (SBM) (Baeverfjord and Krogdahl, 1996; Ingh et al., 1991), and intestinal inflammatory changes have been found in Atlantic salmon when SBM constitutes as little as 7.6% of the total diet (Krogdahl et al., 2003). Saponins in combination with at least one more yet unidentified component appear to initiate the plant-induced inflammation (Bureau et al., 1998; Knudsen et al., 2008). The mechanisms involve an increased intestinal permeability (Nordrum et al., 2000), which likely expose the otherwise shielded basolateral surfaces of the intestinal epithelial cells to inflammation-inducing microbial or food-derived factors present in the intestinal lumen.

Recent studies in our group have shown that inclusion of a bacterial meal (BM) grown on natural gas in extruded salmon diets can prevent development of SBM-induced enteritis (Romarheim et al., 2011). The BM contains mainly (>90%) the gram-negative methanotrophic bacterium *Methylococcus capsulatus* (Bath), and the meal has approximately 95% dry matter, and 70% crude protein and 10% crude fat on an *as is* basis after drying. Strains of the heterotrophic bacteria *Aneurinibacillus* sp., *Brevibacillus agri* and *Ralstonia* sp. are also present to facilitate the growth of *M. capsulatus* by removal of organic carbon from the bioreactor (Bothe et al., 2002). *M. capsulatus* (Bath) is a Type I thermophilic bacterium originally isolated from the hot springs in Bath, England. The bacteria utilize natural gas as carbon and energy source through a series of oxidation steps; methane is oxidized to methanol, and then to formaldehyde, formic acid and finally CO_2 (Hanson and Hanson, 1996).

The full sequencing of the *M. capsulatus* genome (Ward et al., 2004) and proteomic analyses (Berven et al., 2006) have unveiled numerous proteins with both known and yet unknown functions. The bacterium has an outer membrane containing a large number of proteins such as c-type heme proteins, lipoproteins, polypeptides, and lipopolysaccharides (LPS) that play a role in the interaction with the growth medium. These membrane components are central for the nutritional value of BM as a feed ingredient. Increased intestinal weight has been shown in some studies, but no intestinal malfunction has been demonstrated (Aas et al., 2006; Romarheim et al., 2011; Storebakken et al., 2004). The BM is approved as a feeding stuff in the EU (Commission regulation (EU) No 575/2011).

Whether the preventative effect of BM against SBM-induced enteritis is caused by *M. capsulatus*, the helper bacteria, or by a combination of them, remains to be elucidated. The basic BM grown on natural gas,

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and a number of down-stream products such as autolyzed, hydrolyzed and nucleic acid reduced BM have proven to be excellent protein sources for fish and monogastric animals as reviewed by Øverland et al. (2010). The potential preventive effect on SBM-induced enteritis by down-stream products derived from BM, or BM grown on other substrates than natural gas, has not been studied yet. The aim of the present experiment was therefore to identify inflammation-preventing BM fractions and products in Atlantic salmon and thus close in on the mechanisms behind the protective effect.

2. Materials and methods

2.1. Bacterial products and diets

Six bacterial products were prepared: basic BM, autolyzed BM (AUT), permeate (PER) and retentate (RET) from AUT, RNA reduced monoculture of *M. capsulatus* (MCap) grown on methane, and BM grown on methanol (MeOH). The basic BM and AUT were produced by aerobe fermentation of liquid natural gas (LNG; 99% methane) in a semi industrial scale loop fermentor. The bacterial biomass was sterilized by short-time heat treatment at 140 °C, spray dried, and pelleted to obtain a non-dusty biomass (basic BM). AUT was produced by high pressure homogenization, in which the bacterial biomass was subjected to a pressure drop to open the cell walls, followed by 4 h of incubation at 50 to 55 °C to activate and allow the endogenous enzymes to digest cellular components before spray drying. The AUT was the starting point for making PER and RET. Dried AUT was diluted with tap water (100 g L^{-1} water) and filtered through a hollow fiber cartridge with 0.2 μm pores, 1 mm lumen diameter and 30 cm fiber length (GE Healthcare; model no. CFP-2-E-5A) mounted on an Amicon ProFlux M12 crossflow microfiltration system at 20 °C. The PER was collected continuously throughout the filtration process, whereas the RET fraction was washed with 3 volumes of water before collection at the end. The filtration yielded a water soluble and low molecular weight permeate, and a retentate consisting mainly of insoluble molecules and macromolecules that did not pass through the filter, e.g. lipids, larger proteins and cell wall fragments. The PER was pre-dried at 40 °C in a rotational evaporator before both the PER and RET were freeze dried. MCap was made from a *M. capsulatus* monoculture produced in a 15 L bioreactor with pure methane gas as carbon and energy source. Biomass was continuously harvested from the fermentor, and the nucleic acid content was reduced according to the method described by Larsen and Joergensen (1996). In brief, the method includes a 10 s heat shock at 90 °C, pH adjustment to between 7.0 and 7.5, and incubation for 20 min at 60 °C. This procedure led to a MCap with only 15.6 g kg^{-1} nucleic acids (Table 1). The nucleic acid reduced biomass was dried by evaporation followed by freeze drying. MeOH was grown on methanol as carbon source instead of natural gas in a laboratory fermentor and freeze dried.

Eight diets were formulated (Table 2). A diet with 710 g kg^{-1} FM (FM diet) served as a negative control and a diet with 200 g kg^{-1} SBM (SBM diet) substituting FM served as a positive control for SBM-induced enteritis. The remaining diets were made by partly substituting FM in the SBM diet with 150 g kg^{-1} BM (BM diet), 150 g kg^{-1} AUT (AUT diet), 72 g kg^{-1} PER (PER diet), 78 g kg^{-1} RET (RET diet), 150 g kg^{-1} MET (MCap diet), or 150 g kg^{-1} MeOH (MeOH diet). Filtration of AUT gave 52% PER and 48% RET, and the inclusion levels of the bacterial products thus represented the same amount of initial biomass in all diets. A study by Romarheim et al. (2013) showed that Atlantic salmon fed a diet with both 20% SBM and 20% basic BM did not develop SBM-induced enteritis, whereas signs of intestinal inflammation were seen when the BM inclusion was reduced to 15%. The sub-marginal inclusion of 15% BM products was therefore applied in the present experiment to explore differences among the products. The feed ingredients were mixed, and the dough was squeezed through a pasta machine (Italgi P35 SP) with a 3 mm die and rotating cutting knives at the end of the die. Gelatin (75 g kg^{-1} diet) dissolved in heated water (250 g kg^{-1} diet) was used to ensure proper binding of the feed pellets. The feed pellets were dried at 55 °C in a heat chamber to 92–94% dry matter and stored cold until feeding.

Table 1
Individual nucleobases and calculated total nucleic acid content in the bacterial meal products.

	Bacterial meal (BM)	Autolysate (AUT)	Permeate (PER)	Retentate (RET)	Nucleic acid reduced *M. capsulatus* (MCap)	Methanol grown BM (MeOH)
Nucleobases, g kg^{-1} (as is)						
Adenine	7.9	5.1	2.5	7.7	0.8	6.5
Cytosine	7.7	7.1	4.4	10.2	2.2	6.4
Guanine	13.1	13.0	10.1	16.4	1.9	10.5
Thymine	1.2	1.3	0.6	2.2	0.9	1.4
Uracil	5.0	7.0	8.7	5.3	0.3	3.8
Calculated total nucleic acids, g kg^{-1}	88.2	85.5	68.1	105.3	15.6	72.3

2.2. Fish, rearing conditions and sampling

All diets were fed to triplicate fish tanks with Atlantic salmon (*Salmo salar*) for 4 weeks at the Nofima research station at Sunndalsøra, Norway. Salmon with a mean initial weight of 107 g were randomly allocated into tanks (20 fish $tank^{-1}$) with seawater at full salinity (7.5 °C;

Table 2
Ingredients and chemical composition of the experimental diets.

	FM diet	SBM diet	BM diet	AUT diet	PER diet	RET diet	MCap diet	MeOH diet	
Ingredients, g kg^{-1} *as is*									
Fish meal (FM)	710	510	350	350	433	427	350	350	
Soybean meal (SBM)[a]	–	200	200	200	200	200	200	200	
Bacterial meal (BM)	–	–	150	–	–	–	–		
BM autolysate (AUT)	–	–	–	150	–	–	–	–	
BM permeate (PER)	–	–	–	–	72	–	–	–	
BM retentate (RET)	–	–	–	–	–	78	–	–	
Nucleic acid reduced *M. capsulatus* (MCap)	–	–	–	–	–	–	150	–	
Methanol grown BM (MeOH)	–	–	–	–	–	–	–	150	
Gelatin[b]	75	75	75	75	75	75	75	75	
Potato starch[c]	75	75	75	75	75	75	75	75	
Fish oil	135	135	145	145	140	140	145	145	
Vitamin/mineral premix[d]	5	5	5	5	5	5	5	5	
Analyzed chemical composition, g kg^{-1} DM									
Crude protein	600	561	546	542	557	545	546	519	
Crude lipid		204	192	215	228	193	208	198	209
Total ash	120	100	80	90	100	90	80	80	

[a] Denofa, Fredrikstad, Norway.
[b] Rousselot SAS, Courbevoie, France.
[c] Lyckeby Culinar AB, Fjälkinge, Sweden.
[d] Vitamin and mineral premix provided (per kg diet): all-*trans* retinyl acetate, 860 μg; cholecalciferol, 37.5 μg; D,L-α-tocopherol acetate, 200 mg; menadione, 10 mg; thiamin, 15 mg; riboflavin, 25 mg; nicotinic acid, 75 mg; pantothenic acid, 30 mg; pyridoxine, 15 mg; folic acid, 5 mg; cyanocobalamin, 20 μg; ascorbyl monophosphate, 125 mg; biotin, 0.25 mg; Ca, 1.1 g; $ZnSO_4$, 296 mg; $MnSO_4$, 41 mg; $CuSO_4$, 13 mg; $CoSO_4$, 2.6 mg; CaI_2, 3.5 mg; astaxanthin, 175 mg.