

Article

Short-Term Dietary Inclusion of *Methylococcus capsulatus*-Derived Single-Cell Protein Modulates Growth and Immune-Gene Expression in Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

Hannah Malene Jensen ^{1,2,*} , Kaan Kumas ² , Per Walter Kania ² , Kurt Buchmann ² , Eleni Ntokou ¹ 
and Nana Wentzel Thorninger ¹ 

¹ Unibio A/S, Langebjerg 1, DK-4000 Roskilde, Denmark; en@unibiogroup.com (E.N.)

² Laboratory of Aquatic Pathobiology, Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbojlen 7, DK-1870 Copenhagen, Denmark; kumas.kaan@sund.ku.dk (K.K.); pwk@sund.ku.dk (P.W.K.); kub@sund.ku.dk (K.B.)

* Correspondence: hmje@sund.ku.dk; Tel.: +45-3112-6828

Abstract

We here report immunomodulatory effects of feeding with microbial single-cell protein (SCP from *Methylococcus capsulatus*) on female rainbow trout (*Oncorhynchus mykiss*) following replacement of conventional feed proteins in a 30-day feeding period. Conventional protein ingredients (soy protein concentrate, fishmeal (FM), wheat gluten meal and corn gluten meal) were partially replaced with microbial SCP at graded inclusion levels (0, 4, 6 and 8% SCP). Growth performance parameters, such as final body weight (FBW) and final fish length (FL), were elevated at 6% SCP inclusion. In the present investigation we found that immune gene expression in the liver and spleen was regulated following feeding microbial SCP in a concentration-dependent way. Feed with 4% SCP inclusion induced a significant upregulation of the hepatic expression of IL-10 ($p < 0.05$), whereas splenic expression of IL-6 and Cathelicidin-2 genes were downregulated. No significant differences were observed at 6% SCP inclusion. At 8% SCP inclusion, splenic expression of multiple immune-related genes, including those encoding Cathelicidin-2, CD8, IgM, IL-6, and SAA, was significantly downregulated, along with hepatic expression of Cathelicidin-1 ($p < 0.05$). Although immune gene expression was downregulated at 8% SCP inclusion, growth performance was not adversely affected. These findings indicate that up to 8% of conventional feed protein can be replaced with microbial SCP without compromising growth performance, although immune gene regulation varies depending on tissue and inclusion level. Gene regulation and health effects induced by feeding rainbow trout with high microbial SCP inclusion feed and over longer time periods should be further investigated. For this purpose, we recommend studies on functional immune parameters such as vaccine responsiveness and antibody production.

Keywords: *Methylococcus capsulatus*; immune response; gene expression; growth

Key Contribution: Dietary inclusion of microbial single-cell protein induced only minor modulation of immune parameters, with more pronounced responses observed in the spleen than in the liver.



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1. Introduction

Fish aquaculture is regarded as one of the most sustainable methods for producing animal protein, primarily due to a superior feed conversion efficiency in fish compared to terrestrial livestock production. In intensive farming systems using commercial feed types, typical feed conversion ratios (FCRs) are about 6.0–10.0 for beef cattle, 2.7–5.0 for pigs, 1.7–2.0 for chickens, and 1.0–2.4 for farmed fish and shrimp [1]. As the global population continues to grow, demand for animal protein is increasing, which places a severe pressure on environmental resources [2,3]. The low FCR of farmed fish makes fish aquaculture well-suited to meet this rising demand for a rapidly growing global population [3,4]. However, reliance on conventional protein sources, such as fishmeal (FM) and soy protein, in the aquaculture feed industry, may in some cases challenge ecological stability. Thus, fishmeal production, derived from wild-capture fisheries, contributes to overfishing, stock depletion, and marine ecosystem disruption [5], while soy-based alternatives are frequently linked to deforestation, land-use change, and elevated greenhouse gas emissions [6,7]. These issues emphasize the urgent need for development of sustainable protein alternatives, which can reduce the ecological footprint of aquafeed production and ensure long-term food security.

Microbial single-cell proteins (SCPs), particularly those derived from methanotrophic bacteria, such as *Methylococcus capsulatus*, which use methane as a sole carbon and energy source, represent a novel and planet-friendly type of raw material for aquaculture feed production [8]. This approach decouples protein production from marine and terrestrial resources and aligns with circular economy principles by leveraging methane, a potent greenhouse gas, into a valuable feed ingredient. Novel feed ingredients must, however, be critically risk-assessed to secure the health of the aquacultured fish. Numerous studies have been conducted on various aspects of physiological performance following feeding [9–13], but very few studies [14,15] have investigated the levels of expression of immune-relevant genes during dietary trials and these have focused on fish species such as tilapia and largemouth bass, respectively. One study by Ruiz et al. [16] suggested that a moderate level of dietary SCP from *M. capsulatus* may confer some protection and decrease mortality of rainbow trout following exposure to the bacterial pathogen *Aeromonas salmonicida*. However, immune mechanisms involved in such SCP-associated protection were not investigated. We have therefore analyzed the expression of a wide array of immune-relevant genes covering innate and adaptive immune factors in rainbow trout following feeding with SCP derived from methanotrophic bacteria at different inclusion levels. For the study, we used microbial SCP as a partial replacement for conventional protein ingredients (soy protein concentrate, FM, wheat gluten meal, and corn gluten meal) in compound diets for juvenile rainbow trout (*Oncorhynchus mykiss*). Along with the assessment of potential immune regulations, we evaluated growth performance and feed utilization.

2. Materials and Methods

2.1. Ethics Statement

The study was designed to minimize stress and handling, with animal welfare prioritized throughout the experimental period. All procedures were conducted in accordance with institutional and national guidelines for the care and use of experimental animals and run under license 2024-15-0201-01697 issued by the Danish Experimental Animal Inspectorate under the Ministry of Food, Agriculture and Fisheries of Denmark.

2.2. Experimental Animals

A total of 1200 certified virus-free, female rainbow trout (*O. mykiss*) were hatched and reared at a certified pathogen-free hatchery (Aqua Baltic, Nexø, Denmark). The virus-free status is confirmed by routine sampling twice a year (Danish Food Administration, Ministry

of Food, Agriculture and Fisheries). The fish were randomly divided into twelve tanks (100 fish in each) with an initial tank weight of 3687–4294 g, or a mean initial body weight (IBW) of $39.73 \text{ g} \pm 2.03$ (S.D.). Sample size was based on previous studies and standard practice for nutritional feeding trials in rainbow trout, balancing statistical requirements with available experimental facilities and animal welfare considerations. Group allocation was known to all personnel at all stages of the experiment, including allocation, conduct, outcome assessment, and data analysis. Fish were acclimatized in the tanks for 14 days prior to the feeding experiment.

2.3. Rearing Conditions

Prior to and throughout the feeding trial, experimental fish were maintained in 700-L tanks supplied with aerated, recirculated municipal water at $12.47 \text{ }^{\circ}\text{C} \pm 1.11$ S.D., with dissolved oxygen levels of $9.02 \text{ mg/L} \pm 0.19$ S.D. (oxygen saturation: $84.52\% \pm 1.06$ S.D.). Water flow was maintained at 500 L/hour. Fish were maintained under continuous light throughout the experiment.

Feeding

The fish biomass in each tank was calculated, and the amount of feed was adjusted weekly. All fish tanks were offered 2.0% of their biomass daily by automatic feeders. The biomass was measured on day 0 and day 30 to secure an initial and final total body weight for each tank. Throughout the trial, the fish were fed their respective experimental diets, and no other treatments or interventions were performed until the trial was terminated.

2.4. Diets

The microbial SCP (Uniprotein[®], Unibio A/S, Roskilde, Denmark) is derived from the methanotrophic, gram-negative bacterium *M. capsulatus* (Bath), which uses methane gas as its carbon source, leading to the production of biomass with a high protein content. Production is achieved by aerobic fermentation using the bacterial strains *M. capsulatus* (Bath), *Ralstonia* sp., *Aneurinibacillus danicus* and *Bacillus firmus*. Four experimental diets (2 mm pellet size) with microbial SCP inclusion levels of 0, 4, 6 and 8% were manufactured by the Danish Technological Institute (DTI, Sønder Stenderup, Denmark). Diets were formulated to be isoproteic and isoenergetic. The non-protein nitrogen content was calculated to account for 0.22%, 0.33%, and 0.44% of the 4%, 6% and 8% SCP diets, respectively. The diets were formulated with SCP replacing various protein sources, reflecting a typical commercial feed formulation (Table 1). The feeding experiment was conducted for 30 days using triplicate tanks (in total, 12 tanks). The four experimental diets are referred to as 0%, 4%, 6%, and 8% SCP throughout the paper.

Table 1. Diet composition. Formulation and calculated proximate composition of the four experimental diets.

Diet Ingredients		Diets			
		0%	4%	6%	8%
Soy protein concentrate ¹	%	29.86	28.43	27.72	27.00
Wheat grain, standard ²	%	15.40	15.47	15.51	15.54
Fishmeal ³	%	13.00	12.00	11.50	11.00
Wheat gluten meal ⁴	%	13.00	12.50	12.25	12.00
Corn gluten meal ⁵	%	10.00	9.00	8.50	8.00
SCP ⁶	%	0.00	4.00	6.00	8.00
Canola oil (rapeseed) ⁷	%	12.67	12.43	12.31	12.19

Table 1. Cont.

Diet Ingredients		Diets			
		0%	4%	6%	8%
Fish oil ⁸	%	6.44	6.55	6.61	6.67
Monoammonium phosphate ⁹	%	1.64	1.65	1.65	1.65
Vitamins premix ¹⁰	%	0.50	0.50	0.50	0.50
Minerals premix ¹¹	%	0.20	0.20	0.20	0.20
Choline chloride, 60% choline ¹²	%	0.17	0.16	0.15	0.15
DL-Methionine ¹³	%	0.12	0.11	0.10	0.10
Astaxanthin supplement (10% astaxanthin) ¹⁴	%	0.05	0.05	0.05	0.05
L-Lysine HCL ¹⁵	%	0.04	0.02	0.02	0.01
Nutritional composition					
Dry matter	%	95.00	95.00	95.00	95.00
Crude protein	%	45.50	45.70	45.79	45.89
Crude lipids	%	22.00	22.00	22.00	22.00
Crude fiber	%	1.83	1.77	1.74	1.71
Crude ash	%	5.55	5.36	5.27	5.17
Carbohydrates, calculated ¹⁶	%	21.95	21.95	21.94	21.94
Starch	%	12.48	12.36	12.30	12.24
Gross energy (MJ/kg), calculated ¹⁷	MJ/kg DM	23.20	23.25	23.27	23.29

¹ Crude protein 62.0%, Crude lipid 1.57%, Sementes Selecta S/A, Goiania, Brazil. ² Crude protein 12.6%, crude lipids 1.7%, Roland Mills United GmbH, Germany. ³ Crude protein 69.5%, crude lipid 12.1%, Köster Marine Proteins GmbH, Hamburg, Germany. ⁴ Crude protein 78.2%, fat 2.4%, Kröner-Stärke GmbH, Ibbenbüren, Germany. ⁵ Crude protein 59.9%, crude lipids 1.6% Roquette, Lestrem, France. ⁶ Crude protein 68.45%, crude lipids 8.3%, Uniprotein®, Unibio A/S, Roskilde, Denmark. ⁷ Brökelmann & Co, Oelmühle GmbH + Co, Germany. ⁸ Fóðurverksmiðjan Laxá hf, Akureyri, Iceland. ⁹ Yara GmbH & Co. KG, Dülmen, Germany. ¹⁰ Vitamin premix for aqua meeting requirements of fish as specified by NRC [17], VDS NV, Deerlijk, Belgium. ¹¹ Mineral premix for aqua meeting requirements of fish as specified by NRC [17], VDS NV, Deerlijk, Belgium. ¹² Impextraco NV, Belgium, Kolvit®, 60 CC. ¹³ MetAMINO DL-methionine, 99%, Evonic Industries AG, Essen, Germany. ¹⁴ Carophyll Pink 10%, DSM-Firmenich, Maastricht, NL. ¹⁵ L-lysine hydrochloride 98.5%, Meihua holdings group Co., Ltd., China. ¹⁶ Calculated as carbohydrates = 100 – moisture (%), crude protein (%), crude fat (%), crude ash (%). ¹⁷ Calculated as GE = (protein (%) × 23.6 MJ/kg + crude fat (%) × 39.5 MJ/kg + carbohydrates (%) × 17.2 MJ/kg)/100 [17].

2.5. Sample Collection

At day 30, 10 fish/tank ($n = 30$ per diet) were euthanized in tricaine methanesulfonate (MS-222) (cat. no. A5040, Merck Life Science ApS, Denmark) for subsequent determination of final body weight (FBW), final fish length (FL), spleen weight, liver weight and for calculation of hepatosomatic index (HSI) and spleen somatic index (SSI).

An additional 10 fish/tank ($n = 30$ per diet) were euthanized by an overdose of MS-222 (300 mg/L). Final body weight and final fish length were measured before liver and spleen samples were taken from each fish and preserved in RNAlater (cat. no. R0901, Merck Life Science ApS, Søborg, Denmark) in RNase-free tubes. The tissue samples were incubated for 24 h at 4 °C and subsequently stored at −20 °C until further processing for gene expression studies. This resulted in 20 measurements of FBW and FL per tank, and 10 tissue samples per tank for gene expression analysis.

2.6. RNA Isolation and cDNA Synthesis

RNA purification and cDNA synthesis were performed as described by Polke-Pedersen et al. [18], with some modifications. The purified RNA was stored at −80 °C until cDNA synthesis in a T100 Thermocycler (Bio-Rad Laboratories, Copenhagen, Denmark) using a 20 µL reaction volume with 1000 ng of RNA, oligo d(T)16 primer and TaqMan®

reverse transcription reagents (N8080234, Thermo Fisher Scientific, Roskilde, Denmark). Reactions were run at 25 °C for 10 min, 37 °C for 60 min and 94 °C for 5 min. The cDNA (10× diluted to 200 µL with RNase-free H₂O (10977049, Thermo Fisher Scientific, Denmark) was stored at −20 °C until further use.

2.7. Quantitative Real-Time PCR (qPCR)

Gene expression analyses were performed according to Polke-Pedersen et al. [18]. Details on primers and probes are described in Supplementary Table S1. In the current study, 24 immune-related target genes were analyzed. These included genes encoding innate immune cytokines (IFN-γ, IL-1β, IL-2, IL-4/13A, IL-6, IL-8, IL-10, IL-12, TGF-β, TNF-α), acute-phase proteins (serum amyloid A (SAA), complement C3), and antimicrobial peptides (Cathelicidin-1, Cathelicidin-2), lysozyme). Additionally, the following genes encoding adaptive immune markers were examined: T-cell receptor β (TCRβ), T-cell surface antigens (CD4, CD8, MHC I, MHC II), and immunoglobulin heavy chains (IgM, IgT, and the trout-specific IgD isotypes; IgDm and IgDs).

2.8. Calculations

The initial weight for each replicate tank was calculated by dividing the total tank biomass by the number of fish per tank ($n = 100$). The mean IBW for each dietary treatment (0%, 4%, 6% and 8% SCP) was subsequently calculated from the means of the three replicate tanks.

The FBW was calculated as the mean body weight of 20 fish sampled from each replicate tank. The mean FBW for each dietary treatment was subsequently calculated as the mean of the three replicate tanks.

The daily feed intake (DFI) for each experimental tank was calculated as the amount of feed given (g) per fish per day. The mean DFI for each dietary treatment was subsequently calculated based on the means of the three replicate tanks:

$$\text{DFI (g/fish)} = \text{dry feed fed (g)} / 100 \text{ fish} / 30 \text{ days} \quad (1)$$

The average daily gain (ADG) for each experimental tank was calculated based on 20 fish per tank using the equation below. The mean AGD for each dietary treatment was subsequently calculated based on the means of the three replicate tanks:

$$\text{ADG (g/d)} = (\text{FBW (g)} - \text{IBW (g)}) / 30 \text{ days} \quad (2)$$

The body weight gain (BWG), the weight gain rate (WGR) and the specific growth rate (SGR) were calculated based on 20 fish per tank. The means for each dietary treatment was subsequently calculated based on the means of the three replicate tanks and calculated as follows:

$$\text{BWG (g)} = \text{FBW (g)} - \text{IBW (g)} \quad (3)$$

$$\text{WGR (\%)} = 100 \times (\text{FBW (g)} - \text{IBW (g)}) / \text{IBW (g)} \quad (4)$$

$$\text{SGR (\%/day)} = 100 \times (\text{Ln (FBW (g))} - \text{Ln (IBW (g))}) / 30 \text{ days} \quad (5)$$

The condition factor (CF) for each experimental tank was calculated based on the body weight and length of 20 fish per tank. The mean CF for each dietary treatment was subsequently calculated based on the means of the three replicate tanks:

$$\text{CF (g/cm}^3\text{)} = (\text{FBW (g)} / \text{FL}^3 \text{ (cm)}) \times 100 \quad (6)$$

The feed conversion ratio (FCR) was calculated based on the total weight gain of each experimental tank. The final tank biomass was calculated as the total weight of the 20 sampled fish plus the biomass of the remaining 80 fish. The initial tank biomass was then subtracted from the final tank biomass to determine total tank weight gain. The FCR was calculated by dividing the total amount of feed provided during the 30-day feeding period by the corresponding tank weight gain. The mean FCR for each dietary treatment was subsequently calculated based on the means of the three replicate tanks:

$$\text{FCR} = \text{Feed given per tank (g)} / \text{weight gain per tank (g)} \quad (7)$$

The hepatosomatic index (HSI, %) was calculated for 10 fish from each replicate tank. The mean HSI for each dietary treatment was subsequently calculated as the mean of the three replicate tanks:

$$\text{HSI (\%)} = 100 \times (\text{liver weight (g)} / \text{FBW (g)}) \quad (8)$$

The spleen somatic index (SSI, %) was calculated for 10 fish from each replicate tank. The mean HSI for each dietary treatment was subsequently calculated as the mean of the three replicate tanks:

$$\text{SSI (\%)} = 100 \times (\text{spleen weight (g)} / \text{FBW (g)}) \quad (9)$$

2.8.1. Statistical Analysis of Performance Parameters

All statistical analyses were performed in GraphPad Prism (version 10 for Windows). The data was tested for normality using the Shapiro–Wilk test and homogeneity of variances was evaluated with the Brown–Forsythe test. Normally distributed data with equal variances were analyzed with one-way ANOVA with Tukey’s post hoc test when relevant. Non-normally distributed data with equal or unequal variances were analyzed using the Kruskal–Wallis test followed by Dunn’s post hoc test. Statistical significance was determined at a threshold of $p < 0.05$.

Final body weight, spleen weight and HSI were analyzed using the Kruskal–Wallis test, with these as response variables and experimental diet as the predictor. Pairwise differences between diets were assessed using Dunn’s post hoc test.

For each parameter, IBW, FL, CF, BWG, ADG, SGR, WGR, DFI, liver weight, SSI and FCR were analyzed using one-way ANOVA, with these as response variables and experimental diet as the predictor, respectively. If necessary, this was followed by a Tukey’s post hoc test.

2.8.2. Statistical Analysis of Gene Expression

Using the software NormFinder (version 0953) [19], the average of the three genes encoding the elongation factor (ELF) 1- α , β -actin and ARP genes was found suitable as an endogenous control (reference genes). Gene expression was assessed using the simplified $2^{-\Delta\Delta C_q}$ method [20]. All SCP diets (4%, 6% and 8%) were compared to 0% SCP control using one-way ANOVA and Dunnett’s post hoc test. Gene regulation was considered significant relative to the 0% SCP control group when a ≥ 2 -fold change in expression was observed together with a statistical significance threshold of $p < 0.05$, following the approach described by Kania and Buchmann [21], which has been commonly applied in immunological studies. Due to their exponential nature, folds are presented as geometrical means multiplied/divided by GSEM (geometric standard error of the mean); and statistical tests were performed using the $-\Delta\Delta C_q$ values as they represent the $\log_2$ transformation of the folds. When comparing exponential data, such as fold-change values,

the relative difference is expressed as a ratio by dividing one value by the other rather than by subtracting the values.

During data analysis, genes that could not be reliably quantified due to consistently high Cq values or insufficient amplification were excluded from further analysis. The criterion was at least $n \geq 3$ Cq per dietary treatment. Individual samples with no detectable amplification (no Cq value) for a specific gene were excluded from the analysis of that gene only. During tissue sampling, one liver sample from the 8% SCP group was excluded due to a handling error, resulting in a maximum sample size of $n = 29$ for hepatic gene expression analysis in this group. All other diet groups had a maximum of $n = 30$.

2.9. Study Protocol

A study protocol, including research questions, experimental design, and sampling strategy, was prepared prior to the initiation of the experiment. However, the protocol was not formally registered in a public repository.

3. Results

3.1. Performance and Organ Parameters

The growth performance of juvenile rainbow trout is shown in Table 2. We found a significantly improved FBW at 87.57 g at 6% SCP inclusion, which was 9.5% higher than the control diet ($p < 0.05$). Although BWG at 6% SCP inclusion was 19.91% higher than the control group, no overall significant effect of dietary treatment was detected by one-way ANOVA ($p = 0.0578$). However, Tukey's post hoc test indicated a significant difference between the 0% and 6% SCP groups (adjusted $p = 0.0425$). Likewise, ADG did not differ significantly among dietary treatments (one-way ANOVA, $p = 0.0578$), although Tukey's post hoc test also indicated a difference between the 0% and 6% SCP groups (adjusted $p = 0.0425$).

Table 2. The effects of the dietary microbial SCP on juvenile rainbow trout (*O. mykiss*) after 30 days of feeding. All samples from 0%, 4%, 6% and 8% diets were taken at day 30.

Parameters	SCP Inclusion Levels				<i>p</i> -Value	Adjusted Post Hoc <i>p</i> -Value
	0%	4%	6%	8%		
IBW ¹ (g)	39.86 ± 0.91	40.35 ± 1.37	39.47 ± 1.81	39.23 ± 1.10	n.s. ★	
95% CI	35.94–43.78	34.47–46.22	31.7–47.24	34.49–43.96		
FBW ² (g)	79.98 ^b ± 2.12	85.39 ^{ab} ± 1.27	87.57 ^a ± 0.79	84.56 ^{ab} ± 0.35	0.0135 ★★	0.0274
95% CI	70.88–89.08	79.94–90.85	84.15–90.99	83.07–86.06		
FL ³ (cm)	18.23 ^b ± 0.18	18.84 ^{ab} ± 0.09	18.88 ^a ± 0.10	18.78 ^{ab} ± 0.17	0.0391 ★	0.0493
95% CI	17.44–19.01	18.43–19.25	18.44–19.32	18.03–19.52		
CF ⁴ (g/cm ³)	1.32 ± 0.01	1.27 ± 0.00	1.30 ± 0.01	1.27 ± 0.03	n.s. ★	
95% CI	1.27–1.36	1.26–1.29	1.25–1.34	1.14–1.40		
BWG ⁵ (g)	40.12 ^b ± 2.51	45.05 ^{ab} ± 1.68	48.10 ^a ± 1.04	45.34 ^{ab} ± 1.18	0.0578 ★	0.0425
95% CI	29.33–50.90	37.83–52.26	43.61–52.59	40.26–50.41		
ADG ⁶ (g/d)	1.34 ^b ± 0.08	1.50 ^{ab} ± 0.06	1.60 ^a ± 0.03	1.51 ^{ab} ± 0.04	0.0578 ★	0.0424
95% CI	0.98–1.70	1.26–1.74	1.45–1.75	1.34–1.68		
SGR ⁷ (%/day)	2.28 ± 0.13	2.46 ± 0.10	2.62 ± 0.12	2.52 ± 0.10	n.s. ★	
95% CI	1.72–2.85	2.01–2.91	2.11–3.13	2.07–2.96		
WGR ⁸ (%)	100.90 ± 7.94	112.10 ± 7.13	122.60 ± 8.03	115.90 ± 6.29	n.s. ★	
95% CI	66.75–135.10	81.42–142.80	88.05–157.10	88.87–143.00		
DFI ⁹ (g/fish/day)	1.13 ± 0.03	1.14 ± 0.04	1.12 ± 0.05	1.11 ± 0.03	n.s. ★	
95% CI	1.01–1.24	0.97–1.31	0.90–1.33	0.97–1.24		

Table 2. Cont.

Parameters	SCP Inclusion Levels				<i>p</i> -Value	Adjusted Post Hoc <i>p</i> -Value
	0%	4%	6%	8%		
Liver weight (g)	1.47 ^b ± 0.04	1.62 ^{ab} ± 0.06	1.75 ^a ± 0.06	1.54 ^{ab} ± 0.08	0.0545 ★	0.0468
95% CI	1.30–1.64	1.37–1.88	1.48–2.01	1.20–1.87		
Spleen weight (g)	0.131 ^{ab} ± 0.01	0.125 ^{ab} ± 0.00	0.150 ^a ± 0.00	0.108 ^b ± 0.00	0.0014 ★★	0.0134
95% CI	0.11–0.16	0.11–0.14	0.13–0.17	0.10–0.12		
HSI ¹⁰ (%)	1.88 ± 0.09	1.99 ± 0.03	2.06 ± 0.06	1.89 ± 0.05	n.s. ★★	
95% CI	1.49–2.28	1.84–2.14	1.82–2.29	1.67–2.11		
SSI ¹¹ (%)	0.167 ^{ab} ± 0.01	0.154 ^{ab} ± 0.00	0.175 ^a ± 0.01	0.134 ^b ± 0.00	0.0222 ★	0.0209
95% CI	0.13–0.21	0.14–0.17	0.13–0.22	0.11–0.15		
FCR ¹²	0.741 ± 0.03	0.740 ± 0.03	0.723 ± 0.02	0.706 ± 0.05	n.s. ★	
95% CI	0.63–0.85	0.62–0.86	0.64–0.80	0.50–0.92		
<i>n</i> (tanks)	3	3	3	3		

Note: Data represent the mean ± SEM of triplicate groups. Values within the same row that have different superscript letters are significantly different ($p < 0.05$), whereas values without superscript letters do not differ significantly ($p > 0.05$). n.s. Not statistically significant. ★ Ordinary one-way ANOVA. ★★ Kruskal–Wallis test. ¹ Initial body weight (g). ² Final body weight (g). ³ Fish length (cm), total fish length. ⁴ Condition factor (g/cm³). ⁵ Body Weight Gain (g). ⁶ Average daily gain (g/d). ⁷ Specific growth rate (%/day). ⁸ Weight gain rate (%). ⁹ Daily feed intake (g/fish/day). ¹⁰ Hepatosomatic index (%). ¹¹ Spleen somatic index (%). ¹² Feed conversion ratio. 95% CI (confidence intervals).

No statistically significant differences were measured for FCR, CF, SGR, WGR, DFI and HSI among the four dietary treatments (Table 2).

Significant differences in spleen weights were measured among the four dietary treatments ($p < 0.05$). Mean spleen weight was elevated at 6% inclusion (0.150 g); however, at 8% inclusion, mean spleen weight declined to 0.108 g, corresponding to a 17.18% reduction relative to the control diet ($p < 0.05$). Liver weight did not differ significantly among dietary treatments (one-way ANOVA, $p = 0.0545$), although Tukey's post hoc test indicated a difference between the 0% and 6% SCP groups (adjusted $p = 0.0468$).

The hepatosomatic index (HSI) and spleen somatic index (SSI) were calculated as the relative weights of the liver and spleen to FBW, respectively. Fish fed an 8% inclusion diet exhibited a reduced SSI (0.134%), corresponding to a 20.07% decrease relative to the control diet. In contrast, no significant differences in HSI were measured among the four dietary treatments. No adverse effects were observed.

3.2. Gene Expression

Gene expression was significantly regulated in 7 out of the 24 tested immune genes compared to the control diet (0% SCP inclusion) (Figure 1). For Cathelicidin-1, we observed a downregulation in the liver at 8% SCP inclusion. For Cathelicidin-2 and IL-6, we measured a downregulation in the spleen for the 4 and 8% SCP inclusion. In the spleen, CD8, IgM, and SAA were downregulated for the 8% SCP inclusion. In the liver, IL-10 was upregulated for the 4% SCP inclusion ($p < 0.05$). Detailed results for all qPCR analyses are provided in Supplementary Table S2.

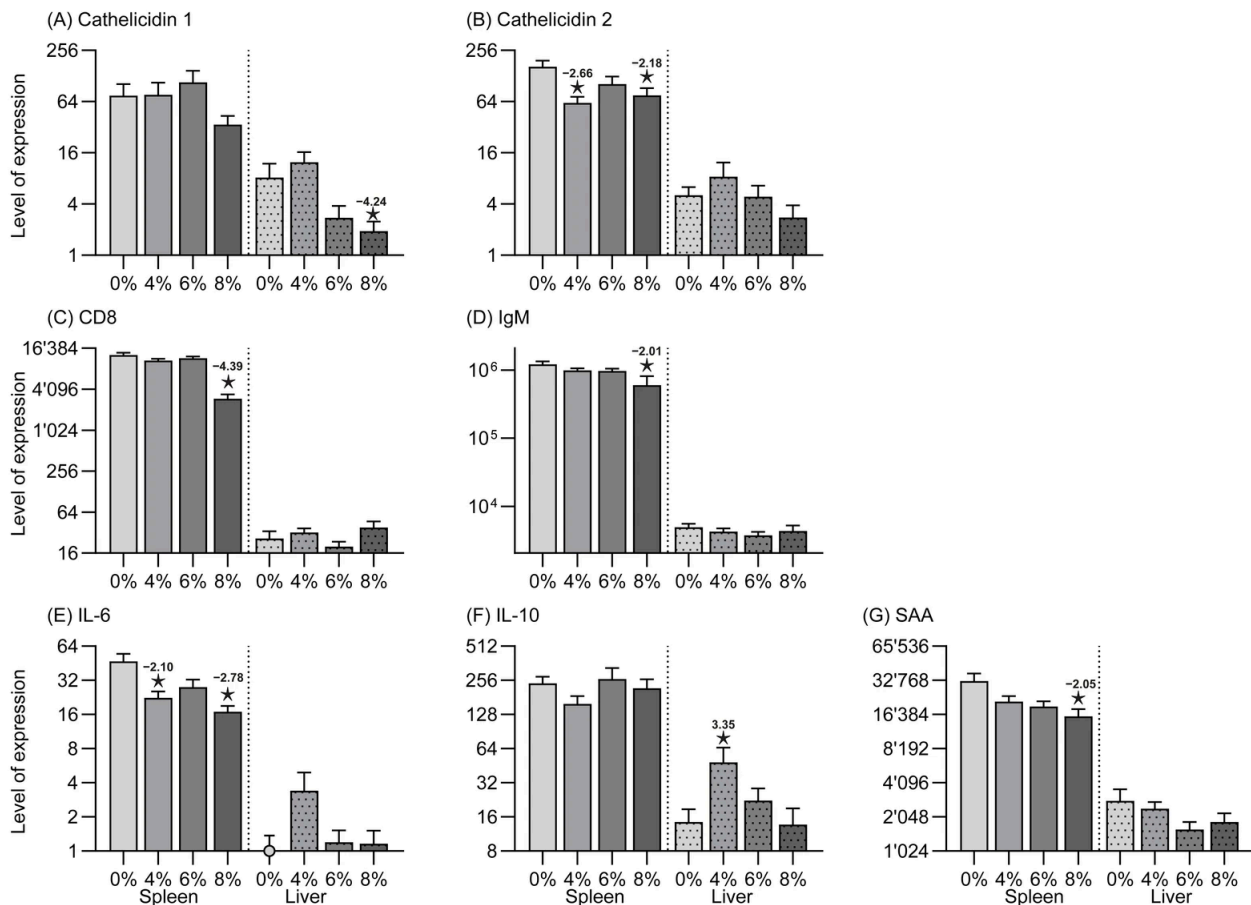


Figure 1. Gene expression levels of central immune-relevant genes in the spleen and liver (A–G) of rainbow trout fed diets containing 0%, 4%, 6%, and 8% of microbial SCP. Levels are expressed as geometric means with geometric SEM. The asterisk indicates a significant difference relative to the 0% diet. Numbers above asterisks indicate fold change relative to 0% SCP diet. Expression levels are relative to the least-expressed group (0% SCP for liver IL-6), indicated by a grey-filled circle.

4. Discussion

4.1. Organ Parameters

We placed focus on the liver due to its central physiological role. The liver in fish performs a wide range of essential functions, including metabolism, digestion, detoxification, storage, immune defense, reproduction, nitrogen catabolism and blood cell turnover [22]. The liver is a major site for the production of acute-phase proteins and complement components, and it has the capacity to initiate innate immune responses [23]. Single-cell protein effects on this organ were reported by Romarheim et al. [24], who found that microbial meal from *M. capsulatus* increased liver and gut weights in Atlantic salmon. They attributed this to the bioactive composition of SCP, including nucleotides and phospholipids, which may stimulate tissue growth and immune modulation. In the present study, liver weight was significantly increased in rainbow trout fed the diet containing 6% SCP compared to the control, but this effect was not observed at the 8% inclusion level. Importantly, no significant differences were detected in HSI among experimental diets over the 30-day trial. These findings suggest that the increased liver weight at 6% SCP inclusion may be attributed to proportional increases in fish body weight, rather than hepatic hypertrophy or other pathological changes. The lack of variation in HSI indicates that liver size remained consistent relative to overall body weight across all dietary treatments. These results are consistent with the findings of Ruiz et al. [16], who reported no significant differences among dietary treatments when rainbow trout were fed experimental diets containing 0,

3.75, 7.5, 11.25 and 15% SCP for 83 days. Feeding even higher inclusion rates (15, 20 and 40% SCP) may affect liver performance [10]. Beyond salmonids, Woolley et al. [12] found a significantly higher HSI in juvenile barramundi (*Lates calcarifer*) fed diets containing 20 and 30% SCP, while Xu et al. [13] reported no significant differences in juvenile Black Sea Bream (*Acanthopagrus schlegelii*) fed diets containing 4.13, 8.27, 16.53, and 24.80% SCP. Similarly, Zhang et al. [15] found no significant effect on HSI when juvenile largemouth bass (*Micropterus salmoides*) were fed diets containing up to 21.5% SCP. Collectively, these studies suggest that SCP inclusion at low to moderate levels does not affect HSI, whereas higher inclusion levels may induce hepatic enlargement, irrespective of species.

We also included spleen performance in the study given its role as a secondary lymphoid organ involved in immune surveillance and inflammatory responses. Thus, variations in spleen size are commonly associated with changes in immune cell proliferation and systemic inflammation [23,25].

While spleen weight did not differ significantly from the control diet, significant differences were observed between the 6% and 8% SCP inclusion levels. Specifically, spleen weight increased by 14.79% at 6% SCP inclusion but decreased by 17.18% at 8% SCP inclusion relative to the control. Consistent with these findings, the SSI was reduced (−20.09%) at the 8% SCP inclusion compared to the control, though this reduction was not statistically significant when compared directly to the control diet alone. However, the SSI at 8% SCP inclusion was significantly lower than that observed at the 6% inclusion level. These results suggest a dose-dependent effect of SCP on spleen size and potentially immune function. The reduced spleen weight and SSI at 8% SCP inclusion, coupled with the observed downregulation of splenic immune-related genes (Cathelicidin-2, CD8, IgM, IL-6 and SAA), may indicate immune modulation or mild exhaustion. Further investigation is warranted to determine whether these changes reflect adaptive responses, compensatory mechanisms, or early signs of immune dysfunction in rainbow trout fed high-inclusion SCP diets.

4.2. Gene Expression

Overall, a more pronounced immune response was measured in the spleen than in the liver (Figure 1). In the spleen, several immune-related genes, including Cathelicidin-2, SAA, CD8, IgM, and IL-6 were moderately downregulated ($p < 0.05$). These genes encode antimicrobial peptides (AMPs), acute-phase proteins, glycoproteins, antibodies, and cytokines involved in innate and adaptive immunity [23,26–28]. In contrast, the liver exhibited a more limited response, with a downregulation of Cathelicidin-1 at 8% SCP inclusion, and upregulation of IL-10 at 4% SCP inclusion ($p < 0.05$).

The pro-inflammatory gene IL-6 is downregulated in the spleen, leading to reduced inflammation. IL-6 induces the acute-phase protein SAA [23], and a similar pattern is observed with the downregulation of SAA in the spleen. The upregulation of hepatic IL-10 at 4% SCP inclusion is consistent with the findings of Zhang et al. [15]. In a ten-week feeding trial with largemouth bass (*M. salmoides*) fed increasing levels of SCP (derived from *M. capsulatus*), inclusion at 4.3% upregulated the expression of TLR2, RelA, TNF- α , IL-1 β , IL-10, and TGF- β in the intestine. However, an inclusion level of 4.3% and 8.6% resulted in a reduced expression of Caspase-3.

Similarly, Chama and colleagues [14] investigated the gene expression in the intestine of genetically improved farmed tilapia (*Oreochromis niloticus*) after a 56-day feeding trial with SCP (from *M. capsulatus*) and found that several immune parameters (IL-8, IL-10, IL-16, IFN- γ , NF- κ B, TNF- α) were downregulated in the intestine. Zhang et al. [29] also reported significant downregulation of intestinal TNF- α , IL-1 β , and IL-8 in spotted seabass fed diets containing 2% microbial SCP for 56 days. In contrast, TGF- β expression was upregulated at

both 2% and 4% SCP inclusion levels, while IL-4 and IL-10 were upregulated at the 2% SCP inclusion. No significant differences in gene expression were observed at SCP inclusion levels of up to 8% compared with the fishmeal-based control diet.

Overall, the evidence suggests that the immunomodulatory effects of microbial SCP depend on the tissue examined, the fish species and the dietary inclusion level. Responses differ among studies, but moderate SCP inclusion appears to promote immune regulation, particularly through the expression of anti-inflammatory genes.

The present study was designed as a preliminary trial, accounting for its relatively short duration. The aim was to investigate changes in the abundance of selected immune-related mRNAs in rainbow trout in response to dietary microbial SCP before conducting a longer-term trial with additional sampling time points. The generally limited effects observed in the present study should therefore be interpreted cautiously. Nevertheless, the lower abundance of selected immune-related transcripts observed at the highest inclusion level (8% SCP) indicates that the response to dietary SCP may differ according to inclusion level. As gene expression measurements provide a snapshot of cellular processes at a specific sampling time point, the observed differences may represent transient responses rather than sustained changes. Moreover, changes in mRNA abundance cannot by themselves establish functional effects on immune competence or health. The present study was therefore designed as a preliminary assessment of the transcriptional response to dietary SCP over a 30-day feeding period, providing a basis for further investigation while acknowledging the limitations of a short-term study.

The experimental conditions were carefully controlled, with fish maintained under consistent temperature, photoperiod, oxygen levels, and water flow, allowing the observed responses to be evaluated in relation to the feeding regime. However, research should focus on whether the extended feeding regimes ultimately enhance or compromise long-term health outcomes. Special focus should be placed in future studies on innate resistance to infection in general and effects on adaptive immunity in particular. The latter issue could be elucidated by performing studies on combined SCP feeding and vaccination response, as this parameter is associated with antibody titer elevations, which again are correlated to protection [30].

4.3. Growth Performance

In the present study, microbial SCP derived from *M. capsulatus*, replacing conventional protein sources formulated in a commercial diet, improved growth performance during the 30-day feeding trial. Inclusion of microbial SCP at 6% improved several growth performance parameters (FBW, BWG, FL and ADG). Despite these improvements, the DFI and FCR did not differ significantly among the dietary treatments. However, a trend towards an improved FCR (8% SCP) was observed, which, although not statistically significant, may be of potential commercial relevance. The relatively short trial duration and limited sample size may have reduced the statistical power to detect differences in FCR. It should be noted that this study was performed with one specific SCP product, which may differ from other sources used by other authors in previous investigations, simply due to differences in cultivation method, growth medium, purification technique and preparation. While direct comparisons with the effects of other SCP studies should be approached with caution, these findings partially align with previous studies evaluating microbial SCP in rainbow trout diets.

Lund et al. [10] reported no effect on SGR at microbial SCP inclusion levels of up to 40% when replacing FM, although FCR increased with higher inclusion levels. In that study, diets contained microbial SCP and FM as the sole protein sources. Similarly, Ruiz et al. [16] found no significant differences in growth performance, including FBW and SGR,

in rainbow trout fed diets containing microbial SCP at inclusion levels up to 15%. In contrast to the previous study, these diets included multiple commercially available protein sources. Consistent with our findings, Ruiz et al. [16] also reported no significant differences in feed intake (FI) among dietary treatments, although FCR increased with increasing microbial SCP inclusion. These findings are further supported by Chama et al. [14], who reported no significant differences in WGR, SGR, FI or FCR among diets containing microbial SCP at inclusion levels up to 8.5% during a 56-day feeding trial in juvenile tilapia. Although multiple commercially available protein sources were included in the formulation, their specific composition differed from that used by Ruiz et al. [16].

As mentioned earlier, the present study was designed as a preliminary trial, and therefore it has its limitations. However, the results indicate that an inclusion rate of 6% microbial SCP produced the most favorable overall response under the conditions of the present study. In addition to improved growth performance at this inclusion level, none of the immune-related genes investigated were significantly regulated. The favorable growth response and absence of significant regulation of the immune-related genes at this inclusion level may reflect several factors, including ingredient complementarity, amino acid balance, palatability-independent nutrient utilization, and the potential effects of microbial bioactive components. However, the present measurements cannot distinguish among these potential mechanisms, and the possibility of chance variation cannot be excluded. The lack of further improvement at the higher inclusion level suggests that the relationship between microbial SCP inclusion and growth performance may not be linear.

5. Conclusions

This feeding trial shows that replacing conventional protein sources from marine ecosystems and agriculture with SCP derived from *M. capsulatus* is a realistic way to diversify the protein portfolio, at least for rainbow trout (*O. mykiss*) and at lower inclusion levels. While dietary replacement of conventional protein with microbial SCP was associated with an increase in FBW at inclusion levels up to 6%, higher concentrations led to slight shifts in the immune response, as evidenced by gene regulation in the spleen and liver. At present, it is not known how this may affect fish health in large-scale aquaculture settings. Future studies should elucidate to which extent this may influence general innate resistance to disease and the ability of fish to respond to vaccines. In particular, the effect of longer trial durations and, consequently, prolonged exposure to microbial SCP should be examined. The study was designed to evaluate SCP inclusions up to 8% (replacing all main protein ingredients) and full replacement of each of these specific ingredients should be tested as well. Finally, it is crucial to investigate if raising the inclusion level further (>8%) may have adverse effects, whereby it will be possible to set the upper maximum level of inclusions.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fishes11090526/s1>, Table S1: Primers and probes used for qPCR assays; Table S2: qPCR results.

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Institutional Review Board Statement: The study was designed to minimize stress and handling, with animal welfare prioritized throughout the experimental period. All procedures were conducted in accordance with institutional and national guidelines for the care and use of experimental animals and run under license 2024-15-0201-01697 issued by the Danish Experimental Animal Inspectorate under the Ministry of Food, Agriculture and Fisheries of Denmark (approval date: 4 June 2024).

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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