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GLOSSARY OF ACRONYMS

Acronym	**Definition**
VPC	Vegetable protein concentrate
FM	Fish meal
FO	Fish oil
HUFA´s	High unsaturated fatty acids
FPH	fish protein hydrolysates
YM	yeast meal
PAP	processed animal proteins
SBM	Soybean meal
FPH	fish protein hydrolysates
Salmon	*Salmo salar* / Atlantic salmon
Turbot	*Scophthalmus maximus*
Seabream	*Sparus aurata* / gilthead seabream
Trout	*Oncorhynchus mykiss* / rainbow trout
Seabass	*Dicentrarchus labrax* / European seabass
SPC	soy protein concentrate

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Table of Contents

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

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1. Executive summary

This deliverable presents the performance results of finfish species such as Atlantic salmon (*Salmo salar*), gilthead seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*) rainbow trout (*Oncorhynchus mykiss*), and turbot (*Scophthalmus maximus*) after being fed with GAIN aquafeeds (detailed approach and feeds design further detailed on D. 1.4 Report on the Formulation of eco-efficient feed, for overview see Figure A. Performance was assessed according to the key performance indicators given in Figure B.)

The first block of trials occurred in 2019 and comprised four experiments on the following species: seabream, trout, turbot, and salmon. The aim of the first block trial was to assess fish performance on the formulation concepts as shown in figure A. In summary, the diets were: 1) Control: to mimic a current standard commercial diet for each species, including moderate levels of fish meal; 2) PAP diet: rich in processed land animal proteins, which are by-products from livestock production, and vegetable protein concentrates (VPCs) of European origin; 3) NoPAP diet: a combination of emerging ingredients, i.e moderate amounts of insect meal, microbial biomasses, microalgae, macroalgae, and VPCs, thus allowing the reduction of both fish meal and avoiding land-animals PAPs inclusion; 4) MIX diet: an extended combination of emerging ingredients, allowing the reduction of both fish meal and VPCs, which includes small to moderate amounts of land-animals PAPs, insect meal, microbial biomasses, microalgae, and macroalgae. Moreover, moderate levels of fish oil in the control diet were replaced by a combination of salmon, algal, and vegetable oils. Formulations had to be adapted to each species based on their known nutritional requirements and tolerance to different ingredients.

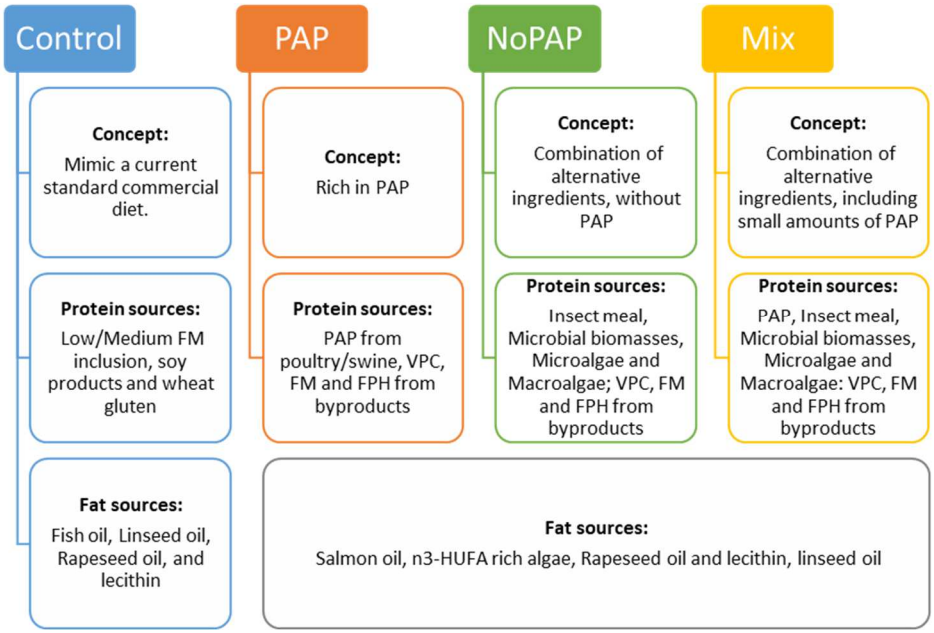

Figure A. Formulation concepts and main ingredients used in GAIN trials with novel aquafeeds. FM – fish meal; PAP – processed animal protein from farmed animals (e.g., poultry meal, feather meal and blood meal); VCP – vegetable (e.g., pea, rapeseed) protein concentrates from European origin; FPH - fish protein hydrolysates from fisheries and aquaculture byproducts (e.g., fish trimmings, heads and frames); Salmon oil - by-product from salmon farming industry.

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The second block of trials occurred in 2020 and comprised five experiments on the following species: seabass, seabream, trout, salmon, and turbot. The second block of trials aimed to assess fish performance, building on the formulation and performance results on the block 1 trials. Moreover, a stronger effort was made in terms of lowering formulation costs, and also ingredients developed under GAIN were used. Fish protein hydrolysates (FPH) from by-products (Task 1.3) were used in order to add further to the circularity concept of the diets. In addition, selenium-rich microalgae (Task 1.1) and macroalgae rich in several minerals (Task. 2.2) were used. To assess larger differences among treatments and concepts, diets were formulated according to the formulation concepts as follows: 1) Control to mimic a current standard commercial diet for each species, followed by 2) NoPAP: a combination of emerging ingredients, thus allowing the reduction of both fish meal and avoiding PAPs inclusion, 3) NOPAP+ an improved version of NoPAP diet with higher protein content, 4) PAP diet comprised several processed land animal proteins, and 5) PAP- diets an economical version of the PAP diet, with lower protein content. For turbot a slight adaption to the above concepts was used, due to its known low tolerance to fish meal replacement; FM level in the Control diet was replaced by 30% and 60% of PAP and NOPAP ingredients, leading to PAP30, PAP60, NOPAP30, and NOPAP60, respectively. For the seabream trial, a special diet (NoPAP SANA) was formulated, using the NOPAP concept, but to which a feed additive promoting gut health was added. It should be noted that FM in all GAIN alternative diets had origin in fisheries by-products, while the Control diets had a high-quality FM.

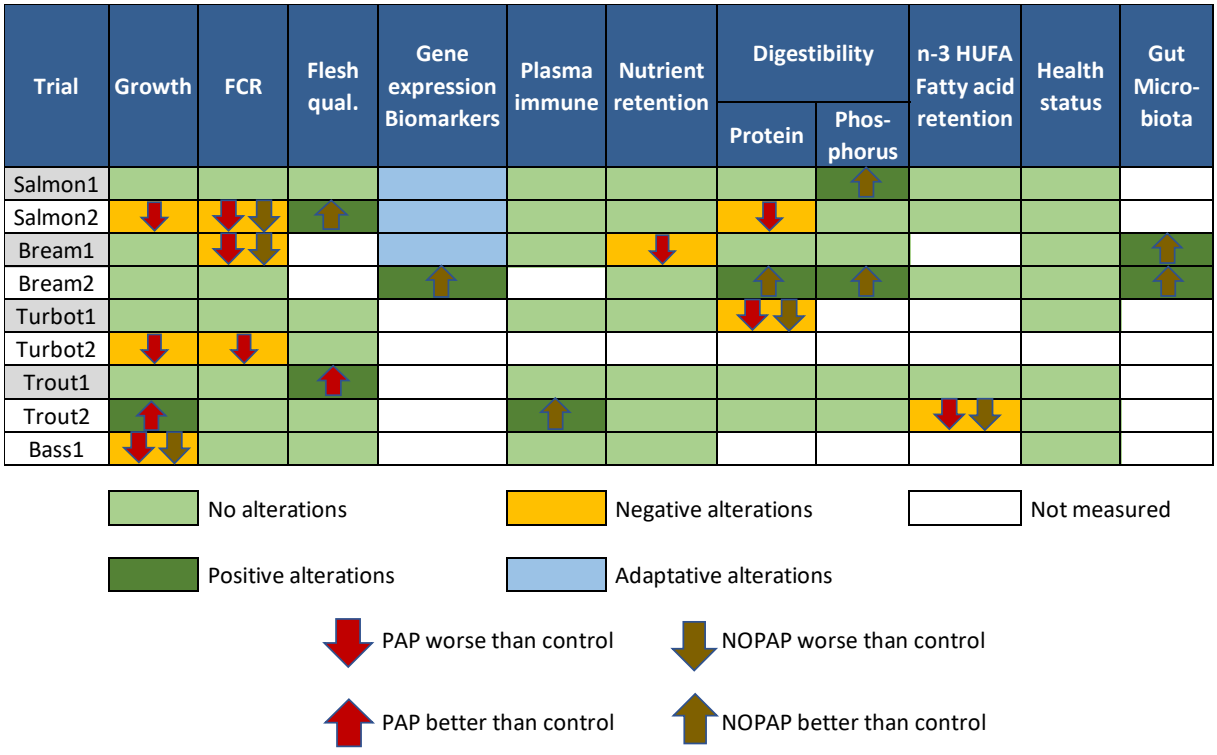

Fig B. Overview on how the different key performance indicators were affected by the GAIN novel aquafeed formulations in the 9 fish trials conducted.

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Atlantic salmon

In trial **Salmon1** growth performance was very similar for the 3 diets tested. All the other parameters analysed, including health scores and those referring to intestinal mucosa status, plasma innate immune defences and oxidative status in the liver were also similar between diets. GAIN novel feed formulations seem to lead to fish with good welfare and health. Nonetheless head kidney gene expression on fish fed with novel feed formulations suggest that there is an adaptation of the inflammatory response profile, which is not a problem in itself. In turn, on the liver gene profile, genes differentially expressed are involved in growth performance, lipid metabolism, and energy metabolism. Moreover, once gene expression was monitored twice; a few weeks after exposure to the diets, and at the end of the trial, it is clear that fish have, and as could be expected, have an adaptation period to the novel diets, but seem to reach a new allostatic balance.

Growth performance was good for the 5 diets tested in Also in the **Salmon2 trial**. No impact of diets could be seen on fish welfare and health status based on the immune parameters measured in the plasma, lipid peroxidation in the liver, and anterior intestine mucosal mapping. However, there was a tendency for worse mucosal status in fish of the PAP diet, but still within normal values for the species. Regarding the gene expression, it followed the trend of the other analyses, with no signs of health and welfare being negatively affected.

In short, feed formulations such as NOPAP and PAP, devoided of fish meal, and containing a basket of alternative protein sources such as microbial biomasses, land-animal processed proteins, insect meal, fish protein hydrolysates (from aquaculture by-products) and vegetable protein concentrates; and replacing 50% of the fish oil by a mix of rapeseed, and algae oils; are likely valuable options to support accelerated growth, good health, and good feed conversion ratio in Atlantic salmon. However, good results will depend on the high protein digestibility of the chosen ingredients. Moreover, positive results on consumer perception may arise due to improvements in flesh quality.

Gilthead seabream

Results of trials **Bream1** and **Bream2** suggest that the novel feed formulations, and in particular the NOPAP diet, give a good growth performance in seabream, and are good alternatives to current gilthead seabream feeds. Still, in Bream1 FCR was worse in both PAP and NOPAP diets compared to Control, and this may be related to lower protein retention. Bream 1 trial suggests that fish fed with NOPAP diet show a slight improvement of innate immunity, as shown by higher IgM, bactericidal, and anti-protease activities. Furthermore, mucosal mapping ™ results agree with the plasma innate immune results where the fish fed with NOPAP and MIX diets presented higher values of barrier status compared to the PAP diet. This result is also supported by the gene expression profile of the head kidney.

In **Bream2** trial the NoPAP SANA diet modulated the expression of several genes in the liver showing the capacity to reduce lipogenesis, mitochondrial activity, and the risk of oxidative stress and, at the same time, promoting an anti-inflammatory gene expression profile in the head kidney, and posterior intestine. All these changes may be seen as adaptations to the novel diet, the fish looking for a new physiological equilibrium. Therefore, from a fish health point of view, no constraints in using novel diets were found for gilthead seabream. In fact, the NoPAP SANA diet may even promote some improved immune competence, and no

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increased susceptibility against an intestinal parasite challenge could be observed as in previous studies using alternative formulations.

Clearly, a feed formulation such as NOPAP SANA, devoided of fish meal, and containing a basket of alternative protein sources such as microbial biomasses, insect meal, fish protein hydrolysates (from aquaculture by-products) and vegetable protein concentrates; and replacing 50% of the fish oil by a mix of salmon, and algae oils; seems to be a valuable option to support accelerated growth, good health, and a very good feed conversion ratio in gilthead seabream.

Turbot

In trial **Turbot1** growth performances and feed conversion ratios of turbot juveniles were very good for the GAIN novel formulations and comparable to the control diet. Moreover, plasma immune parameters and nutrient retention were unaffected in the novel feed formulations, despite protein digestibility being lower in PAP and NOPAP diets compared to control.

The **Turbot2** trial results suggest that pre-adult fish fed with PAP 60 diet had the overall lowest growth and feed conversion performance, followed by PAP 30 and NOPAP 60. The results on condition factors, hepato-somatic index, and the survival rates indicate a good nutritional and health status in all the diets. Moreover, no diet effects on the dressout loss and fillet yield were observed, suggesting no negative effects on flesh quality.

In short, a feed formulation such as NOPAP 30, based on: 28% of a lower quality fish meal (from by-products), and alternative protein sources such as microbial biomasses, insect meal, fish protein hydrolysates (from aquaculture by-products), and vegetable protein concentrates; and replacing 50% of the fish oil by a mix of salmon, algae and rapeseed oils; seems to be a valuable formulation for turbot in the grow-out phase resulting in good growth, feed performance and health.

Rainbow trout

In trial **Trout1** growth performances in rainbow trout were very good and similar between the control and the novel GAIN feed formulations. Moreover, fish were healthy throughout the trial and no difference in plasma lysozyme could be seen, which supports the suitability of the GAIN formulation concepts for eco-efficient farming of healthy trout.

The growth performance and feed conversion in trial **Trout2** were also very good in the 5 diets tested. Protein and energy retentions were also very good in the 5 diets tested, with somewhat lower protein retention for the PAP and PAP- diets. However, DHA retention was lower in the 4 GAIN alternative formulations compared to the control. This leads to suggest that another selection of oils should be tested in future trout trials.

Clearly, feed formulations such as NOPAP and PAP, devoided of fish meal, and containing a basket of alternative protein sources such as microbial biomasses, land-animal processed proteins, insect meal, fish protein hydrolysates (from aquaculture by-products) and vegetable protein concentrates are valuable options to support accelerated growth, good health, and very good feed conversion ratio in rainbow trout. Moreover, consumer perception in terms of flesh quality will be good.

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European seabass

Only one trial was performed in seabass. It suggests that NOPAP and PAP diets lead to a slightly lower growth performance compared to seabass fed the commercial-type diet. Moreover, results showed slightly decreased health parameters for the PAP and MINUS groups, while sensory evaluation was not significantly affected by any of the diets tested, except for consistency after cooking.

Overall, these results seem to support the hypothesis that the NOPAP diet and PLUS diets are viable options for seabass, but further studies are needed to investigate if fish physiology is affected by the different diets.

In general terms, it seems trout was the species that accepted the best new formulations, and turbot the one that accepts them worst. Overall NoPAP diets seem to present better results for all fish species tested during this project. However, the PAP concept seems to be also valid and the less positive results in some species are likely to have to do more with the batch quality of one or more of the ingredients used, namely in terms of protein digestibility, than the PAP concept itself. Moreover, results on sensory evaluation for salmon, trout, and seabass suggest that the novel formulations tested would be well accepted by the consumer. Still, formulation costs tended to be higher in alternative diets, and sustainability evaluation was not favorable (results from WP4, not shown in the present Deliverable).

The 9 trials on fish novel feeds performed during the GAIN project confirmed that it is possible to produce fish using formulation concepts and ingredient baskets that fit into a circular economy framework, which was a main objective of the project. We demonstrated that fish production can be achieved using eco-efficient feeds. For trout, the new formulations even increased production in a cost-effective manner, which may improve the competitiveness of the industry. Furthermore, the very good acceptance of fish fillets after sensorial analysis in salmon and trout reinforces the idea that consumer acceptance for alternative formulations and ingredients will not be a problem. Still, this required that the industry communicates well the pros and cons of eco-intensification, including the circular economy-driven benefits and food safety, of using aquafeed formulations using an alternative ingredient basket including fish meal from by-products, microbial biomasses, insect meals, fish protein hydrolysates (from aquaculture by-products), vegetable protein concentrates, macroalgae, microalgae, salmon oil, algae oils, and rapeseed oil.

These GAIN trials on fish novel feeds also demonstrated that fish protein hydrolysates (FPH) arising from aquaculture side-streams, as well as macroalgae and microalgae, can be used as effective aquafeed ingredients. FPH are valuable to stimulate feed intake due to their high content in free amino acids, while containing peptides with putative bioactivities. Micro and macro -algae were also successfully used as a source of minerals, in particular Selenium. Bioactive peptides from FPH, and pigments, phenolic, polysaccharide, and other compounds from algae, may explain the positive effect on fish immunity observed in some GAIN fish trials.

In short, GAIN feed formulations, including ingredients using aquaculture and fisheries side-streams, and other emerging ingredients adhering to circular economy principles, are viable options for eco-efficient European fish farming, especially once costs of emerging ingredients become price-competitive, and renewable energies are used to produce them.

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2. Introduction

Green Aquaculture Intensification in Europe (GAIN) (www.unive.it/gainh2020_eu) is a project financed by the European Union under the Horizon2020 framework. The project was run by a consortium of 20 partners from a variety of professional backgrounds, spanning across 11 different countries, including Canada and China. GAIN's primary aim is to assist the ecological intensification of aquaculture in the European Union (EU) and the European Economic Area (EEA), with the dual objectives of increasing production and competitiveness of the industry, while ensuring sustainability and compliance with EU regulations on food safety and environment.

Within the scope of this report, we address Task 1.2 "Design and performance assessment of novel feeds" of the GAIN Project, a large part of the field trials and feed investigations were carried out in WP 1. Task 1.2 aims to develop a new generation of sustainable fish feeds specifically designed to facilitate aquaculture eco-intensification through increased circularity and resource utilization, using a set of candidate ingredients such as algae and by-products of aquaculture activities. These ingredients were complemented by emerging commercial ingredients (e.g., processed land animal proteins, heterotrophic microalgae oils, single-cell meals and insect meals, plant protein concentrates of European origin) keeping in mind compliance with the regulatory framework and social acceptance (GAIN Tasks 3.1 and 3.4 respectively).

This deliverable describes the results achieved during Task 1.2, where the main goal was to design feeds based on micro- and macro- algae, fish by-products, and emerging ingredients for specific species such as salmon (Salmo *salar*), turbot (*Scophthalmus maximus [Psetta maxima]*), seabream (*Sparus aurata*), seabass (*Dicentrarchus labrax*) and trout (*Oncorhynchus mykiss*). These results, together with the characterization of ingredients derived from GAIN´s Tasks 1.1 and 2.2, will help formulators to decide on which combinations of emerging ingredients to use, aiming at formulations that are suitable for a sustainable and eco-eficient aquaculture growth.

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3. Methodology

This section presents the fish performance assessment of novel feeds designed for the GAIN project. For detailed formulation concept and ingredients selection please see Deliverable 1.4 Report on the Formulations of eco-efficient feeds.

3.1 Location of the trials

The trials were divided into two blocks (further referred to as Block 1 and Block 2) of experiments which in turn were divided across the partners according to their species expertise and facilities available as well as the selected candidates, as such: SPAROS and CSIC (seabream), AWI (turbot and seabass), FEM (trout), and GIFAS (salmon). Detailed information about the rearing conditions, number of tanks, replicates and water temperature conditions is presented on Annex 4 to this deliverable.

3.2 Ingredients Selection

For Block 1 trials the candidate ingredients selection was based on circularity principles, maximizing resource efficiency, while contributing towards low waste in the agro-food value chain, feed cost-effectiveness, and considering social acceptance, so to optimize sustainability within the current/predictable regulatory framework. For Block 2 trials FPH (from Task 1.3) were used to build further on the circularity concept of the diets. In addition, selenium-rich microalgae (from task 1.1) and macroalgae (from task 2.2) were used to concretize this concept. For more detailed information on FPH and microalgae used in each diet please see below session 3.3.

3.3 Diets formulation and production

All powder ingredients were mixed according to the target formulation in a double-helix mixer (model 500L, TGC Extrusion, France) and ground (below 400 µm) in a micro pulverizer hammer mill (model SH1, Hosokawa-Alpine, Germany). Diets (pellet size changed according to species and fish size) were manufactured with a twin-screw extruder (model BC45, Clextral, France) with a screw diameter of 55.5 mm. Extrusion conditions: feeder rate (80-85 kg/h), screw speed (247-266 rpm), water addition in barrel 1 (345 ml/min), temperature barrel 1 (32-34ºC), temperature barrel 3 (111-117°C). Extruded pellets were dried in a vibrating fluid bed dryer (model DR100, TGC Extrusion, France). After cooling, oils were added by vacuum coating (model PG-10VCLAB, Dinnissen, The Netherlands). Coating conditions were: pressure (700 mbar); spraying time under vacuum (approximately 90 seconds), return to atmospheric pressure (120 seconds). Immediately after coating, diets were packed in bags or sealed plastic buckets and shipped to the research site where they were stored at room temperature, but in a cool and aerated emplacement. Representative samples of each diet were taken for composition analysis.

Diets were formulated according to the known nutritional requirements of the target species (NRC 2011) and manufactured by extrusion at SPAROS facilities. Formulation of all trials are presented in the following tables.

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Table 1: Formulation of the experimental diets for trial Trout 1.

Ingredients, %	**CTRL**	**NoPAP**	**PAP**	**MIX**
Fishmeal LT70	20.000	5.000	5.000	
Fish hydrolysate (by-products)	3.000	3.000	3.000	3.000
Insect meal		5.000	5.000	10.000
Microbial protein meal		5.000	5.000	10.000
Yeast protein meal		3.000	3.000	3.000
Feather meal hydrolysate			5.000	5.000
Porcine hemoglobin			2.500	2.500
Poultry meal 65			20.000	10.000
Microalgae meal (*Spirullina*)		5.000		5.000
Microalgae meal (*Chlorella*)		0.500		0.500
Pea protein concentrate		6.000		
Wheat gluten	8.000	8.500		
Corn gluten meal	5.000	5.000	5.000	4.500
Soy protein concentrate	18.000	5.000		
Soybean meal 48	5.000			
Wheat meal	10.000	9.250	11.950	9.750
Pea starch	5.000	5.000	5.000	5.000
Fish oil	7.400	3.700	3.700	3.700
Salmon oil (by-products)		8.000	8.000	8.000
DHA-rich algae (*Schizochytrium*)		3.200	3.200	3.200
Rapeseed oil	9.700	2.800		0.600
Linseed oil	4.100	4.100	4.100	4.100
Rapeseed lecithin	0.500	1.000	1.000	1.000
Vitamin and mineral premix	1.000	1.000	1.000	1.000
Vitamin C (35%)	0.100	0.100	0.100	0.100
Betaine HCl	0.280	0.280	0.280	0.280
Brewer's yeast		4.000	4.000	4.000
Macroalgae mix		1.000	1.000	1.000
Antioxidant	0.350	0.350	0.350	0.350
Sodium propionate	0.100	0.100	0.100	0.100
Monocalcium phosphate	1.900	2.850	1.300	2.200
L-Lysine	0.300	1.000	0.500	0.950
L-Tryptophan	0.100	0.300	0.200	0.250
DL-Methionine	0.150	0.550	0.400	0.600
L-Taurine		0.400	0.300	0.300
Yttrium oxide	0.020	0.020	0.020	0.020
Total	**100.00**	**100.00**	**100.00**	**100.00**
Feed composition (%DM)				
Crude protein	44.91	46.05	44.46	46.76
Crude fat	24.54	20.36	24.41	19.67
Energy (kJ/g)	23.53	23.40	24.08	23.71
Ash	7.80	6.59	6.21	6.17

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Table 2: Formulation of the experimental diets for trial Salmon 1.

Ingredients (%)	CTRL	PAP	NOPAP
Fishmeal LT70 [a]	5.000		
Fishmeal Super Prime [b]	10.000		
Fishmeal 60 (by-products) [c]		2.500	2.500
Fish hydrolysate (by-products) [d]		5.000	5.000
Krill meal [e]	4.000		
Feather meal hydrolysate [f]		5.000	
Haemoglobin powder [g]		5.000	
Poultry meal 65 [h]		9.000	
Insect meal [i]		10.000	10.000
Microbial protein meal [j]		10.000	10.000
Microalgae meal (Scenedesmus) [k]			0.900
Microalgae meal (Chlorella) [l]			1.100
Soy protein concentrate [m]	15.000		
Pea protein concentrate [n]	10.000		11.500
Wheat gluten [o]	10.000	10.000	11.500
Corn gluten meal [p]	4.500	0.000	2.500
Wheat meal [q]	10.575	7.165	9.925
Pea starch [r]		3.000	
Fish oil [s]	6.500	3.250	3.250
Salmon oil (by-products) [t]		6.000	6.000
DHA-rich algae (Schizochytrium) [u]		3.000	3.000
Rapeseed oil [v]	18.500	11.900	13.400
Vitamin and mineral premix [w]	1.000	1.000	1.000
Vitamin C (35%) [x]	0.100	0.100	0.100
Betaine HCl [y]	0.150	0.150	0.150
Brewer's yeast [z]		2.000	2.000
Macroalgae mix [aa]		2.000	1.000
Antioxidant [ab]	0.200	0.200	0.200
Sodium phosphate [ac]	3.000	2.700	3.000
Astaxanthin (10%) [ad]	0.055	0.055	0.055
L-Histidine [ae]	0.700	0.300	0.800
L-Lysine [af]	0.200	0.200	0.350
L-Threonine [ag]	0.000	0.000	0.100
L-Tryptophan [ah]	0.150	0.060	0.150
DL-Methionine [ai]	0.200	0.250	0.300
L-Taurine [aj]	0.150	0.150	0.200
Yttrium oxide [ak]	0.020	0.020	0.020
Total	100.00	100.00	100.00
Diet composition (as feed basis)			
Crude protein, % feed	48.13	47.45	47.72
Crude fat, % feed	27.82	28.70	29.58
Ash, % feed	9.19	6.89	6.90
Gross Energy, MJ/kg feed	24.55	25.03	24.71
Total P, % feed	1.76	1.47	1.51

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Table 3: Formulation of the experimental diets for trial Bream 1.

Ingredients (%)	**Ctrl**	**Mix**	**PAP**	**NOPAP**
Fishmeal LT70 [a]	20.000			
Fishmeal 60 (by-products) [b]				5.000
Fish hydrolysate (by-products) [c]		5.000	5.000	5.000
Insect meal [d]		10.000	5.000	5.000
Microbial protein meal [e]		10.000	5.000	5.000
Yeast protein meal [f]		2.500	2.500	2.500
Feather meal hydrolysate [g]		5.000	5.000	
Porcine blood meal [h]		3.000	3.000	
Poultry meal 65 [i]	5.000	10.000	20.000	
Microalgae meal (*Spirullina*) [j]		5.000		5.000
Microalgae meal (*Chlorella*)[k]		0.500		0.500
Soy protein concentrate [l]	9.000			
Pea protein concentrate [m]				4.100
Wheat gluten [n]	4.000			4.000
Corn gluten meal [o]	10.000	1.400	4.500	15.000
Soybean meal 48 [p]	12.000			
Rapeseed meal [q]	4.000	7.000	5.700	11.500
Wheat meal [r]	8.470			
Pea starch [s]	3.000	9.000	6.000	7.900
Yellow peas [t]	6.200	7.030	14.580	3.000
Fish oil [u]	6.000			
Salmon oil [v]		3.000	3.000	3.000
DHA-rich algae (*Schizochytrium*) [x]		3.700	3.600	3.200
Rapeseed oil [z]	8.260	6.300	6.000	8.500
Rapeseed lecithin [aa]	0.600	1.000	1.000	1.000
Vitamin and mineral premix *	1.000	1.000	1.000	1.000
Vitamin C (35%) [ab]	0.100	0.100	0.100	0.100
Brewer's yeast [ac]		4.000	4.000	4.000
Macroalgae MIX [ad]		2.000	2.000	2.000
Antioxidant [ae]	0.200	0.200	0.200	0.200
Sodium propionate [af]	0.100	0.100	0.100	0.100
Monocalcium phosphate [ag]	2.000	2.200	1.900	2.500
L-Tryptophan [ah]	0.050	0.150	0.150	0.180
DL-Methionine [ai]		0.300	0.150	0.200
L-Taurine [aj]		0.500	0.500	0.500
Yttrium oxide [ak]	0.020	0.020	0.020	0.020
Total	100.00	**100.00**	**100.00**	**100.00**
Diet composition (as feed basis)				
Dry matter (%)	94.11	93.47	93.23	93.64
Ash (%)	8.47	8.05	8.35	7.19
Crude Protein (%)	44.20	45.30	44.71	44.88
Crude Lipid (%)	17.84	16.41	16.29	17.62
Gross Energy (KJ/g)	21.50	21.20	20.65	21.90

Table 4: Formulation of the experimental diets for trial Turbot 1.

Ingredients (%)	**CTRL**	**NoPAP**	**PAP**	**MIX**
Fishmeal LT70	50.00			

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Fishmeal 60 (by-products)		35.00	35.00	25.00
Fish hydrolysate (by-products)		5.00	5.00	5.00
Insect meal		5.00	5.00	7.50
Porcine hemoblobin			2.50	
Poultry meal			10.20	7.50
Microbial protein meal		2.50	2.50	5.00
Yeast protein meal		2.50	2.50	5.00
Microalgae meal (*Spirullina*)		2.00		3.00
Microalgae meal (*Chlorella*)		0.50		0.60
Microalgae meal (*Tetraselmis*)		0.20		0.20
Soy protein concentrate	10.00			
Pea protein concentrate		12.40	5.00	8.00
Wheat gluten	11.00	11.50	10.00	10.00
Soybean meal 48	4.00			
Wheat meal	8.00			
Pea starch	4.00	8.89	8.99	8.99
Fish oil	11.60	4.64	4.64	4.64
DHA-rich algae (*Schizochytrium*)		1.08	1.08	1.88
Rapeseed oil		4.64	3.44	3.44
Rapeseed lecithin		0.80	0.80	0.80
Vitamin and mineral premix	1.00	1.00	1.00	1.00
Vitamin C (35%)	0.05	0.05	0.05	0.05
Vitamin E (50%)	0.05	0.05	0.05	0.05
Betaine HCl		0.50	0.50	0.50
Macroalgae mix		0.50	0.50	0.50
Antioxidant	0.18	0.18	0.18	0.18
Sodium propionate	0.10	0.10	0.10	0.10
L-Tryptophan		0.15	0.15	0.15
DL-Methionine		0.30	0.30	0.30
L-Taurine		0.50	0.50	0.60
Yttrium oxide	0.02	0.02	0.02	0.02
Total	**100.00**	**100.00**	**100.00**	**100.00**
Proximal composition (as feed basis)				
Crude protein, % feed	55.41	51.92	54.31	55.32
Crude fat, % feed	15.65	17.08	15.08	12.88
Ash, % feed	5.13	9.05	9.68	8.95
Gross Energy, MJ/kg feed	21.53	20.19	19.54	19.03

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Table 5: Formulation of the experimental diets for trial trout 2.

Ingredients (%)	**CTRL**	**NO PAP**	**PAP**	**NO PAP+**	**PAP-**
Fishmeal Super Prime	15.000			12.500	
Krill meal				5.000	
Fish protein hydrolysate	2.000				
FPH-TURBOT-HEAD		0.250	0.250	0.250	0.250
FPH-TURBOT-TF		0.250	0.250	0.250	0.250
FPH-SALMON-HEAD		0.250	0.250	0.250	0.250
FPH-SALMON-TF		0.250	0.250	0.250	0.250
FPH-BREAM/BASS		1.000	1.000	1.000	1.000
Feathermeal hydrolysate			7.500		15.000
Porcine blood meal			2.000		4.000
Poultry meal			5.500		11.000
Insect meal (Black soldier fly)		16.000	16.000	10.000	5.000
Fermentation biomass (M. capsulatus)		16.000	16.000	10.000	5.000
Soy protein concentrate	20.000	10.000		10.000	
Pea protein concentrate		2.550		10.000	
Wheat gluten	6.000	3.000		3.000	
Corn gluten meal	7.000	3.500			
Soybean meal 48	10.000				
Wheat meal	11.200				
Whole peas		12.030	20.840		31.770
Pea starch (raw)	4.000	7.000	4.000	13.350	
Vit & Min Premix - WITH I and Se	1.000				
Vit & Min Premix - NO I and Se		1.000	1.000	1.000	1.000
Macroalgae SHP		2.000	2.000	2.000	2.000
Macroalgae SHP + Se		0.050	0.050	0.050	0.050
Microalgae Se-rich		0.200	0.300	0.200	0.300
Vitamin E50	0.030	0.030	0.030	0.030	0.030
Betaine HCl	0.100	0.100	0.100	0.100	0.100
Antioxidant	0.200	0.200	0.200	0.200	0.200
Sodium propionate	0.100	0.100	0.100	0.100	0.100
Monocalcium phosphate	1.040	2.260	1.800	0.700	1.700
L-Lysine HCl 99%	0.500	0.600	0.450		0.650
L-Tryptophan	0.040	0.010	0.040		0.170
DL-Methionine					0.120
L-Taurine	0.170	0.200	0.120	0.100	0.040
Yttrium oxide	0.020	0.020	0.020	0.020	0.020
Fish oil	5,300	2,650	2,650	2,650	2,650
Salmon oil (by-products)		10,000	10,000	10,000	10,000
Algae oil (Veramaris)		1,000	1,000	1,000	1,000
Rapeseed oil	16,300	7,500	6,300	6,000	6,100
Total	**100,000**	**100,000**	**100,000**	**100,000**	**100,000**
As fed basis	**CTRL**	**NO PAP**	**PAP**	**NO PAP+**	**PAP-**
Crude protein, % feed	39.6	39.3	41.5	43.1	38.4
Crude fat, % feed	21.5	22.1	23.6	22.2	22.1
Fiber, % feed	1.6	1.6	1.4	1.3	2.5
Ash, % feed	6.3	6.2	6.0	5.9	5.8
Gross Energy, MJ/kg feed	23.4	22.8	22.8	23.3	23.5

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Table 6: Formulation of the experimental diets for trial Seabass 1.

Ingredients, %	CTRL	NO PAP	PAP	NOPA P+	PAP-
Fishmeal Super Prime	10.000			15.000	
Fishmeal 60 (by-products)	5.000				
Krill meal				5.000	
Fish protein hydrolysate	3.000				
FPH-TROUT-HEAD		0.500	0.500	0.500	0.500
FPH-TROUT-TF		0.500	0.500	0.500	0.500
FPH-TURBOT-HEAD		0.250	0.250	0.250	0.250
FPH-TURBOT-TF		0.750	0.750	0.750	0.750
FPH-SALMON-HEAD		0.500	0.500	0.500	0.500
FPH-SALMON-TF		0.500	0.500	0.500	0.500
Feather meal hydrolysate			5.000		10.000
Porcine blood meal			2.250		5.000
Poultry meal	10.000		14.000		19.000
Insect meal (Black soldier fly)		15.000	10.000	13.500	
Fermentation biomass (*C. glutamicum*)		5.000	5.000	2.500	
Fermentation biomass (*M. capsulatus*)		15.000	10.000	13.500	
Soy protein concentrate	4.400				
Pea protein concentrate		2.500		2.500	
Wheat gluten	6.000	2.000		2.000	
Corn gluten meal	6.000	2.000		2.000	
Soybean meal 48	15.000				
Sunflower meal 40		5.000			13.600
Wheat meal	11.400	5.700	5.700	5.700	5.700
Whole peas	4.000	13.530	16.210	8.340	14.590
Pea starch (raw)	4.000	4.000	4.000	4.000	4.000
Vit & Min Premix - WITH I and Se	1.000				
Vit & Min Premix - NO I and Se		1.000	1.000	1.000	1.000
GAIN Macroalgae SHP		2.500	2.500	2.500	2.500
GAIN Macroalgae SHP Se-rich		0.100	0.100	0.100	0.100
GAIN Microalgae WUR Se-rich		0.200	0.200	0.200	0.200
Vitamin E50	0.030	0.030	0.030	0.030	0.030
Betaine HCl	0.100	0.100	0.100	0.100	0.100
Antioxidant	0.250	0.250	0.250	0.250	0.250
Sodium propionate	0.100	0.100	0.100	0.100	0.100
Monoammonium phosphate	1.300	2.750	1.850	1.450	1.600
L-Histidine		0.200			
L-Lysine		0.600	0.200		0.550
L-Tryptophan	0.100	0.100	0.100		0.250
DL-Methionine	0.200	0.300	0.250		0.500
L-Taurine		0.170	0.090	0.010	0.060
Yttrium oxide	0.020	0.020	0.020	0.020	0.020

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Lecithin		0.250	0.250		0.250
Fish oil	5.400	2.700	2.700	2.700	2.700
Salmon oil		9.000	9.000	13.500	9.000
Algae oi		1.000	1.000	1.000	1.000
Rapeseed oil	12.700	5.900	5.100		4.900
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
As fed basis (theoretical composition)	**CTRL**	**NO PAP**	**PAP**	**NO PAP+**	**PAP-**
Crude protein, % feed	34.53	36.37	35.54	35.08	32.15
Crude fat, % feed	20.40	19.87	20.23	19.07	19.04
Ash, % feed	7.34	7.50	7.38	7.13	7.24
Gross Energy, MJ/kg feed	21.29	20.85	19.67	20.53	20.72

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Table 7: Formulation of the experimental diets for trial Bream 2.

Ingredients (%)	CTRL	NoPAP SANA
Fish meal Super Prime	10	
Fish meal (by-products)	7	
Fish protein hydrolysate	3	
GAIN FPH-TROUT-HEAD		0.5
GAIN FPH-TROUT-TF		0.5
GAIN FPH-TURBOT-HEAD		0.25
GAIN FPH-TURBOT-TF		0.75
GAIN FPH-SALMON-HEAD		0.5
GAIN FPH-SALMON-TF		0.5
Poultry meal	10	
Insect meal (Black soldier fly)		10
Fermentation biomass (C. glutamicum)		5
Fermentation biomass (M. capsulatus)		10
Soy protein concentrate	6	4.5
Pea protein concentrate		6.1
Wheat gluten	4	3
Corn gluten meal	10	7.5
Soybean meal 48	12.5	
Rapeseed meal	7	
Sunflower meal 40	5	20
Wheat meal	10.61	
Whole peas		6.12
Pea starch (raw)		3.6
Vit & Min Premix - WITH I and Se	1	
Vit & Min Premix - NO I and Se		1
GAIN Macroalgae SHP		2.5
GAIN Macroalgae SHP Se-rich		0.1
GAIN Microalgae WUR Se-rich		0.2
Vitamin E50	0.03	0.03
Betaine HCl	0.1	0.1
Antioxidant	0.2	0.2
Sodium propionate	0.08	0.08
Monoammonium phosphate	0.55	2.6
L-Lysine HCl 99%		0.3
L-Threonine		0.05
L-Tryptophan	0.06	0.1
DL-Methionine	0.05	0.3
Yttrium oxide	0.02	0.02
Fish oil	4.9	2.5
Salmon oil		9.6
Algae oil (Veramaris)		1
Rapeseed oil	7.9	
SANACORE		0.5
Total	100.00	100.00
Diet composition (as feed basis)		
Dry matter (%)	93.2	90.8
Ash (%)	8.3	7.2
Crude Protein (%)	50.5	49.6
Crude Lipid (%)	17.0	17.6
Gross Energy (KJ/g)	22.3	22.6

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Table 8: Formulation of the experimental diets for trial Turbot 2.

Ingredients (%)	**CTRL**	**NO PAP 30**	**PAP 30**	**NO PAP 60**	**PAP 60**
Fishmeal LT70	40.00				
Fishmeal 60 (by-products)		28.00	28.00	16.00	16.00
Fish protein hydrolysate	4.00				
FPH-BLUE WHITING - GAIN		4.00	4.00	4.00	4.00
Krill meal		3.50	3.50	3.50	3.50
Porcine blood meal			6.00		6.00
Poultry meal			15.00		15.00
Insect meal (Black soldier fly)		8.75	8.75	13.70	13.70
Fermentation biomass (M. capsulatus)		8.75	8.75	13.70	13.70
Soy protein concentrate	10.00	7.50		7.50	
Wheat gluten	14.20	10.65		10.65	
Wheat meal	17.83	11.65	12.17	12.18	12.65
Vit & Min Premix PV01 - With Se	1.00				
Vit & Min Premix PV01 - No Se		1.00	1.00	1.00	1.00
Vitamin E50	0.10	0.10	0.10	0.10	0.10
Betaine HCl	0.05	0.05	0.05	0.05	0.05
Macroalgae SHP - GAIN		2.00	2.00	2.00	2.00
Macroalgae SHP + Se - GAIN		0.05	0.05	0.05	0.05
Microalgae Se-rich - GAIN		0.30	0.30	0.30	0.30
Antioxidant	0.20	0.20	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10	0.10	0.10
Monoammonium phosphate	1.00	1.50	0.30	2.30	1.20
L-Lysine HCl 99%		0.80		0.95	0.10
L-Taurine		0.08	0.01	0.20	0.13
Yttrium oxide	0.02	0.02	0.02	0.02	0.02
Fish oil	7.00	3.50	3.50	3.50	3.50
Salmon oil		3.80	3.80	5.60	5.50
Algae oil		1.00	1.00	1.20	1.20
Rapeseed oil	4.50	2.70	1.40	1.20	0.00
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**
As fed basis (theoretical composition)	**CTRL**	**NO PAP 30**	**PAP 30**	**NO PAP 60**	**PAP 60**
Crude protein, % feed	52.0	52.0	52.0	52.0	52.0
Crude fat, % feed	16.0	16.0	16.0	16.0	16.0
Crude fat (no oils)	4.5	5.0	6.3	4.4	5.8
Fiber, % feed	1.0	0.9	0.5	1.0	0.6
Ash, % feed	9.2	10.2	11.2	9.3	10.5
Gross Energy, MJ/kg feed	21.0	20.4	20.6	20.7	20.9

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Table 9 Formulation of the experimental diets for trial Salmon2

Ingredients (% feed)	CTRL	NOPAP	PAP	NOPAP+	PAP-
Fishmeal LT70	12.50			12.50	
Krill meal	2.50			2.50	
Fish protein hydrolysate	2.00				
FPH-BLUE WHITING		2.00	2.00	2.00	2.00
Feather meal hydrolysate			8.00		11.25
Porcine blood meal			5.00		7.50
Poultry meal			8.50		16.00
Insect meal (Black soldier fly)		12.50	12.50	6.25	
Fermentation biomass (*M. capsulatus*)		12.00	12.00	6.00	
Soy protein concentrate (Soycomil P)	20.00	10.00		10.00	
Pea protein concentrate (Lysamine GPS)		5.25		11.25	
Wheat gluten	14.00	7.00		7.00	
Wheat meal	16.98				
Whole peas		8.16	12.25		30.34
Pea starch (raw)		8.25	7.16	9.48	
Vit & Min Premix - WITH I and Se	1.00				
Vit & Min Premix - NO I and Se		1.00	1.00	1.00	1.00
GAIN Macroalgae SHP		1.75	1.75	1.75	1.75
GAIN Macroalgae SHP Se-rich		0.05	0.05	0.05	0.05
GAIN Microalgae WUR Se-rich		0.20	0.30	0.25	0.30
Vitamin E50	0.10	0.10	0.10	0.10	0.10
Betaine HCl	0.05	0.05	0.05	0.05	0.05
Antioxidant	0.20	0.20	0.20	0.20	0.20
Sodium propionate	0.10	0.10	0.10	0.10	0.10
Monoammonium phosphate	1.50	2.20	1.50	1.30	1.35
Carophyll Pink 10% - Astaxanthin	0.06	0.06	0.06	0.06	0.06
L-Histidine	0.40	0.60	0.50	0.60	0.40
L-Lysine HCl 99%	0.70	0.65	0.40	0.70	0.58
L-Tryptophan	0.05	0.02	0.07	0.07	0.25
DL-Methionine	0.25	0.25	0.20	0.35	0.37
L-Taurine	0.10	0.20	0.10	0.13	0.04
Yttrium oxide	0.02	0.02	0.02	0.02	0.02
Fish oil (Sopropeche)	7.00	3.50	3.50	3.50	3.50
Algae oil (Veramaris)		2.35	2.35	2.75	1.45
Rapeseed oil	20.50	21.55	20.35	20.05	21.35
Total	**100.00**	**100.00**	**100.00**	**100.00**	**100.00**

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3.4 Biochemical analysis

Analysis of diets, whole-fish, and feces was made with analytical duplicates, and following in most cases the methodology described by AOAC (2006). Dry matter after drying at 105ºC for 24 h; total ash by combustion (550ºC during 6 h) in a muffle furnace; crude protein (N×6.25) by a flash combustion technique followed by a gas chromatographic separation and thermal conductivity detection with a Leco N Analyzer (Model FP-528, Leco Corporation, USA); Following an acid hydrolysis step, crude lipid was determined by dichloromethane extraction (40-60ºC) using a Soxtec™ 2055 Fat Extraction System (Foss, Denmark). Gross energy was measured in an adiabatic bomb calorimeter (Werke C2000 basic, IKA, Germany). Following an acid hydrolysis step, crude lipid was determined by dichloromethane extraction (40-60ºC) using a Soxtec™ 2055 Fat Extraction System (Foss, Denmark). Gross energy was measured in an adiabatic bomb calorimeter (Werke C2000 basic, IKA, Germany). For fatty acid analysis of feeds, lipids were first extracted according to the method of Folch et al. (1957), and afterward, the fatty acid composition was determined by gas-chromatography analysis of methyl esters, according to the procedure of Lepage and Roy (1986). For mineral analysis, dry samples were weighed (50–200 mg) in quartz vessels. Samples were then digested in 3 mL of nitric acid (HNO3 tracer grade, 70%) in a Discovery SP-D microwave digestion unit according to the following program: 200ºC; 4min ramp; 3 min hold. The samples were then cooled to room temperature and a final volume of 10 mL was achieved by adding ultrapure water. The samples were subsequently diluted 16x in ultrapure water and standard curves were prepared in ultrapure water. Mineral quantification was performed by MP-AES (Agilent, model 4200). Blank samples, containing only the decomposition acid were included to measure the matrix effects of decomposition, which were subtracted from every element in each sample

3.5 Growth Evaluation criteria

Growth performance parameters were calculated accordingly the following equations:

IBW (g): Initial mean body weight.
FBW (g): Final mean body weight.
Relative growth rate, RGR (%/day): (e^{g}-1) x 100, with g= (Ln FBW – Ln IBW)/days.
Feed conversion ratio, FCR: crude feed intake / weight gain (corrected for mortalities and sampling).
Feed intake, FI (%BW/day): (crude feed intake / (IBW+FBW) / 2 / days) x 100.
Protein efficiency ratio, PER: wet weight gain / crude protein intake.

Nutrient retention for the different nutrients (i.e., crude protein, crude fat, energy, fatty acid, or mineral) was calculated as:

$$\text{Nutrient Retention } (\%) = 100 \times \frac{(\text{FBW} \times \text{NFF}) - (\text{IBW} \times \text{NIF})}{\text{Nutrient intake}}$$

NFF: Nutrient content of fish at the end of the trial
NIF: Nutrient content of fish at the start of the trial

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Apparent digestibility coefficients (ADC) of dietary nutrients and energy in the experimental diets were calculated according to NRC (2011):

$$\text{ADC, \%} = 100 \times \frac{\text{\% marker diet}}{\text{\% marker feces}} \times \frac{\text{\% nutrient feces}}{\text{\% nutrient diet}}$$

3.6 Lysozyme

Lysozyme was performed using protocol previously established by Ellis (1990) with some modifications. Briefly, plasma from Trout´s blood was sampled from caudal vein with heparinized syringes, centrifuged at 2000 g for 10 minutes at 7°C. Plasma was stored at -20°C until and shipped to AWI where the samples were processed. A standard curve was performed using 20 ul of lysozyme at concentration of 0.001 g/ml in serial dilutions 1:2 in buffer (Na2HPO4 (hydrate); 0.05 M; pH 6.2) and 130 µL *Micrococcus luteus* (0.6 mg / mL). An aliquot of 10 ul of the samples were plated in a flat bottom microplate together with 10 ul of buffer and 130 µL *M. luteus*. Plates were read at 450 nm at each 1 min for a total of 10 min. The decrease or absorbance is induced by bacterial lysis by lysozyme. Thus, calculations were performed using the following equation below. The analysis was performed in analytical triplicate for each sample and results are expressed in average of absorbance.

Lyzozyme (abs)= Final abs – Initial abs – blank of buffer – blank of *M.luteus*.

3.7 Flesh quality analysis

This analysis was performed in Trout1 and Trout2 trials.

Marketable Traits

An experimental pool from each tank were collected at the end of the trial. After slaughter the fish were stored at 1°C covered with ice for 24h. The day after fish were eviscerated, and carcass, whole viscera, liver and mesenteric fat were weighted. Then, carcass yield,

cY (%)= 100 x [W(g) - visceral weight (g) / W(g)]

hepatosomatic index,

hsi (%)= 100 x [liver weight (g) / W(g)]

viscerosomatic index

vsi (%)= 100 x [visceral weight (g) / W(g)]

and mesenteric fat index

VFI (%)= 100 x [mesenteric fat (g) / W(g)]

were calculated. Fish were fileted afterwards.

Texture Profile Analysis (TPA)

The right fillet from each sample was assigned to the Texture Profile Analysis. Textural features were measured on a sample of muscle (with the section of 4×4 cm), withdrawn from the epiaxial region of the fillet. TPA was carried out using a Zwick Roell® 109 texturometer (Ulm, Germany) equipped with a 1 kN load cell and a cylindrical probe (10 mm), and the Text Expert II software version 3.0. Two consecutive cycles (downstroke and upstroke), with a five second break between them, were set and the deformation was limited at 50% of total thickness by a crosshead speed of 100 mm/min (Veland and Torrissen, 1999). From the resulting curve, the following parameters were determined:

- hardness (N), maximum force required to compress the sample;

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- cohesiveness, The area of work during the second compression divided by the area of work during the first compression (Area 2/Area 1)
- Gumminess, Gumminess applies only to semi-solid products and is Hardness * Cohesiveness (which is Area 2/Area 1).
- Resilience (Nmm) it is calculated by dividing the upstroke energy of the first compression by the downstroke energy of the first compression (Area 4/Area 3)
- Adhesiveness (Nmm) the negative force area under the baseline between compression cycles.

Colour

A Konica-Minolta CR-400 colourimeter was utilised for colourimetric measurement carried out according to the CIELab system (CIE, 1976). Flesh colour was measured in triplicate on the cranial, dorsal and caudal positions from the left fillet, then Chroma/Hue (C*L*h color space) and Entire Colour Index (Pavlidis et al., 2006) were calculated.

Colour differences (ΔE_{lab}) between samples was calculated according to the following formula:
$\Delta E (\beta - \alpha) = [(L*\beta - L*\alpha)2 + (a*\beta - a*\alpha)2 + (b*\beta - b*\alpha)2]0.5$
where α and β represent alternatively the mean colour values of different diets (Mokrzycki & Tatol, 2011).

3.8 Sensorial Analysys

Fifty fish trout and sixty salmon were collected from the tanks fed CTRL, PAP, and NoPAP diets, were slaughtered and shipped in ice at Sense Test Lab (V.N. Gaia, Portugal) for the sensorial analysis.

Essay Condition: All tests took place at Sense Test - Society for Studies of Sensory Analysis to Food, Lda, in a dedicated tasting room for sensory testing of food products (ISO 8589 Sensory analysis - General guidance for the design of test rooms). One hundred consumers were accustomed to the products that were going to be tested. Thus, the effects of physiological factors and physical conditions on human judgment were reduced. All features and products were analyzed using close to natural light (6500K). Before each test session, a preliminary explanation to the tasters on the conditions and rules of the sensory test were given. Moreover, all rules were handed out with the proof sheets.

The trout were baked in the oven for approximately 12 min at 170° C, and a small portion was served to each taster. Each sample was served on a white dish. In the tasting booth a knife, a fork, paper napkins, a glass of water, a spittoon, and crackers to the tasters, were made available. The tasters were told that both the crackers should only be used between tasting sessions to easily free the taste from the mouth.

After tasting the tester replied to a questionnaire in which the parameters appearance, odor, taste and texture as well as global acceptance of the flesh were evaluated. The evaluation consisted on giving scores from 1 to 9 where 1 referred to “extremely dislike” and 9 referred to “extremely liked”. Means and standard deviation from the scores given were calculated and statistical analysis were performed.

Seabass was also analyzed for sensory analysis in the IBEN Lab (Bremerhaven, Germany). The analysis were done according to ASU L 00.90-6 2015 -06 standards. Specially trained experts anaylysed the fish in the following parameters: Apperance before and after cooking, smell before and after cooking, consistency before and after cooking and taste after cooking.

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3.9 Humoral Immune parameters

Protease activity

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Guardiola et al. (2014) Briefly, 10 µl of plasma was incubated with 100 µl of ammonium bicarbonate buffer and 125 µl of azocasein (Sigma) for 24 h at room temperature in orbital shaker (100 rpm). The reaction was stopped by adding 250 µl of 4.6% trichloro acetic acid (TCA) and the Mixture centrifuged (10,000 x g for 5 min). The supernatants were transferred to a 96-well plate in duplicate containing 100 µl of 1N NaOH, and the optical density (OD) read at 450 nm using a plate reader. Plasma were replaced by trypsin (5 mg/L Sigma), as positive control, corresponding to 100% of protease activity, or by buffer, as negative controls equivalent to 0% activity.

Antiprotease activity

Antiprotease activity was determined according to the method described by Machado et al, (2015). Briefly, 10 µl of plasma samples were incubated with the same volume of a trypsin solution (5 mg/ml in $NaHCO_3$, 5 mg/ml, pH 8.3) for 10 min at 22 ºC. To the incubation Mixture, 100 µl of phosphate buffer (PBS) (NaH_2PO_4, 13.9 mg/ml, pH 7.0) and 125 µl of azocasein (20 mg/ml in $NaHCO_3$, 5 mg/ml, pH 8.3) were added and incubated for 1 h at 22 ºC. Lastly, 250 µl of 10% TCA were added to each microtube and incubated for 30 min at 22 ºC. The Mixture was centrifuged at 10 000 $\times$ *g* for 5 min at room temperature. Afterwards, 100 µl of the supernatant was transferred to a 96-well plate in duplicate containing 100 µl of 1 M NaOH per well. The OD was read at 450 nm in a microplate reader (Synergy HT). PBS instead of plasma and trypsin served as blank. Then the percentage inhibition of trypsin activity compared to the reference sample was calculated.

Bactericidal Activity

Plasma bactericidal activity was measured according to Graham & Secombes (1988), adapted by Machado et al, (2015) and validated and adjusted to the experimental species. Briefly, 20 µl of plasma were added in duplicate wells in a U-shaped 96-well microplate. Positive control was prepared by adding HBSS instead of plasma to chosen wells. Each well received 20 µl of Phdp (1 x 106 cfu ml^{-1}) and then incubated for 2.5 h at 25 ºC. Each well was added 25 µl of MTT (3-(4,5 dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide) (1 mg ml^{-1} ; 33 Sigma) and then incubated for 10 min at same temperature to allow formation of a precipitate. After incubation, the microplates were centrifuged at 2000 x g for 10 min and the precipitate was dissolved in 200 µl of dimethyl sulfoxide (Sigma). Absorbance was read at 540 nm. Bactericidal activity was obtained by the difference between the surviving bacteria in the positive control (100 %) and the surviving bacteria in plasma samples and was expressed as percentage.

IgM

Total serum IgM levels were evaluated using the enzyme-linked immunosorbent assay (ELISA) Cuesta et al, (2004). First, 100 µl per well of 1:100 diluted plasma in NaHCO3 (50mM, pH=9.6) was placed in flat-bottomed 96-well plates in triplicate. Then the proteins were coated for an hour incubation at room temperature. After the sample was removed and added 300 µl per well of blocking buffer (5% low-fat milk in T-TBS (0.1% Tween 20) follow by incubation of an hour at room temperature. After this time the blocking buffer was removed, and the plate was washed three times with T-TBS. Then 100 µl per well of diluted (1:200 in blocking buffer)

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anti-salmon IgM monoclonal antibody (Aquatic Diagnostics Ltd) was added and incubated for 1 hour. The antibody then is removed, and the plate was washed three times with T-TBS. 100 µl per well of the diluted (1:1000 in blocking buffer) mouse secondary antibody were added and incubated for 1 h. once again the antibody was removed, and the plate was washed three times with T-TBS. Finally, 100 µl per well of "TMB substrate solution for Elisa" were added and incubated for 5 minutes then the reaction was stopped with 100 µl per well of H2SO4 (2M), and the plates were read at 450 nm. Negative controls consisted of samples without plasma, then these OD values were subtracted for each sample value.

3.10 Fish welfare

Fish welfare was examined in the Salmon trial using the GIFAS Animal Welfare Scoring, described on Annex 1 of the GAIN Task 1.3 and resumed on Table 9.

Table 10: GIFAS Animal Welfare Scoring

Parameter	**Welfare score**				
	0	**1**	**2**	**3**	**4**
Condition	Good	Normal (wild salmon-like)	Slender/ malnourished		
Spine	None	Deformed – normal weight	Deformed - malnourished		
Fins	Normal	Slight damage	Moderate damage	Severe damage with visible sore and inflammation	
Skin	None	Slight scale loss	Significant scale loss	Surface wound <1cm^2	Large, life-threatening open wounds
Eyes	Healthy	One eye damaged: cataracts, bulging eye, bleeding	Both eyes damaged: cataracts, bulging eye, bleeding		
Gills	Healthy	Slight visible defects – pale lamellae and damage	Serious defects – bleached gills, severe erosion, etc		

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Operculum	Normal	Slight erosion (gills slightly exposed)	Servere erosion		
Snout	Normal	Minor surface wound	Wound with bleeding		
Underbite	None	Minor deformity	Clear deformity	Extreme deformity	
Overbite	None	Minor deformity	Clear deformity	Extreme deformity	

3.11 Oxidative Stress

Liver homogenization

Liver homogenization followed the procedures described in Fernandes et al. (2017) with minor modifications. Briefly, samples of liver with 76.2 ± 9.5 mg were homogenized with 1 600 µl of ultrapure H_2O on a tissue homogenizer (Precellys 24 homogenizer, Bertin) for 2 cycles of 15 seconds each at 6 000 x g. Then for the lipid peroxidation analyses a 2 ml eppendorf with 200 µl of homogenize liver added 4 µl of 4% BHT (2,6-Di-tert-butyl-4-methyl phenol, dissolved in methanol. Samples were immediately frozen at -80 ºC, until necessary again.

Protein quantification

Protein quantification of liver samples were done based on the protocol of Pierce ® BCA Protein Assay Kit (Scientific, 2015). Briefly, is necessary prepare diluted albumin standards (BSA): i) 0 mg/mL, ii) 0.025 mg/mL, iii) 0.25 mg/mL, iv) 0.5 mg/mL, v) 0.75 mg/mL, vi) 1 mg/mL, vii) 1.5 mg/mL, viii) 2 mg/mL. Pipette 25 µL of each standard or sample in duplicate into a 96-well plate, then add 200 µL of the reaction reagent to each well and mix plate thoroughly on a plate shaker for 30 seconds. Cover the plate and incubate at 37 °C for 30 minutes. Finally cool the plate at room temperature and measure the absorbance at 562 nm.

Catalase (CAT)

CAT activity was measured following the method described by Oliveira et al. 2010 (Oliveira et al., 2010). The degradation rate of the substrate H_2O_2, monitored at 240 nm for 2 minutes (each read every 15 seconds interval). The reaction mixture consisted of 248 µl of 30% H_2O_2 (substrate), 30 ml of 0.05 M K-phosphate buffer, pH=7.0 and 10 µl of each sample. Enzymatic activities were determined in triplicate.

Lipid peroxidation (LPO)

LPO levels were determined following the method described by Oliveira et al. 2015 (Oliveira, Cardoso, Soares, & Loureiro, 2015). Briefly, 100 µl of cold TCA 100% were added to each LPO sample and vortex, then 1mL of TBA 0.73% were added to all samples, blanks and vortex. After that samples where incubated for 1 hour at 100 ºC in an oven, and then centrifuge for 5minutes at 11500 rpm. Finally, the supernatant was pipette to a microplate (200 µl) in triplicate. The absorbance was measured at 535 nm and LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein (Oliveira et al., 2015).

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3.12 Mucosal Mapping

Tissue (0.5 cm) from the dorsal skin and foregut were sampled and placed in cassettes. The cassettes were stored in 4% phosphate buffered formalin in order to fix the samples and then sent to Quantidoc (Bergen, Norway). Mucosal Mapping™ was applied to detect possible changes in the fish tissues. Detailed methodology of samples processing, and staining are described in Pitman et al. (2013). Two measurements were obtained from intestinal samples (I and II) and then the barrier status was calculated following the equation bellow (III).

I. Mucous cell area (A): Average size of mucous cells from >100 cells/section
II. Mucous cell density (D): % of tissue area (epithelium) filled with mucous cells
III. Barrier status = (1/ (A:D))x1000

3.13 Apparent digestibility measurements

At the end of the trial feed and feces were analyzed to determine the apparent digestibility coefficients (ADCs) using yttrium an inert tracer as an indirect method.
ADCs of the dietary nutrients were calculated as follows:

$$\mathrm{ADC} = 100 - 100 * \left(\left(\frac{\%\ \text{dietary marker}}{\%\ \text{faecal marker}} \right) * \left(\frac{\%\ \text{faecal nutrient}}{\%\ \text{dietary nutrient}} \right) \right)$$

3.14 Nutrient retention

a) Nutrient Gain

$$\text{Nutrient Gain} = (\text{final weight} * \%\ \text{final nutrient}) - (\text{initial weight} * \%\ \text{initial nutrient})$$

b) Nutrient Retention

$$\text{Nutrient Retention} = 100 * \left(\left(\frac{\text{Nutrient Gain}}{\text{Feed Consumption } (\text{g}\frac{\text{DM}}{\text{fish}})} \right) * (\%\ \text{nutrient in feed}) \right)$$

3.15 PCR-array analysis

Total RNA from liver and head kidney was extracted using a TRIzol RNA Isolation (Invitrogen by Thermo Fisher Scientific, Carlsbad, CA, USA).

Two different 96-well PCR-array layouts were designed for the simultaneous profiling of several genes. The qPCR reactions were performed using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA). Diluted RT reactions were conveniently used for qPCR assays in a 25 µL volume in combination with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and specific primers at a final concentration of 0.9 µM. The program used for PCR amplification included an initial denaturation step at 95ºC for 3 min, followed by 40 cycles of denaturation for 15 s at 95ºC and annealing/extension for 60 s at 60ºC. All the pipetting

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operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>92%) was checked, and the specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5ºC/10s over a temperature range of 55-95ºC) and linearity of serial dilutions of RT reactions (>0.99). Fluorescence data acquired during the extension phase were normalized by the delta-delta CT method (Livak and Schmittgen, 2001) using beta-actin as housekeeping gene due to its stability among different experimental conditions (average CT among experimental groups varied less than 0.2 in liver and head kidney). Data on multigene expression were analysed by one-way and two-way analysis of variance (ANOVA), followed by Student Newman-Keuls (SNK) test. Fold changes in gene expression between experimental and control fish were analysed by Student t-test, using SigmaPlot v14 (Systat Software Inc., San José, CA, USA). Genesis software (Sturn et al, 2002) was used to generate heat maps of data gene expression.

3.16 Gene expression analysis

Total RNA from L, HK, and PI was extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA). The RNA yield was higher than 3.5 µg with absorbance measures ($A_{260/280}$) of 1.9-2.1. Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) using random decamers and 500 ng of total RNA in a final volume of 100 µL. Reverse transcription (RT) reactions were incubated 10 min at 25°C and 2 h at 37°C. Negative control reactions were run without the enzymeFor each array, qPCR reactions were performed using an iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA). Diluted RT reactions (×6) were used for qPCR assays in a 25 µL volume in combination with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and specific primers at a final concentration of 0.9 µM. The program used for PCR amplification included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (> 92%) was checked, and the specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5°C/10 s over a temperature range of 55-95°C), and linearity of serial dilutions of RT reactions (r^2 > 0.98). Fluorescence data acquired during the extension phase were normalized by the delta-delta C_T method (Livak and Schmittgen, 2001), using beta-actin as housekeeping gene due to its stability in different experimental conditions (average C_T between experimental groups varied less than 0.2).

3.17 DNA extraction from mucus samples

Posterior intestine mucus samples (200 µl) were treated with 250 µg/ml of lysozyme (Sigma) for 15 min at 37°C. Then, DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) following the manufacturer's instructions. DNA concentration, quality and purity were measured using a Nanodrop 2000c (Thermo Scientific) and agarose gel electrophoresis (1% w/v in Tris-EDTA buffer). DNA was stored at -20°C until sequencing.

3.18 Illumina MiSseq sequencing and bioinformatics analysis

The V3-V4 region of the 16S rRNA gene (reference nucleotide interval 341-805 nt) was sequenced using the Illumina MiSeq system (2 x 300 paired-end run) at the Genomics Unit

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from the Madrid Science Park Foundation (FPCM). The details on the PCR and sequencing of amplicons are described elsewhere (Piazzon et al., 2019). Raw forward and reverse reads were quality filtered using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and pre-processed using Prinseq (Schmieder and Edwards, 2011). Terminal N bases were trimmed in both ends and sequences with > 5% of total N bases were discarded. Reads that were < 150 bp long, with Phred quality score < 28 in both of the sequence ends and with a Phred average quality score < 26 were excluded. Then, forward and reverse reads were merged using fastq-join (Aronesty, 2013).

Bacteria taxonomy assignment was performed using the Ribosomal Database Project (RDP) release 11 as a reference database (Cole et al., 2014). Reads were aligned with a custom-made pipeline using VSEARCH and BLAST (Altschul et al., 1990; Rognes et al., 2016). Alignment was performed establishing high stringency filters (≥ 90% sequence identity, ≥ 90% query coverage). Taxonomic assignment results were filtered and data were summarized in an operational taxonomic units (OTUs) table. Sample depths were normalized by total sum scaling and then made proportional to the total sequencing depth, following the recommendations previously described (McKnight et al., 2019).

3.19 Inferred metagenome and pathway analysis

Piphillin was used to normalize the amplicon data by 16S rRNA gene copy number and to infer metagenomics contents (Iwai et al., 2016). This analysis was performed with the OTUs significantly driving the separation by diet in the PLS-DA analysis (described in the Statistics section). For the analysis, a sequence identity cut-off of 97% was used, and the inferred metagenomics functions were assigned using the Kyoto Encyclopedia of Genes and Genomes database (KEGG, Oct 2018 Release). Raw KEGG pathway output from Piphillin was analyzed with the R Bioconductor package DESeq2 using default parameters, after flooring fractional counts to the nearest integer (Piazzon et al., 2020; Love et al., 2014; Bledsoe et al., 2016).

3.20 Statistical analysis

Data for growth performance parameters, immune parameters, digestibility and retentions are presented as mean of four replicates ± standard deviation. Prior to ANOVA, values expressed as percentage were subjected to arcsin square root transformation. Statistical significance was tested at 0.05 probability level. All statistical tests were performed using the R statistics software (version 21) and STATISTICA (version 13, TIBCO Software Inc.).
For gene expression analysis data on multigene expression were analysed by one-way and two-way analysis of variance (ANOVA), followed by Student Newman-Keuls (SNK) test. Fold changes in gene expression between experimental and control fish were analysed by Student t-test, using SigmaPlot v14 (Systat Software Inc., San José, CA, USA). Genesis software (Sturn et al, 2002) was used to generate heat maps of data gene expression

For microbiome analysis rarefaction curves (plotting the number of observed taxonomic assignations against the number of sequences), species richness estimates and alpha diversity indexes were obtained using the R package phyloseq (McMurdie and Holmes, 2013). Differences in species richness, diversity indexes and phylum abundance were determined by Kruskal-Wallis test using the Dunn's post-test, with a significance threshold of $P < 0.05$. Beta diversity across groups was tested with permutational multivariate analysis of variance

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(PERMANOVA) using the non-parametric method adonis from the R package Vegan with 10,000 random permutations. To study the separation among groups, supervised partial least-squares discriminant analysis (PLS-DA) and hierarchical clustering of samples were sequentially applied using EZinfo v3.0 (Umetrics, Umea, Sweden) and R package ggplot2, respectively. The contribution of the different genes to the group separation was determined by the minimum variable importance in the projection (VIP) values achieving the complete clustering of the conditions with a VIP value of 1. Hotelling's T2 statistic was calculated by the multivariate software package EZinfo v3.0. The quality of the PLS-DA model was evaluated by the parameters R2Y (cum) and Q2 (cum), which indicate the fit and prediction ability, respectively. To assess whether the supervised model was being over-fitted, a validation test consisting on 500 random permutations was performed using SIMCA-P+ (v11.0, Umetrics). For the OTU-gene correlations, the expression values of the differentially expressed genes ($P < 0.05$) from the three tissues and the normalized counts values from the OTUs driving the separation in the PLS-DA model (VIP ≥ 1) for each individual fish were used (18 samples in total, 9 CTRL and 9 NoPAP SANA). The Spearman rank correlation coefficients and the corresponding P values were calculated with the cor.test() function from the corrplot R package (Wei, 2013) with two-sided alternative hypothesis. Significant gene-OTU correlations were accepted at a $P < 0.01$ and visualized with corrplot package. A correlation network was built using Cytoscape v3.8.2 (Smoot et al., 2011).

For the parasite challenge of Bream2 trial, parasitological variables studied were prevalence of infection (percentage of infected fish in a sampled group) and intensity of infection (median Ct values of fish that were PCR positive for the parasite). Each individual was treated as a replicate and each group included all the fish (replicate tanks were not treated individually, as no tank effect was detected). Kruskal-Wallis test (Dunn's post-test) was used to determine differences in intensity values. Statistical significance was considered at $P < 0.05$. To evaluate the recovery trend observed between the intermediate (40 dpe) and final samplings (78 dpe) the individual ΔCt values (Cts at 78 dpe – Ct at 40 dpe) were plotted against the Ct values at 40 dpe and the regression lines for each diet group were calculated.

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4. Performance of Block 1 trials

This section will present main results of growth performance, body composition, nutrient retention, and apparent digestibility of four trials with Trout, Salmon, Seabream and Turbot, respectively. Additionally, specific analyses depending on the trial objective were performed and are presented in section *5 Additional specific analysis Block1.*

4.1 Trout 1

4.1.1 Growth performance parameters

At the end of the trial (97 days of experimental feeding; Table 11), just one dead fish was observed in PAP treatment. Final body weight (FBW) ranged between 335 and 353 grams, which represents an average of 5.4-fold increase of initial body weight (IBW). No statistical difference was found in the growth parameters calculated (P>0.05).

Table 11: Growth performance after 77 days of feeding.

Parameter	Ctrl	NoPAP	PAP	Mix
IBW (g)	62.36±1.06	63.33±0.60	64.07±1.96	62.85±1.11
FBW (g)	353.04±17.50	341.86±16.45	349.22±13.62	335.14±14.33
Weight gain (g)	290.68±16.58	278.53±16.07	283.34±12.14	272.29±13.65
RGR (%/day)	1.79±0.04	1.74±0.04	1.75±0.01	1.72±0.04
FCR	0.76±0.02	0.78±0.02	0.78±0.01	0.79±0.02
FI, %ABW/d	1.10±0.01	1.11±0.01	1.11±0.01	1.12±0.01
PER	3.06±0.08	2.97±0.07	2.97±0.03	2.93±0.06

Values are means and standard deviation (n=4).

4.1.2 Whole body composition

Data on the whole body composition of fish at the end of the trial are presented in Table 12. Dietary treatments had no effect on the whole-body composition of fish in terms of moisture, ash, protein, fat and energy (P>0.05).

Table 12: Whole body composition (DM) of fish fed the various dietary treatments.

	Ctrl	NoPAP	PAP	MIX
DM, %	33.54±0.44	34.77±0.19	34.46±1.22	34.10±0.57
Ash, %	4.69±0.73	4.50±0.64	4.50±0.36	4.55±0.55
Protein, %	48.50±0.47	47.45±1.11	46.40±0.87	46.44±1.13
Fat, %	41.23±1.19	42.54±1.65	38.81±3.01	41.37±1.80
Energy, kJ/g	27.12±0.57	27.93±0.38	27.95±0.37	27.41±0.81

Values are means and standard deviation (n=4).

Initial fish: dry matter 23.55%; ash 6.25%; protein 67.61%; fat 23.52%; energy 24.50kJ/g.

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4.1.3 Nutrient retention

Values for nutrient and energy retention (expressed as percentage of intake) are presented in Table 13. Fish fed new formulated diets did not present statistical differences in Fat and Energy retention (P>0.05). However, fish fed Mix diet presented lower protein retention when compared to control diet.

Table 13: Nutrient and energy retention of trout fed the various dietary treatments.

Retention	Ctrl	NoPAP	PAP	MIX
Protein, %	50.07±1.56[a]	48.58±1.50[a]	48.18±0.80[a]	44.85±1.51[b]
Fat, %	103.11±4.28[a]	107.70±4.74[a]	87.65±6.09[b]	82.74±2.65[b]
Energy, %	57.25±3.01	61.03±1.52	58.55±1.71	56.89±1.47

Different letters indicate statistical differences among dietary groups. Values are means and standard deviation (n=4).

4.1.4 Mineral retention

Values for mineral retention (expressed as percentage of intake) are presented on Table 14. Fish fed new formulated diets presented lower retention of cupper (Cu) and Iron (Fe), when compared to control. On the other hand, fish fed new formulations presented higher retention for potassium (K), mafnesium (Mg), manganese (Mn), Phosphorous and selenium (Se).

Table 14: Mineral retention for trout fed various dietary treatments.

Minerals	CTRL	NoPAP	PAP	MIX
As	68.17±5.11	<LOQ	<LOQ	<LOQ
Ca	17.18±8.96	31.12±13.80	26.59±11.53	27.44±12.96
Cu	11.24±1.47[a]	6.35±0.42[bc]	7.67±0.47[b]	5.81±0.34[c]
Fe	6.22±0.09[a]	3.95±0.35[c]	4.54±0.21[b]	4.14±0.21b[c]
K	48.15±2.80[d]	87.57±2.41[c]	111.37±4.43[a]	100.53±5.88[b]
Mg	19.76±0.28[a]	23.83±1.31[b]	28.79±2.51[a]	22.88±1.12[bc]
Mn	1.05±0.40	0.91±0.32	1.10±0.26	0.79±0.23
Na	19.70±0.57[b]	20.24±1.65[b]	25.36±0.98[a]	25.59±1.67[a]
P	31.61±4.49	35.16±6.14	41.67±7.89	34.95±4.89
Se	53.21±1.75[ab]	59.99±9.97[a]	61.57±8.09[a]	44.90±4.67[b]
Zn	11.85±1.62	14.53±2.88	13.34±1.08	14.43±0.80

Different letters indicate statistical differences among dietary groups. Values are means and standard deviation (n=4).

4.1.5 Fatty acid retention

Fatty acid retention (expressed as percentage of intake) are shown on Table 15. Results revel there was difference in fatty acids retentions among groups. Fish fed NoPAP diet had higher retention for total saturated ans monounsaturated fatty acids when compared to PAP and MIX group.

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Table 15: Fatty acid retention for trout fed various dietary treatments.

Fatty acid	CTRL	NoPAP	PAP	MIX
Total saturated	171.6±18.7[ab]	194.0±27.8[a]	141.3±7.2[b]	141.8±20.3[b]
Total monounsaturated	124.43±21.33[ab]	154.99±25.4[a]	112.89±8.53[b]	106.3±12.1[b]
20:5n-3	7.4±0.66	13.17±2.68	9.77±4.72	12.93±4.52
22:6n-3	10.41±3.45	13.56±2.01	6.71±3.27	19.73±12.81
n-3 HUFA (DHA+EPA)	8.56±1.7	13.34±1.74	8.36±3.9	15.83±7.99
Total n-3 PUFA	11.16±4.62	20.23±4.65	11.52±3.4	16.43±8.26
Total n-6 PUFA	52.56±6.19	65.61±16.94	46.83±10.21	54.62±12.72
Total PUFA	30.11±5	42.28±8.96	28.32±6.63	33.43±10.1
20:4n-6	10.28±20.56	10.02±20.05	7.82±15.64	22.4±31.53
C18:2n-6	49.23±7.38	61.66±13.77	44.25±7.71	51.95±10.25
C18:3n-3	14.78±6.97	23.6±3.27	14.31±2.54	16.47±6.55

Different letters indicate statistical differences among dietary groups. Values are means and standard deviation (n=4).

4.1.6 Apparent digestibility

Apparent digestibility for protein, fat and energy was not different in trout fed formulated diets when compared to control (Table 16).

Table 16: Percentage of protein, energy and fat apparent digestibility of fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NoPAP – Circular economy driven formula algae without processed animal protein.

ADC (%)	Ctrl	NoPAP	PAP	MIX
Protein, %	88.4±4.1	89.2±0.3	86.7±0.6	85.1±0.3
Fat, %	97.2±1.1	96.6±2.5	98.1±1.2	98.2±0.8
Energy, %	85.8±2.0	86.3±2.8	89.1±1.3	88.1±0.6

Values are means and standard deviation (n=4).

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4.2 Bream 1

4.2.1 Growth performance parameters

At the end of the trial (77 days of experimental feeding; Table 17), no mortality was observed. Final body weight (FBW) ranged between 128 and 136 grams and which represents an average of 2.4-fold increase of initial body weight (IBW) without significan diferences (p=0.885). However, fish fed diet control showed a significantly lower FCR and higher PER than those fed diets with PAP and MIX diets (p<0.0001). NOPAP also had a somewhat higher FCR. No statistical differences were found amongst the various NOPAP, PAP, Mixed, and control in terms of FBW (p=0.070), RGR (p=0.104), weight gain (p=0.090) and voluntary feed intake (VFI) (p=0.082).

Table 17. Growth performance of fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NOPAP – Circular economy driven formula algae without processed animal protein.

	CTRL	MIX	PAP	NoPAP
IBW (g)	56.22±0.80	55.91±0.53	55.80±0.35	55.81±0.33
FBW (g)	136.69±3.27	132.04±0.85	128.43±3.88	134.63±6.35
Weight gain (g)	80.47±2.88	76.13±0.77	72.63±4.20	78.82±6.48
RGR (% BW/d)	1.16±0.03	1.12±0.01	1.09±0.05	1.15±0.07
VFI (%/day)	1.63±0.06	1.76±0.03	1.76±0.07	1.70±0.11
PER	1.57± 0.01^{a}	1.39± 0.04^{b}	1.33± 0.01^{bc}	1.50± 0.03^{a}
FCR	1.40± 0.02^{a}	1.56± 0.02^{b}	1.62± 0.02^{c}	1.48± 0.03^{d}

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

4.2.2 Whole-body composition

Data on the whole-body composition of fish at the end of the trial is presented in Table 18. Dietary treatments had no effect on the whole-body composition of fish (P>0.05).

Table 18. Whole-body composition of fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NOPAP – Circular economy driven formula algae without processed animal protein (n=4).

Composition (DM%)	CTRL	MIX	PAP	NoPAP
DM, %	36.16±0.67	35.90±2.38	36.67±0.32	36.71±0.90
Ash, %	9.12±1.18	10.43±0.94	10.00±1.28	10.60±1.14
Protein, %	47.50±0.91	47.01±1.25	45.77±1.28	47.08±0.84
Lipid, %	40.90±0.63	40.15±1.72	40.10±1.70	40.47±1.02
Energy, kJ/g	26.10±0.35	25.74±0.61	25.55±0.19	26.04±0.35
Phosphorus, %	1.88±0.17	1.91±0.06	1.97±0.08	1.81±0.08

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test) n=4.

4.2.3 Apparent digestibility

Apparent digestibilities coeficients (ADC) of energy, protein and phosphorus of fish fed with four different dietary groups are displayed on Table 19. Dietary treatments had no effect on

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the apparent digestibility of energy and phosphorous (p>0.05), but affected protein ADC (p=0.002). Fish fed with the control diet presented a significantly lower protein apparent digestibility when compared to the novel feed formulations.

Table 19. Protein, energy, and phosphorus apparent digestibility coefficients of fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NoPAP – Circular economy driven formula algae without processed animal protein.

	CTRL	MIX	PAP	NoPAP
Protein	58.32± 5.32^{a}	72.07± 6.23^{b}	73.20± 3.36^{b}	74.02± 4.34^{a}
Energy	71.50±1.57	68.06±1.81	67.87±2.55	68.35±3.47
Phosphorus	40.43±5.83	46.47±8.24	42.30±6.63	48.19±2.05

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test) n=4.

4.2.4 Nutrient Retention

Results of protein, lipids, energy, and phosphorous retention of fish fed with different diets are shown in Table 20. Dietary treatments affected the retention levels of protein. Fish fed with the control diet presented higher percentages of retention when compared to the novel feed formulations, and significantly higher when compared to the Mix and PAP diet.

Table 20. Protein, lipids, energy, and phosphorous retention of fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NoPAP – Circular economy driven formula algae without processed animal protein (n=16). Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

Retention (%)	CTRL	MIX	PAP	NoPAP
Protein	28.19±1.15^{ac}	24.42±2.46^{bc}	23.20±1.39^{b}	27.31±0.66^{abc}
Lipids	73.32±1.88	73.08±12.40	69.69±4.29	76.61±5.01
Energy	33.96±0.67	30.61±4.50	30.32±0.32	33.14±2.04
Phosphorus	31.23±5.31	29.70±4.22	29.40±1.76	32.77±1.80

4.2.5 Mineral Retention

Mineral retention (expressed as percentage of intake) is shown o Table 21. Differences were detected among the treatments. Fish fed MIX and PAP diets presented lower retention for Iron (Fe), magnesium (Mg) and sodium (Na) when compared to control. Fish fed MIX diet also presented lower cupper (Cu) retention. Otherwise, seabream fed NoPAP diet had higher potassium (K) retention when compared to control.

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Table 21: Mineral retention for bream fed different dietary treatments. Values are means and standard deviation (n=4).

Mineral	CTRL	MIX	PAP	NO PAP
P	31.23±5.31	29.70±4.22	29.40±1.76	32.77±1.80
As	24.69±19.18	16.75±13.50	12.02±4.88	35.45±28.47
Ca	36.44±9.06	43.79±8.21	37.06±3.72	49.29±2.42
Cu	3.22±0.58a	1.58±0.35c	2.12±0.08bc	2.70±0.78ab
Fe	3.09±0.78a	1.49±0.49b	1.76±0.36b	2.30±0.53ab
K	24.40±2.73b	24.98±2.90b	24.92±0.46b	34.17±3.31a
Mg	12.68±1.25a	9.38±1.41 b	10.45±0.27b	9.99±0.55b
Mn	1.87±0.39	1.49±0.29b	1.67±0.59ab	1.35±0.22b
Na	14.04±1.65a	10.30±2.37b	11.97±0.84b	10.23±0.27ab
Zn	4.66±0.36	3.97±1.04	4.47±0.43	4.67±0.26

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

4.3 Salmon 1

4.3.1 Growth performance parameters

Growth performance details after 96 days of experimental feeding are displayed in Table 23. No statistical differences were found amongst the diets for all parameters under study.

Table 22. Growth performance of fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein.

Parameter	CTRL	PAP	NoPAP
IBW (g)	151.96±1.78	151.74±1.57	152.60±1.51
FBW (g)	801.86±32.57	795.89±42.07	799.06±20.74
Weight gain (g)	649.90±32.13	644.15±43.43	646.46±21.78
RGR (% BW/day)	1.75±0.04	1.74±0.06	1.74±0.04
FCR	0.86±0.03	0.89±0.01	0.90±0.02
VFI (%/day)	1.51±0.08	1.55±0.05	1.56±0.04
PER	2.03±0.00	1.98±0.04	2.00±0.04
Survival (%)	98.41±2.61	98.64±1.57	99.32±1.36

Values are mean and standard deviation (n=4; one-way ANOVA).

4.3.2 Whole-body composition

Data on the whole-body composition of fish at the end of the trial is presented in Table 24. Dietary treatments did not affect the whole-body composition parameters.

Table 23. Whole-body composition of fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein.

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	CTRL	PAP	NoPAP
DM, %	32.22±2.01	32.19±1.78	32.26±2.62
Ash, %	7.70±0.61	8.62±0.74	6.12±1.24
Protein, %	49.29±0.51	49.78±0.57	51.08±0.94
Lipid, %	42.49±0.79	42.46±1.16	42.67±1.91
Energy, kJ/g	26.87±0.29	27.17±0.39	27.01±0.46

Values are mean and standard deviation (n=4; one-way ANOVA).

4.3.3 Apparent digestibility

Dietary treatments did not affect the protein and lipid apparent digestibility but affected the energy and phosphorous apparent digestibility (Table 25). Data show that energy apparent digestibility is significantly higher in CTRL when compared to PAP diet. Regarding phosphorous, the apparent digestibility is higher on fish fed with the NOPAP diet when compared to fish fed with the control and PAP diets.

Table 24. Protein, energy, and phosphorus apparent digestibility of fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy -driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein.

ADC (%)	CTRL	PAP	NoPAP
Protein	88.64±0.36	88.45±0.63	88.92±0.45
Lipids	99.05±0.07	99.06±0.25	99.27±0.16
Energy	87.32±0.46 [a]	86.00±0.86 [b]	86.94± 0.31[ab]
Phosphorous	55.54± 4.72[a]	62.78±10.74 [ab]	73.02± 2.31[b]

Different letters indicate statistical differences among dietary groups. Values are mean and standard deviation (n=4; one-way ANOVA; Tukey post hoc test).

4.3.4 Nutrient Retention

Results of protein, lipids, and energy retention of fish fed with different diets are shown in Table 26. Dietary treatments did not affect the nutrient retention levels.

Table 25. Percentage of protein, lipids, energy, and phosphorous retention of fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein

Retention (%)	CTRL	PAP	NoPAP
Protein	33.23±2.55	33.99±2.02	35.44±3.86
Lipids	57.72±3.38	55.74±3.12	55.18±6.90
Energy	39.05 ±2.76	38.68±2.69	39.45±4.48

Different letters indicate significant differences (one-way ANOVA; $p< 0.05$; Tukey post hoc test). (n=16).

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4.3.5 Fatty acid Retention

Fatty acid retention (expressed as percentage of intake) is shown on Table 27. Salmon fed PAP and NoPAP diets had higher total saturated fatty acid retention when compared to fish fed control diets.

Table 26: Fatty acid retention of salmon fed different dietary treatments.

Fatty acids	CTRL	PAP	NOPAP
Total saturated	61.47±6.78[a]	73.64±4.74[b]	74.03±6.44[b]
Total monounsaturated	69.56±7.43	68.74±5.78	72.60±6.86
20:5n-3	42.86±5.16	44.01±8.14	47.69±3.90
22:6n-3	57.81±4.32	61.14±6.30	67.92±5.80
n-3 HUFA (DHA+EPA)	49.15±4.31	51.85±6.37	56.90±4.70
Total n-3 PUFA	58.23±4.20	57.05±5.60	58.43±5.77
Total n-6 PUFA	62.63±6.90	60.98±4.40	68.27±6.66
Total PUFA	60.82±5.56	59.35±4.58	64.00±5.12
20:4n-6	35.44±37.91	48.82±49.58	12.03±53.41
C18:2n-6	*	*	*
C18:3n-3	55.89±7.18	48.56±4.79	50.48±1.60

Different letters means statistical differences among treatments. Values are means and standard deviation (n=4). *not detected.

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4.4 Turbot1

4.4.1 Growth performance parameters

At the end of the trial (112 days of experimental feeding) final body weight (FBW) ranged between 81.9 and 85 grams (Table 28), which represents an average of 4.1-fold increase of initial body weight (IBW). No statistical differences were found in the growth parameters calculated (P>0.05). However, differences were found on condition factor (CF) and hepatosomatic index (HIS).

Table 27: Growth performance after 112 days of feeding.

	Ctrl	NoPAP	PAP	Mix
IBW (g)	20.1±0.3	20.3±0.4	20.4±0.3	20.3±0.4
FBW (g)	85.2±9.7	82.0±9.5	82.9±6.1	81.9±7.3
Weight gain (g)	65.2±9.9	61.7±9.8	62.6±6.3	61.6±7.4
SGR (%/day)	1.26±0.10	1.22±0.12	1.23±0.08	1.22±0.08
FCR	0.85±0.0	0.89±0.03	0.90±0.02	0.85±0.08
FI, %ABW/d	1.05±0.04	1.07±0.05	1.08±0.05	1.05±0.08
PER	2.12±0.10	2.04±0.08	2.00±0.05	2.13±0.19
CF	2.11±0.01 [a]	2.07±0.02 [ab]	2.10±0.01 [a]	2.04±0.05 [b]
HSI	1.82±0.20 [a]	1.48±0.13 [b]	1.53±0.10 [b]	1.47±0.12 [b]

Different letters indicate statistical differences among dietary groups.

4.4.2 Whole body composition

Whole-body composition of turbot for dry matter (DM), ash, protein, fat, and energy are shown in Table 29. Fish fed PAP and MIX diet presented lower values for lipid composition when compared to control (p<0.05).

Table 28: Whole-body composition of fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein.

	CTRL	NoPAP	PAP	MIX
DM, %	25.87±1.26	25.55±3.53	23.42±0.89	23.31±1.21
Ash, %	14.35±0.59	16.75±1.16	16.53±0.61	16.88±0.81
Protein, %	62.44±2.09	62.73±2.04	65.19±1.83	63.01±2.06
Lipid, %	17.34±1.44 [a]	15.38±1.07 [ab]	13.56±0.66 [b]	15.04±0.83 [b]
Energy, kJ/g	20.18±1.74	20.89±0.55	20.86±0.51	19.94±0.36

Different letters indicate statistical differences among dietary groups. Values are mean and standard deviation (n=4; one-way ANOVA).

4.4.3 Apparent digestibility

The apparent digestibility coefficients (ADC) for dry matter, protein, and energy were significantly affected by dietary treatments (P < 0.05). Table 30 shows a significantly lower digestibility of protein, energy, and dry matter (P<0.05) in experimental diets when compared to control (based on commercial diets).

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Table 29: Apparent digestibility for dry matter, protein and energy Turbot fed with i) Control, ii) Mix, iii) PAP– Circular economy driven formula with processed animal protein, iv) NOPAP – Circular economy driven formula algae without processed animal protein. Different letters indicate significant differences (one-way ANOVA; p< 0.05, Tukey post hoc test).

ADC (%)	CTRL	PAP	NoPAP	MIX
Dry matter	83.2 ± 1.1[a]	77.1 ± 1.7[b]	77.2 ± 1.9[b]	77.3 ± 1.2[b]
Protein	92.2 ± 0.6[a]	90.2 ± 0.7[b]	90.1 ± 0.6[b]	89.3 ± 0.9[b]
Energy	88.0 ± 1.1[a]	85.3 ± 1.4[b]	85.7 ± 1.2[b]	85.4 ± 0.5[b]

*There was not enough sample to analyze fat in feces, thus no lipid digestibility was calculated.

5. Additional specific analysis Block1

5.1 Trout 1

5.1.1 Lysozyme

Trout fed with formulated diets did not present statistical differences in plasma´s lysozyme after the experiment (Figure 1).

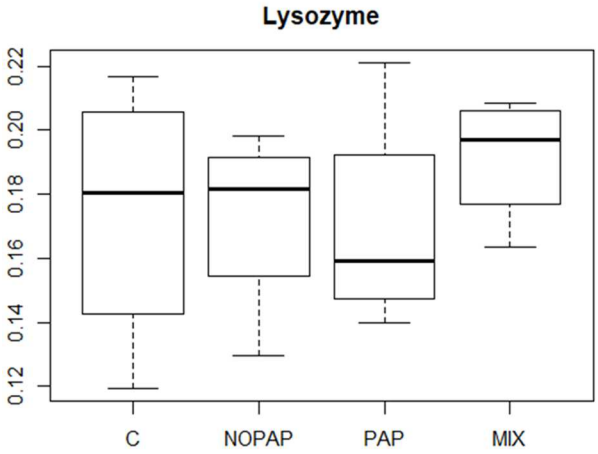

Figure 1: Boxplot for lysozyme results (absorbance average) of Trout´s plasma against *Micrococcus luteus*.

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5.1.2 Flesh quality

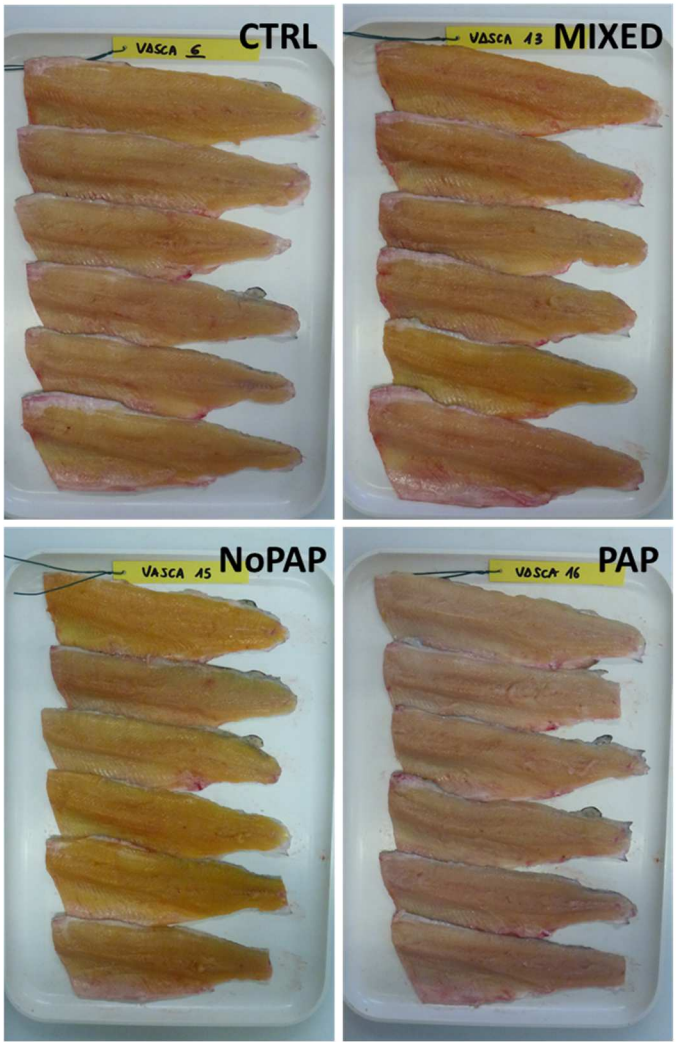

Figure 2: Morphological differences are detected on trout carcass (table 6), where gutted fish differ between diets. The control group reached the best yield with 88.78%, followed by NoPAP (87.92%), PAP (87.29%), and MIXED diet (86.77%).

Carcass yield (cY) is directly influenced by the viscera weight, in fact viscerosomatic index (vsi) is inversely related to cY (Table 31). So, Mixed diet reached the highest vsi (13.23%), then PAP (12.71%), NoPAP (12.08%), and CTRL, the lowest (11.22%). The same significative trend was observed in the hepatosomatic index (hsi). The liver was proportionally bigger in the MIXED diet compared to other experimental groups. No statistical differences were measured in the visceral fat. Textural features were measured on 96 filets by Texture Profile Analysis and are presented in table 32. The variables (hardness, cohesiveness, gumminess, resilience, and adhesiveness) showing no statistical differences between the four experimental diets. The most evident difference was the hardness of fish flesh fed PAP diet compared with the other groups.

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Table 30: Morphological indexes of trout after the experiment

Morphological indexes	NoPAP	CTRL	PAP	MIXED
tW	386.7± 56.06	409.8± 53.52	414.8± 55.77	397.9± 52.23
cY %	87.92± 1.43 ^{ab}	88.78± 1.46 ^{a}	87.29± 1.53 ^{bc}	86.77± 1.24 ^{c}
vsi %	12.08± 1.43^{bc}	11.22± 1.46 ^{c}	12.71± 1.53 ^{ab}	13.23± 1.24 ^{a}
hsi %	1.45± 0.27 ^{b}	1.20± 0.17 ^{c}	1.64± 0.24 ^{ab}	1.73± 0.26 ^{a}
VFI %	3.95± 1.21	3.56± 1.06	3.91± 0.94	4.16± 0.91

*Different subscripts refer to statistical differences (p<0.05) among diets.

Table 31: Texture of trout´s flesh after the experiment.

Texture	NoPAP	CTRL	PAP	MIXED
Hardness	4.89± 1.14	4.95± 1.42	5.38± 1.01	4.83± 0.75
cohesiveness	0.21± 0.02	0.21± 0.04	0.19± 0.02	0.20± 0.01
Resilience	0.026± 0.012	0.022± 0.017	0.026± 0.016	0.020± 0.011
Gumminess	1.00± 0.18	0.99± 0.24	1.05± 0.24	0.95± 0.17
Adhesiveness	0.50± 0.15	0.60± 0.25	0.54± 0.20	0.61± 0.16

Colour parameters (CIELab values) were significantly different among dietary treatments (Table 33). The lightness (L*) of PAP diet was statistically higher than other three diets, in contrast NoPAP has the lowest lightness but the highest value of red (a*) and yellow (b*) indexes. In the L*h*c* color space NoPAP obtained the most vivid chroma, followed by the Mixed and Control diets and last the PAP, where the colour intensity is dullest. About the colour Hue, NoPAP and CTRL have a similar shade, and statistically different from MIXED and PAP diet, the last one has the smallest Hue angle of the group. A pale grey or brown colour is one feature of the flesh of farmed rainbow trout fed without carotenoids. In this experiment, an evident yellow pigmentation of filets has been observed in trout fed with NoPAP diet compared to the other experimental groups. In CTRL and MIXED diet is evident the presence of the yellow hue too, but a less vivid chroma softened the colour shift in the eyes of observer. In contrast, trout fed PAP diet shows a pale pink-gray flesh colour, considered a more natural colour by consumers. Ingredients such as corn meal, micro and macro algae mix are rich in natural pigments, in particular xanthophylls (lutein and zeaxanthin) that could be responsible for the yellow colour, whereas other carotenoids, like astaxanthin and canthaxanthin, account for orange/pink hues, more favourable according to consumer acceptance.

Table 32: Colour outputs of trout´s flesh after the experiment.

Colour	NoPAP	CTRL	PAP	MIXED
L*	42.61± 2.11 ^{c}	43.94± 2.53 ^{b}	45.14± 2.27 ^{a}	43.76± 2.05 ^{b}
a*	4.35± 2.30 ^{a}	2.69± 2.37 ^{c}	3.13± 2.33 ^{bc}	3.46± 2.14 ^{b}
b*	15.46± 4.58 ^{a}	9.19± 3.27 ^{b}	5.26± 2.59 ^{c}	9.42± 3.47 ^{b}
Chroma ab	16.12± 4.94 ^{a}	9.69± 3.75 ^{b}	6.24± 3.27 ^{c}	10.13± 3.82 ^{b}
HUE° ab	74.88± 4.98 ^{a}	76.04± 8.77 ^{a}	61.17± 11.61 ^{c}	70.64± 8.91 ^{b}
ECI	8.37± 4.08 ^{a}	5.03± 4.05 ^{c}	5.32± 3.44 ^{c}	6.50± 3.69 ^{b}

*Different subscripts refer to statistical differences (p<0.05) among diets.

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Pigments deposition into the filet was not homogeneus because carotenoids are lipophilic molecules. Differences are evident along the filet cranial-caudal axis. In this case, the Entire Colour Index (ECI) could be the most appropriate colour index (Pavlidis et al., 2006). In fact Control diet and PAP has an ECI index similar (5.03 and 5.32 respectively) and not statistically different, and this couple of diets is statistically different from Mixed diet (6.50) and NoPAP (8.37).

4.2 Bream 1

4.2.1 Humoral immune parameters

Data concerning humoral immune parameters measured on plasma are displayed on Table 34. Protease activity and bactericidal activity parameters showed no significant differences among diets. Anti-protease activity was significantly lower in MIX compared to CTRL and PAP. Immunoglobulin activity was highest in MIX diet, with significant differences between PAP and MIX diets.

Table 33. Humoral parameters activity in fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NOPAP – Circular economy driven formula algae without processed animal protein (n=40).

Parameters	CTRL	MIX	PAP	NoPAP
Protease (%)	7.27±3.07	7.69±3.52	7.73±3.35	8.02±3.01
Anti-protease (%)	81.33± ^{a}2.07	78.96± 5.31^{b}	81.13± ^{a}1.40	79.86± 3.60^{ab}
IgM (absorbance values)	0.29 ±0.08^{ab}	0.35± 0.11^{a}	0.27± 0.14^{b}	0.33± 0.16^{ab}
Bactericidal activity (%)	30.93±7.48	29.47±6.06	31.38±6.70	35.69±18.91

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

4.2.2 Mucosal Mapping

Results of mucosal mapping are displayed in Table 35. There where were no significant differences between diets on the average size of mucous cells from >100 cells per section in the fish foregut. The highest percentage of mucus cell density was found in the fish fed with PAP diet and the lowest in the ones fed with the NoPAP diet. NoPAP presented significantly lower mucus density compared to Control and PAP. NOPAP presented a significantly lower barrier status compared to Control and PAP.

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Table 34. Mucosal mapping data regarding fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NOPAP – Circular economy driven formula algae without processed animal protein (n=15).

Mucosal Mapping	CTRL	MIX	PAP	NOPAP
Mucous Cell Area	138.00±12.82	125.36±16.40	128.73±11.97	126.00±17.19
Mucous Density (%)	0.06±0.01^{a}	0.05±0.01^{ab}	0.07±0.01^{b}	0.04±0.01^{ac}
Barrier Status	0.44±0.06^{a}	0.41± .09^{ab}	0.51±0.12^{b}	0.35±0.08^{ac}

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

4.2.3 Oxidative status

Data on oxidative status biomarkers are displayed in Table 36. Catalase presented significant differences between the control diet and the novel ones, being higher on the control one. Results of lipid peroxidation measured in the liver of fish from experimental groups did not present significant differences.

Table 35. Oxidative status biomarkers in fish fed with four different dietary groups: i) Control, ii) MIX, iii) PAP– Circular economy driven formula with processed animal protein, iv) NOPAP – Circular economy driven formula algae without processed animal protein (n=40).

	CTRL	MIX	PAP	NOPAP
Catalase (U/mg protein)	46.37± 9.35^{a}	28.67± 10.71^{b}	32.05± 15.19^{b}	36.42± 24.21^{b}
LPO (nmol/g)	12.81±1.25	13.54±2.15	12.68±0.98	13.14±1.44

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

4.2.4 Hepatic gene expression profiling

Data were in reference to the expression level of *cyp7a1* of control fish with an arbitrarily assigned value of 1. Ten out of 42 genes in the array were differentially expressed (DE) at P<0.05, including this set of genes markers of growth performance (*igf-i*), energy metabolism (*cpt1a, cs*), antioxidant defense (*sod2*) as well as a wide representation of lipid-related genes, including elongases (*elovl4, elovl5, elovl6*), desaturases (*scd1a, scd1b*), lipases (*hl*) and nuclear transcription factors (*pparα*).

Overall, the gene expression level was highest in NOPAP fish, whereas a down-regulated response was found in PAP fish with values in the MIX group that are closer to the control group than in the other two experimental groups. This was visualized by heat map analysis (Figure 3) that cluster together control and MIX groups, whereas PAP and NOPAP groups exhibited an opposite response with increased expression levels of *elovl6, scd1a, scd1b, fads2* and a consistent down-regulated expression of *pparα* in NOPAP fish. By contrast, PAP fish showed a down-regulated response in comparison to control fish that was

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statistically significant for *elovl4, elovl5, hl, cpt1a* and *cs*. A similar trend, but not statistically significant at P<0.05 was found for *ghr-i*, *fads2* and *scd1a*.

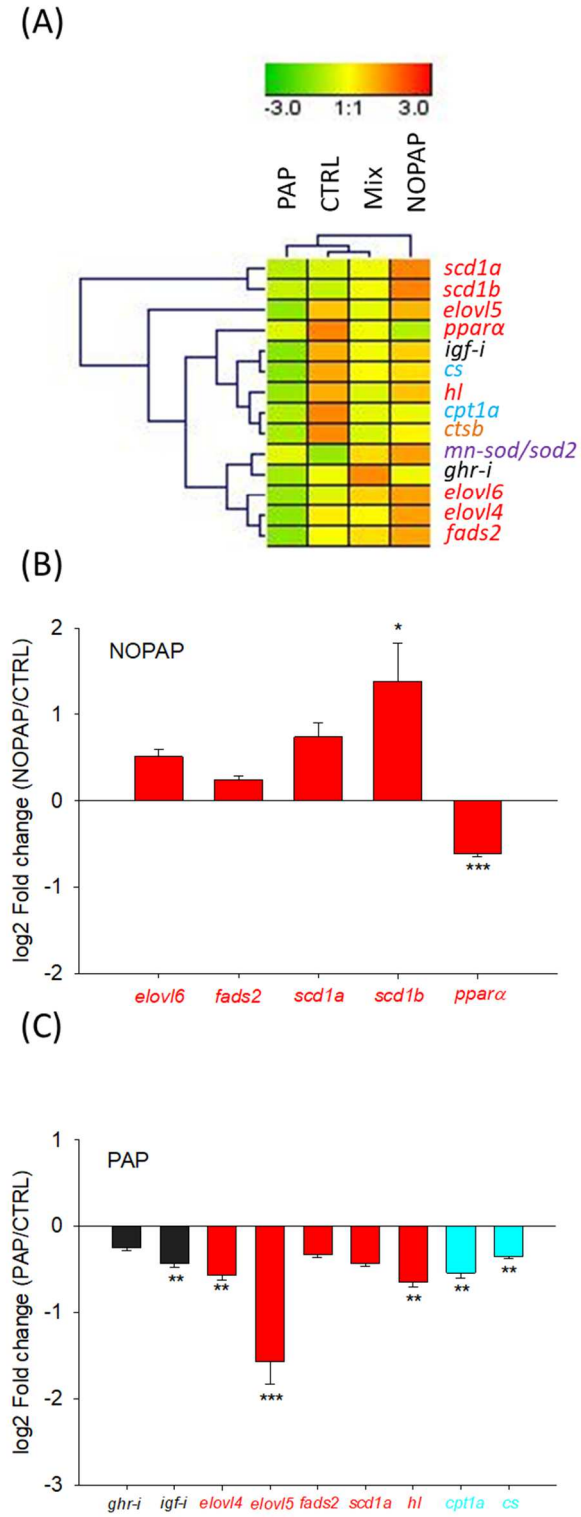

Figure 3. Heat map of liver gene expression profile after filtering for most nutritionally regulated genes (A). Fold changes of changing expressed genes (experimental/control fish) in NOPAP (B) and PAP (C) fish. Values are mean ± SEM of 10-12 fish. Statistically significant differences by Student-t test (*, P<0.1; **, P<0.05; ***, P<0.001).

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4.2.5 Head kidney gene expression profiling

Nine out of 29 genes were DE at P<0.05 by nutritional regulation in the head kidney of seabream juveniles fed control, NoPAP, PAP or MIX diets (Figure 4). As a general trend, *il-8* was up-regulated in NoPAP, PAP and MIX groups. However, a more divergent expression pattern was evidenced for the genes of the array. Thus, PAP group shared a clear pro-inflammatory profile evidenced by the up regulation of *il-8* and other cytokines (*il-1β, tnf-α*) and chemokines (*ck8*) and chemokine receptors (*ccr3)*. The same pattern was found for T-cell markers (*cd3x, cd4-full, cd8a*), whereas the expression of the mucosal *igt-m* was consistently down-regulated. Both MIX and NOPAP MIX fish showed a more attenuated response, being reduced the number of DE genes to *il-8* in NoPAP fish, and to *il-8*, *il-1β* and *ck8* in MIX fish. The magnitude of change was also reduced in these two groups of fish as evidenced heat map analyses and fold change representations in Figure 26.

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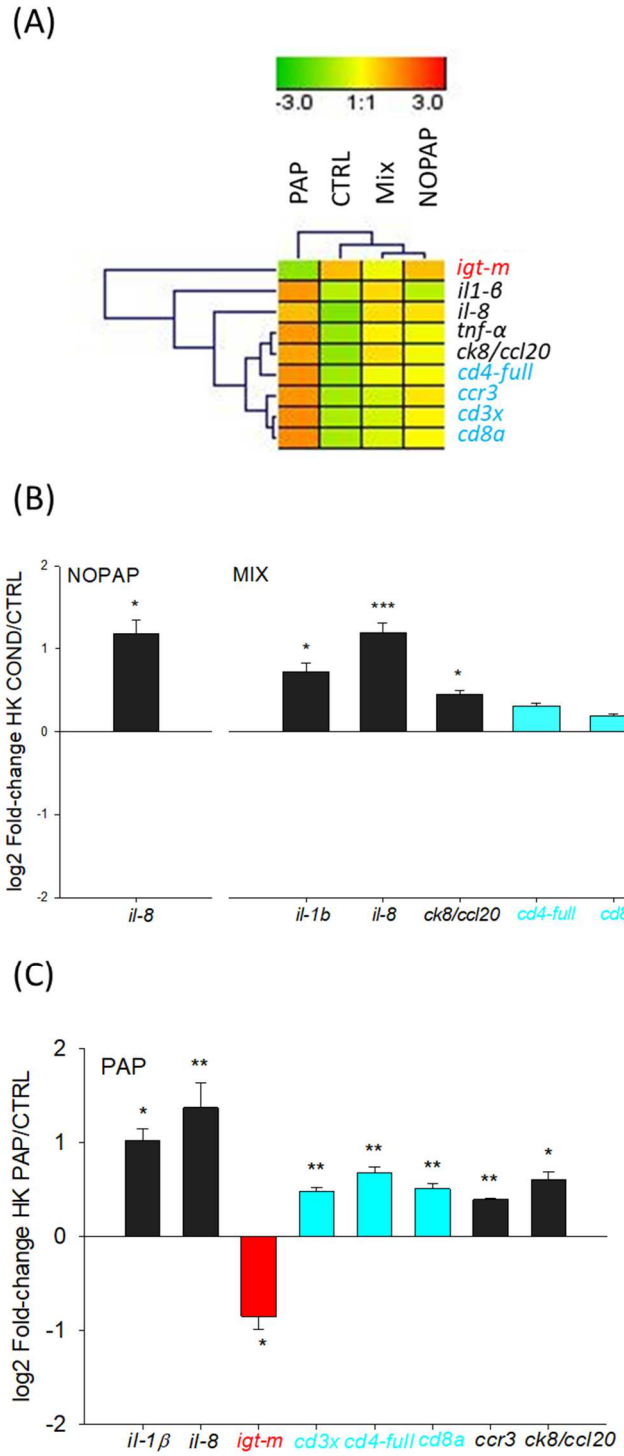

Figure 4. Heat map of head kidney gene expression profile after filtering for most nutritionally regulated genes (A). Fold changes of changing expressed genes (experimental/control fish) in NoPAP/MIX (B) and PAP (C) fish. Values are the mean ± SEM of 10-12 fish. Asterisks indicate statistically significant differences by Student-t test (*, P<0.1; **, P<0.05; ***, P< 0.001).

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4.2.6 Alpha diversity and gut microbiota composition

As shown in Figure 5, the NoPAP diet induced a significant decrease in richness and alpha diversity in comparison to the CTRL group, when regarding the ACE and Shannon estimators ($P < 0.05$), respectively. A total of 747, 621 and 539 OTUs were assigned to CTRL, NoPAP and PAP fish, respectively (Figure 6A). From them, 176 OTUs were present in all dietary groups, representing more than 60% of the overall bacterial composition in all groups, whereas 385 (16.2% of the total microbiota), 262 (11.8%) and 184 (7.3%) were present exclusively in CTRL, NoPAP and PAP fish, respectively. No significant differences among groups (Kruskal-Wallis test, followed by Dunn's post-test. $P < 0.05$) were detected when taxonomic assignations were collapsed to the phylum level (Figure 6B). Proteobacteria was the most abundant phylum reaching values between 35 to 50% of the total bacterial composition, followed by Firmicutes (19-29%), Actinobacteria (11-18%) and Bacteroidetes (2.5-3%). Verrucomicrobia was relatively abundant in the control diet (5.7%), but decreased in the NoPAP and PAP fish (<0.5%).

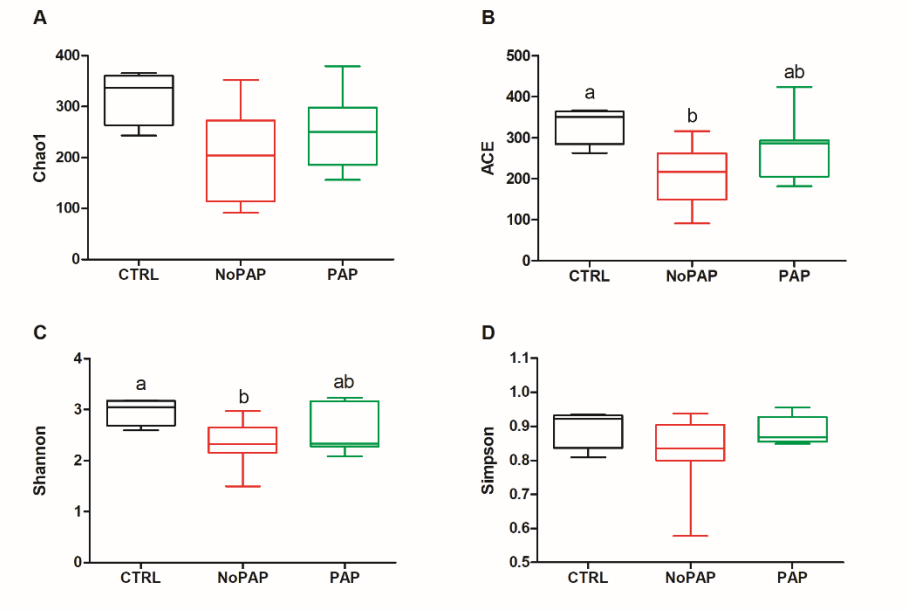

Figure 5: Box plots representing the mean (min-max) of richness estimates ((A) Chao1 and (B) ACE) and diversity indexes ((C) Shannon and (D) Simpson) of the intestinal microbial populations found in fish fed CTRL (n = 4), NoPAP (n = 9) and PAP (n = 7) diets. Different letters indicate significant differences among groups (Kruskal-Wallis with Dunn's post-test, $P < 0.05$).

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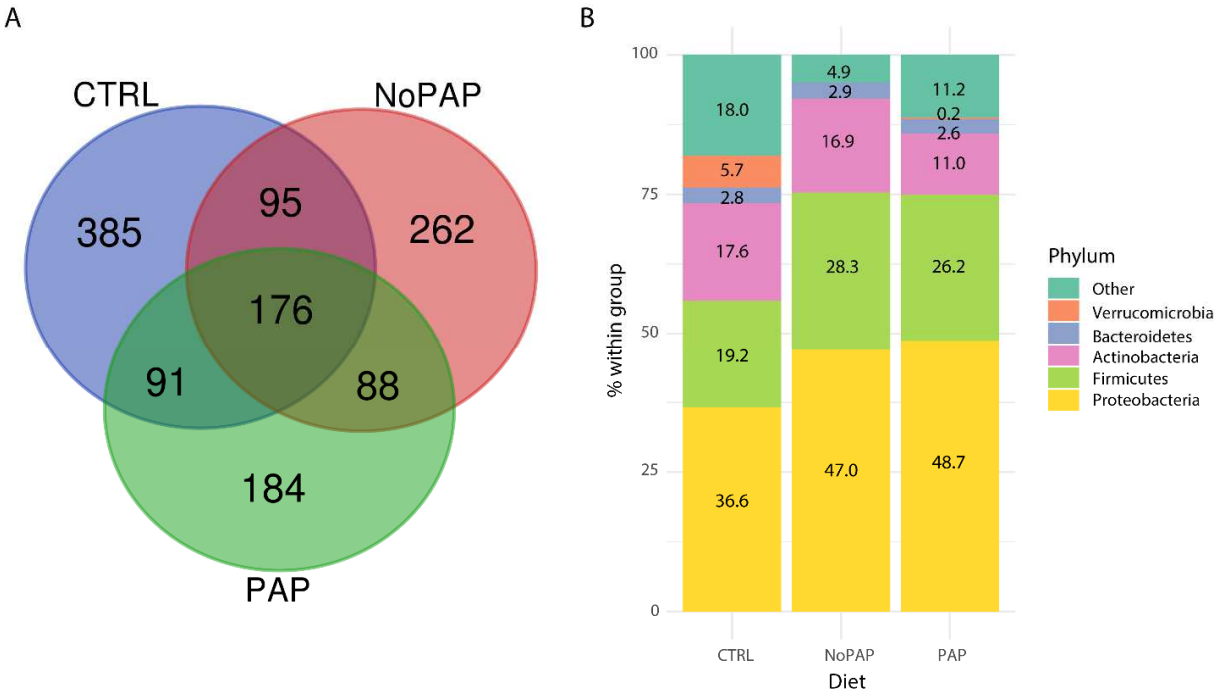

Figure 6: (A) Venn diagram showing unique and shared OTUs in the intestines of fish fed the three experimental diets. The 176 common OTUs represent the 63.5%, 69.4% and 60.5% of the overall microbiota in CTRL, NoPAP and PAP groups, respectively. Unique OTUs for CTRL, NoPAP and PAP groups represent the 16.25%, 11.8% and 7.3% of the overall bacterial composition, respectively. (B) Stacked bar chart representing the relative abundance of bacterial phyla in the three dietary groups. Only the phyla that are present in at least 1% in one of the groups are represented. No significant differences were found among groups (Kruskal-Wallis + Holm-Sidak tests, $P > 0.05$).

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4.2.7 Beta diversity and discriminant analyses

Regarding beta diversity, statistically significant differences among dietary groups were found (PERMANOVA P = 0.049, F = 1.0514, R2 = 0.1101). To further evaluate differences in the bacterial composition among groups, a partial least squares discriminant analysis (PLS-DA) was performed. The discriminant model was based on four components, which explained 98% [R2Y(cum)] and predicted 47% [Q2Y(cum)] of the total variance (Figure 7A). During the statistical processing to construct the model, one fish from the CTRL group appeared as outlier and was excluded from the model. The final model clearly separated the CTRL from the NoPAP and PAP fish (43% explained variance), whereas the second component mainly separated the fish fed the PAP diet from the other two groups (>46% explained variance). According to this, hierarchical clustering putted together CTRL and PAP fed fish, and all samples were properly classified in their respective experimental group (Figure 7B). Filtering by a VIP ≥ 1, a total of 135 OTUs mainly drove the separation among experimental groups.

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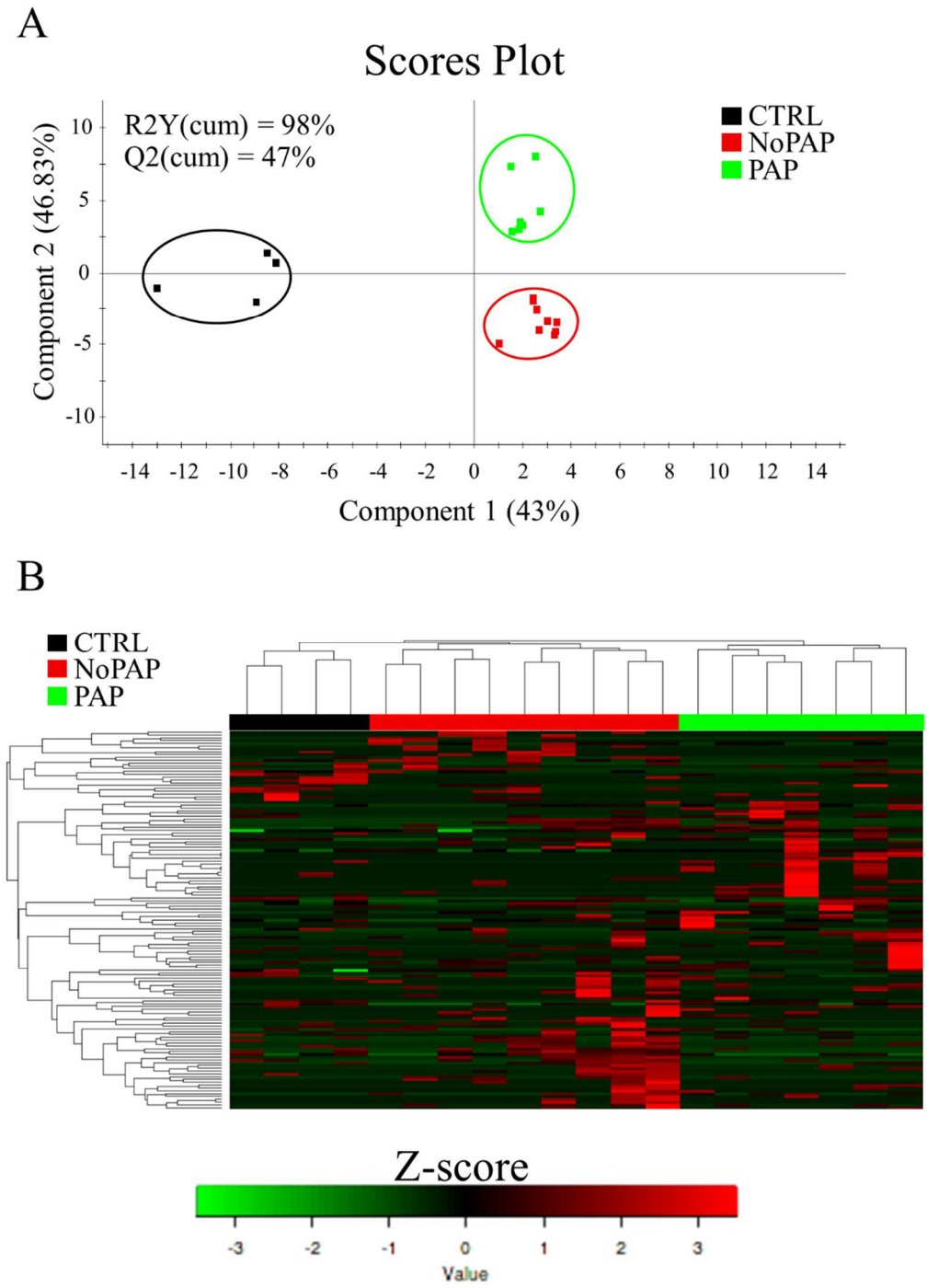

Figure 7: (A) Two-dimensional PLS-DA score plot constructed using the variable diet representing the distribution of the samples between the first two components in the model. (B) Heatmap representing the abundance distribution (Z-score) of the OTUs identified to drive the separation by diet (VIP ≥ 1).

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4.2.8 Inferred pathways

The sequences of the 135 OTUs, driving the separation of dietary groups, were used to discern the potential implication of microbiota in KEGG pathways through an inferred metagenome analysis. This analysis displayed a total of 38 OTUs (VIP ≥1) whose genomes were potentially associated to the expression of genes involved in the differentially represented pathways (FDR < 0.05). When compared to the CTRL fish, 20 and 24 pathways showed to be potentially changing in NoPAP and PAP fish, respectively (Figure 8). Of those, ten pathways were simultaneously changing in both NoPAP and PAP fish, sharing these two groups the up-regulation of routes tailoring immune response and inflammation (C-type lectin receptor, VEGF, TNF and NFκ-B signalling pathways), with a lower degree of activation in fish fed the NoPAP diet. Cholesterol metabolism and the neuroactive ligand-receptor interaction pathways were also over-represented at a similar extent in both conditions. Fish fed the NoPAP and PAP-based feed formulations also displayed an exclusive type of response at this level, with the differential representation of 14 and 10 inferred pathways, respectively.

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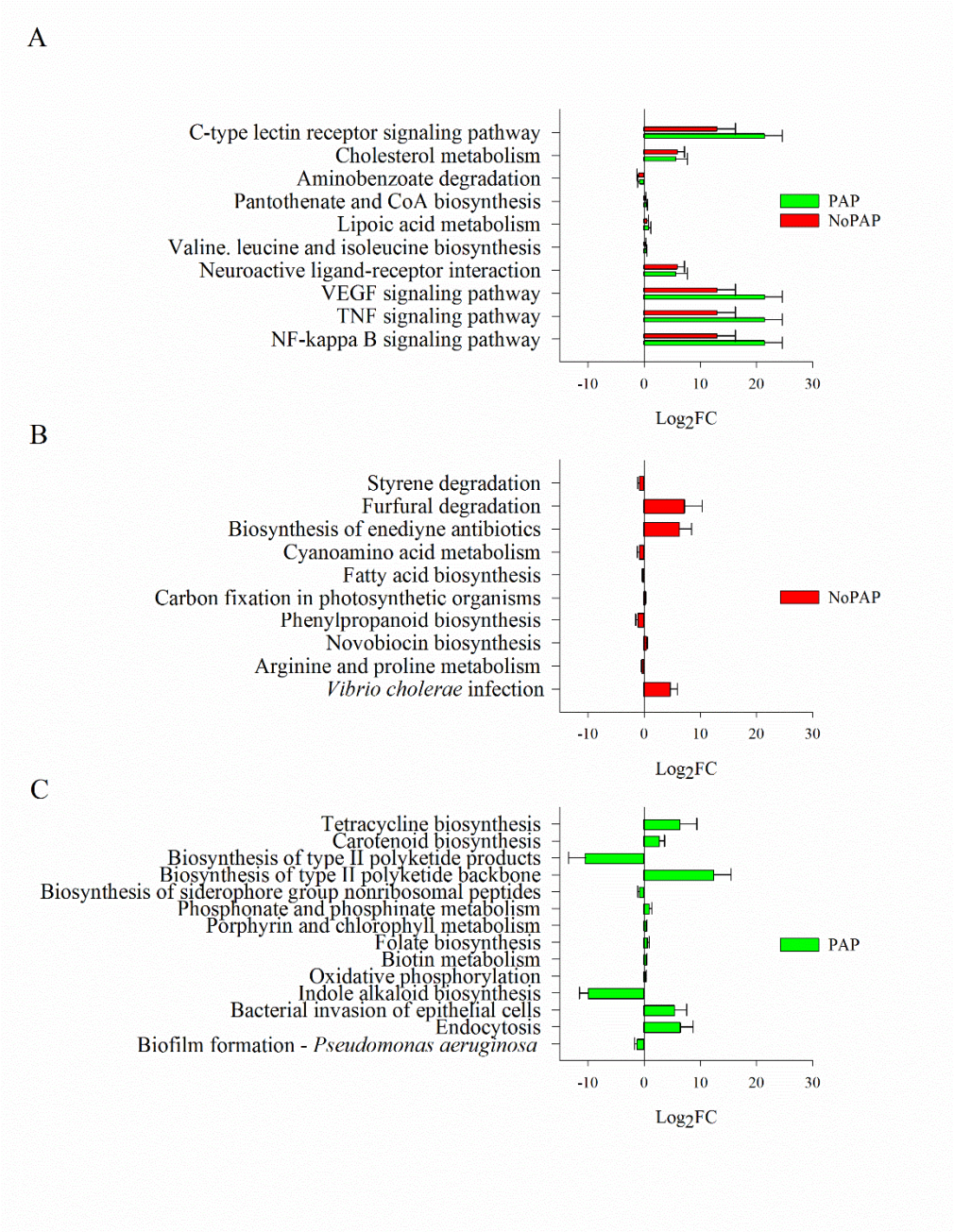

Figure 8: Results from the pathway analysis performed with the predicted metagenome obtained from the discriminant OTUs with VIP ≥ 1. (A) Differentially and common represented pathways (padj < 0.05) when comparing PAP or NoPAP diets against the CTRL diet. (B) Exclusively differentially represented pathways in the NoPAP vs. CTRL. Comparison. (C) Exclusively differentially represented pathways in the PAP vs. CTRL comparison. Bars show the Log_2 fold change of differentially over- or under-represented pathways (± standard error of the calculated fold change).

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5.3 Salmon 1

5.3.1 Lice Count

The Lice count on both sampling points is displayed on Table 37 and. No significant differences were found within diets of the same sampling point.

Table 36. Lice count of fish fed with: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein. No significant differences were observed (n=4; one-way ANOVA).

		CTRL	PAP	NOPAP
1st Sampling	Lice Count	8.25±3.59	12.00±2.45	11.00±4.97
	Lice Count/fish	0.41±0.18	0.60±0.12	0.55±0.25
2nd Sampling	Lice Count	136.50±22.65	152.50±23.98	144.25±27.66
	Lice Count/fish	6.83±1.13	7.63±1.20	7.21±1.38

5.3.2 Fish Welfare

Tables 38 and 39 are related to the number of fish with alterations according to the GIFAS Animal Welfare Scoring. Tables 40 and 41 show this alteration. No significant differences were found in the number of fish with alteration after applying a non-parametric Kruskal-Wallis test, both for intermediate sampling or final sampling.

Table 37. Number of fish with alterations according to the GIFAS Animal Welfare Scoring after being exposed to three different dietary groups on the first sampling point (2 weeks). No significant differences were observed (one-way ANOVA).

First Sampling	Body Condition	Fin erosion	Scale loss	Gills	Operculum erosion	Snout erosion
Control	1	6	11	0	0	0
PAP	2	2	9	1	1	0
NoPAP	2	7	12	1	1	1

Table 38. GIFAS Animal Welfare Scoring of fish after being exposed to three different dietary groups on the first sampling point (2 weeks).

First Sampling	Body Condition	Fin erosion	Scale loss	Gills	Operculum erosion	Snout erosion
Control	Good	Slight damage	Slight	Healthy	Normal	Normal
PAP	Normal (wild salmon-like)	None	Slight	Slight visible defects	Slight erosion (gills slightly exposed)	Normal
NOPAP	Normal (wild salmon-like)	Slight damage	Slight scale loss	Slight visible defects	Slight erosion (gills slightly exposed)	Minor surface wound

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Table 39. Number of fish with alterations according to the GIFAS Animal Welfare Scoring after being exposed to three different dietary groups on the final sampling point. No significant differences were observed (one-way ANOVA)

Final Sampling	Body Condition	Fin erosion	Scale loss	Operculum erosion	Snout erosion
Control	2	12	25	3	0
PAP	1	6	7	3	1
NoPAP	2	10	11	2	0

Table 40. GIFAS Animal Welfare Scoring of fish after being exposed to three different dietary groups on the final sampling point.

Final Sampling	Body Condition	Fin erosion	Scale loss	Operculum erosion	Snout erosion
Control	Normal (wild salmon-like)	Moderate damage	Surface wound <1cm^2	Slight erosion (gills slightly exposed)	Normal
PAP	Normal (wild salmon-like)	Severe damage with visible sore and inflammation	Significant scale loss	Slight erosion (gills slightly exposed	Minor surface wound
NoPAP	Normal (wild salmon-like)	Severe damage with visible sore and inflammation	Surface wound <1cm^2	Slight erosion (gills slightly exposed	Normal

5.3.3 Humoral Immune parameters

Data of humoral immune parameters measured on plasma is displayed in table 42. The different dietary groups didn't affect the humoral parameters analyzed on the plasma.

Table 41. Humoral parameters activity in fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein. No significant differences were observed (n=40; one-way ANOVA).

	CTRL	**PAP**	**NoPAP**
Protease (%)	31.99±3.70	32.42±2.54	32.49±2.43
Anti-protease (%)	61.47±9.67	61.29±7.85	62.31±3.70
IgM (absorbance values)	1.34±0.21	1.44±0.18	1.37±0.27
Bactericidal activity (%)	34.29±1.14	34.67±0.83	34.18±1.64

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5.3.4 Mucosal Mapping

Results of mucosal mapping are displayed in Table 43. The mucosal mapping parameters such as mucous cell area, mucous density, and barrier status were not affected by the dietary groups under study.

Table 42. Mucosal mapping data regarding fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein. No significant differences were observed (n=15; one-way ANOVA).

		CTRL	PAP	NoPAP
Foregut	Mucous Cell Area	179.71±33.22	162.96±37.66	157.56±22.94
	Mucous Density (%)	0.10±0.03	0.10±0.03	0.09±0.02
	Barrier Status	0.58±0.15	0.58±0.11	0.59±0.10
Dorsal	Mucous Cell Area	180.66±33.15	200.52±44.95	186.65±40.00
Skin	Mucous Density (%)	0.16±0.06	0.18±0.05	0.17±0.06
	Barrier Status	0.89±0.23	0.91±0.15	0.92±0.18

5.3.5 Oxidative status

Data on oxidative status biomarkers are displayed in Table 44. Results of lipid peroxidation measured in the liver of fish from experimental groups did not present significant differences.

Table 43. Oxidative status biomarkers in fish fed with three different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein. significant differences were observed (n=4; one-way ANOVA).

	CTRL	PAP	NoPAP
LPO (nmol MDA/g tissue)	40.56±5.51	36.98±2.69	38.07±4.27

5.3.6 Hepatic gene expression profiling

Data were in reference to the expression level of lpl from the CTRL group at 2 weeks with an arbitrary assigned value of 1. Data regarding the gene expression is displayed on heat mapanalysis, Figure 9.

Regarding gene expression inside each sampling point, only the second sampling point (after 96 days of trial) presented genes differentially expressed (DE) at $p<0.05$ on 4 out of 38 genes in the array, including growth performance (*igf2*), lipid metabolism, elongases (*elovl4*) and energy metabolism (*ucp2l* and *sirt1*).

When we use a two-way ANOVA to compare the gene expression between the sampling points we actually see that 19 of 38 genes were significantly expressed, including growth performance (*ghr1, ghr2, igf1, igf2, igfbp1b, igfbp2b, igfr1*), lipid metabolism, including elongases (*elovl2, elovl4, elovl6*), desaturases (*scd1, fads2*), nuclear transcription factors (*pparα*), energy metabolism (*h-fabp, ucp2l* and *sirt1*), antioxidant defence (*gpx1*), immune

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response (*ptrx, bd3*) and for last proteolysis (*ctsb*).
When we use a two-way ANOVA to cross the gene expression between sampling points and diets the count of gene significantly differed decreases to 2 out of 38 genes including growth performance (*igf2*) and lipid metabolism, elongases (*elovl4*).
Overall, the gene expression level was up-regulated in fish fed with novel feed formulations when compared to the control group after 2 weeks and down-regulated after 96 days of trial, on the same genes. After 96 days of trial, we start to see differences between the novel feed formulations. Regarding elovl4 and scd1 we see that when compared to the control group we see an up-regulation on NoPAP on the first gene and a down-regulation on the second. Fish fed with PAP diet by its turn presented an opposite response on the same genes. As a general trend we can see that fish passed by an adaption period to the diets.

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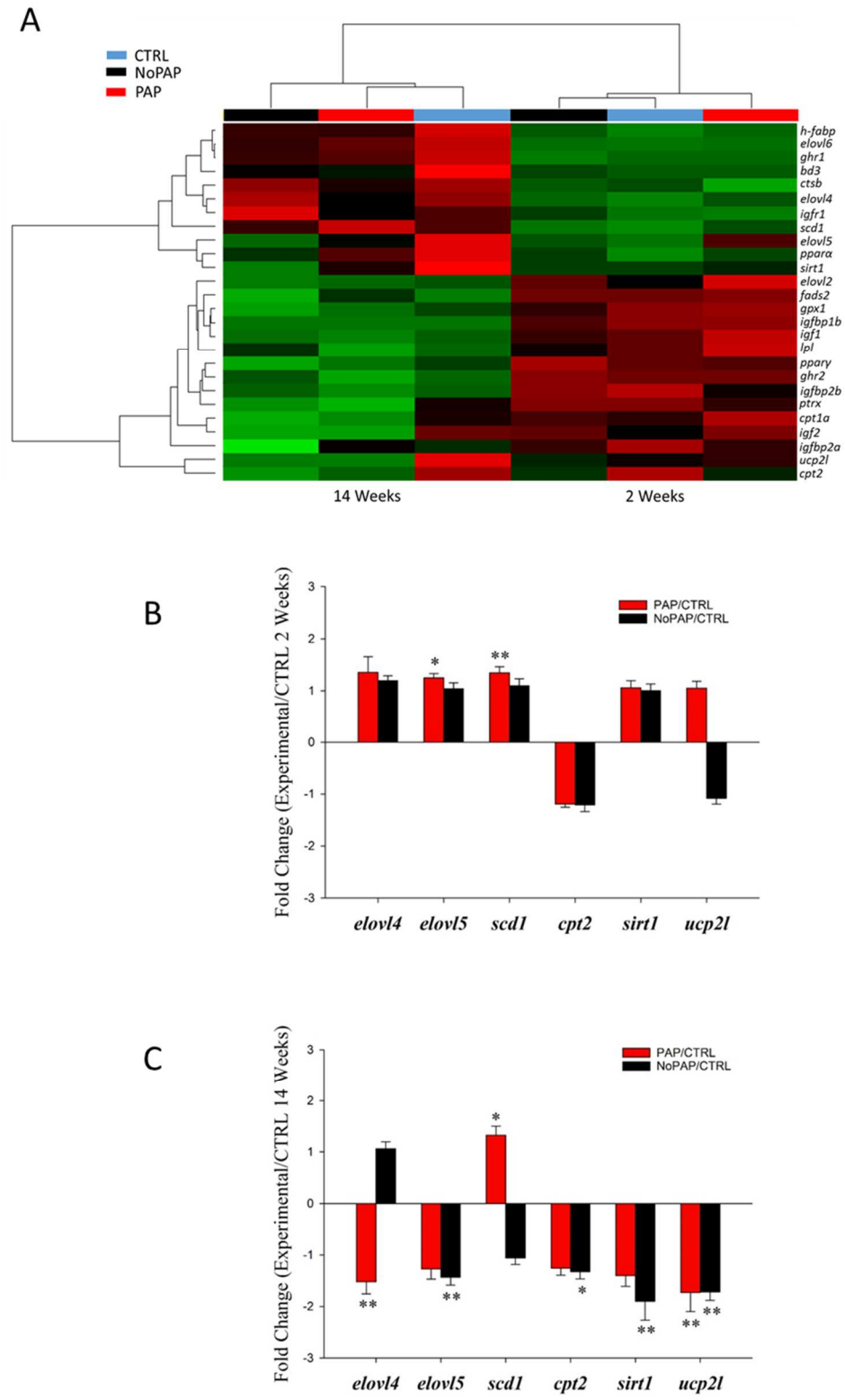

Figure 9. Heat map of liver gene expression profile after filtering for most nutritionally regulated genes (A). Fold changes of changing expressed genes (experimental/control fish) after 2 weeks (B) and after 14weeks (C) of trial. Values are the mean ± SEM of 10-12 fish. Asterisks indicate statistically significant differences by Student-t test (*, P<0.1; **, P<0.05).

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5.3.7 Head kidney gene expression profiling

Data were in reference to the expression level of *c3* from the CTRL group at 2 weeks with an arbitrarily assigned value of 1. Data regarding the gene expression is displayed on heat map analysis, Figure 10.

Regarding gene expression inside each sampling point, the first sampling point (after 2 weeks) presented genes differentially expressed at $p<0.05$ on 2 out of 28 genes in the array. Gene's expression showed a pro-inflammatory profile evidenced by the up-regulation of *il-8* but with a down regulation of *il-10* on fish fed with novel formulations. After 96 days of trial, we see that the same genes continue to be differentially expressed the same way, but we also see another gene DE the *clec1b* membrane protein that is up-regulated.

When we use a two-way ANOVA to compare the gene expression between the sampling points we actually see more that 15 of 28 genes were significantly expressed, including cytokines (*il-6,il-10, il-12* and *il-34*), *igm*, complement factor 3, caspase3, T-cell markers (*cd4* and *csflr1)* and pathogen-associated microbial pattern (PAMP*) (tlr2, tlr9, clec1b, hepc, bd4* and *bd5b).*

When we use a two-way ANOVA to cross the gene expression between sampling points and diets the count of gene significantly differed decreases to 2 out of 28 genes on genes related to the PAMP (*clec10a* and *clec1b*).

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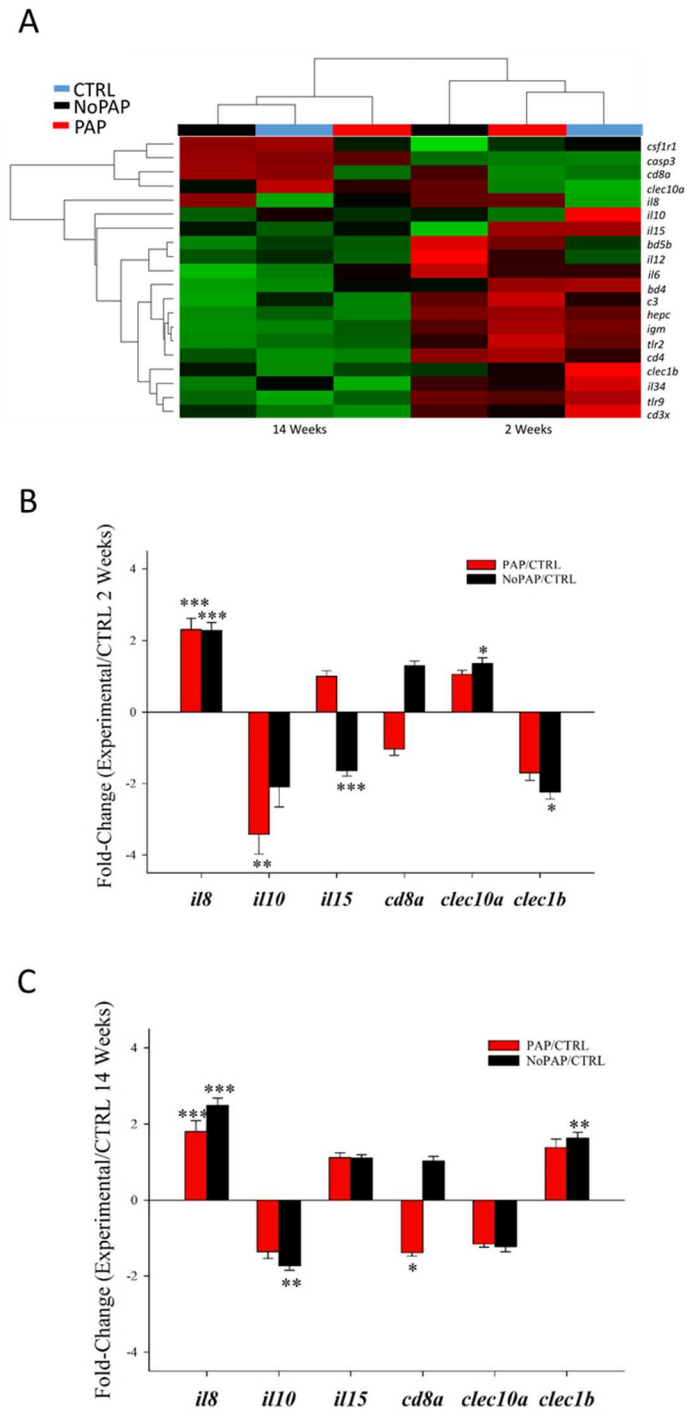

Figure 10. Heat map of head kidney gene expression profile after filtering for most nutritionally regulated genes (A). Fold changes of changing expressed genes (experimental/control fish) after 2 weeks (B) and after 14weeks (C) of trial. Values are the mean ± SEM of 10-12 fish. Asterisks indicate statistically significant differences by Student-t test (*, P<0.1; **, P<0.05).

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6. Performance of Block 2 trials

This section will present the main results of growth performance, body composition, nutrient retention, and apparent digestibility of five trials for Trout, Salmon, Seabream, Seabass, and Turbot, respectively. Additionally, specific analyses depending on the trial objective were performed and are presented in section *7 Additional specific analysis Block2.*

6.1 Trout 2

6.1.1 Growth performance parameters

During the whole test (91 days of experimental feeding; Table 45), ten dead fish over the 20 experimental units were registered (4 NoPAP+; 2 PAP-; 0 CTRL; 1 PAP; 3 NoPAP), the daily death rate was 0.01%, lower than the physiological rate of 0.2%. Final body weight (FBW) ranged between 335.6 ± 6.2 g in NoPAP+ and 291.9 ± 7.3 g in CTRL diet. This means an individual weight gain over 200 g compared to initial body weight (IBW). NoPAP+ and PAP diet led to better growth compared to the CTRL diet, whereas no statistical differences are registered between the couple PAP-/NoPAP and CTRL. In contrast, PAP- diet showed the worst FCR (0.87) and was statistically different from the groups CTRL-PAP-NoPAP (0.82-0.84) and NoPAP+ (0.76).

Table 44: Growth performance (mean ± SD) of fish fed with five different dietary groups for 91 days: i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy driven formula algae without processed animal protein, iv) NOPAP+: a diet similar to NOPAP but with higher protein content, and last, v) PAP-: a diet similar to PAP but with lower protein content. Values are means and standard deviation.

	CTRL	NoPAP	NoPAP+	PAP	PAP-
IBW (g)	58.2±1.81	58.1±1.43	58.6±1.38	59.4±1.40	59.9 ±0.69
FBW (g)	292±7.25 ^{c}	297±11.3 ^{bc}	336±6.17 ^{a}	309±5.30 ^{b}	297±4.45 ^{bc}
Weight gain (g)	234±5.97 ^{c}	239±10.0 ^{bc}	277±4.79 ^{a}	250±4.70 ^{b}	237±3.95 ^{bc}
RGR (%BW/day)	1.79±0.02 ^{c}	1.8±0.02 ^{bc}	1.94±0.01^{a}	1.83±0.02 ^{b}	1.78±0.01^{c}
FCR	0.816±0.01 ^{b}	0.837±0.02^{b}	0.764±0.01^{c}	0.830±0.01^{b}	0.871±0.003^{a}
VFI (%/day)	1.46±0.03 ^{c}	1.51±0.02 ^{ab}	1.48±0.01 ^{bc}	1.52±0.01 ^{ab}	1.55±0.01^{a}
PER	3.09±0.04 ^{a}	3.04±0.06 ^{ab}	3.04±0.03 ^{ab}	2.90±0.05	2.99±0.01 ^{bc}

Different letters refer to statistical differences ($p<0.05$) among diets (n=4; one-way ANOVA).

6.1.2 Whole-body composition

Data on the whole body composition of fish at the end of the trial are presented in Table 46. Dietary treatments did not affect the whole-body composition of fish in terms of moisture, ash, protein, fat, energy, and phosphorus ($p>0.05$).

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Table 45: Whole body composition (mean ± SD) of fish fed the five different dietary groups: i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy driven formula algae without processed animal protein, iv) NOPAP+: a diet similar to NOPAP but with higher protein content, and last, v) PAP-: a diet similar to PAP but with lower protein content. (n=4; one-way ANOVA).

	CTRL	PAP	NoPAP	NoPAP+	PAP-
DM, %	29.33±1.06	29.10±0.36	28.15±0.37	28.55±0.56	28.75±0.44
Ash, %	4.70±0.00	4.70±0.00	4.61±0.17	4.87±0.34	4.61±0.17
Protein, %	53.52±1.04	51.68±0.82	52.77±1.17	54.45±0.50	51.51±2.46
Fat, %	40.10±1.79	41.78±0.80	38.93±2.14	38.51±1.91	40.77±1.24
Energy, kJ/g	28.78±0.41	29.03±0.19	28.49±0.42	28.46±0.41	28.82±0.32
Phosphorus, %	0.84±0.06	0.81±0.03	0.84±0.00	0.91±0.05	0.82±0.03

Initial fish: DM 23.73%; ash 6.61%; protein 68.48%; fat 24.44%; energy 25.94kJ/g; phosphorous 1.15%.

6.1.3 Apparent digestibility

The apparent digestibility coefficients are presented in Table 47. Fish fed PAP- diets presented lower digestibility for Protein and total phosphorous when compared to fish fed other diets. On the other hand, fish fed NoPAP+ and PAP had higher fat digestibility when compared to control; while higher energy digestibility was observed in fish fed NoPAP+ when compared to control and PAP-.

Table 46: Protein, fat, energy, and phosphorus apparent digestibility of fish fed with five different dietary groups i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy driven formula algae without processed animal protein, iv) NOPAP+: a diet similar to NOPAP but with higher protein content, and last, v) PAP-: a diet similar to PAP but with lower protein content.

	CTRL	PAP	NoPAP	NOPAP +	PAP -
Protein	89.25±0.96 ^{a}	88.88±0.48 ^{a}	89.80±0.34 ^{a}	90.33±0.75 ^{a}	86.17±1.28 ^{b}
Fat	97.84±0.47 ^{b}	98.77±0.14 ^{a}	98.20±0.30 ^{ab}	98.74±0.19 ^{a}	98.20±0.53 ^{ab}
Energy	82.35±1.63 ^{b}	84.37±1.08 ^{ab}	83.33±1.39 ^{ab}	86.08±1.25 ^{a}	81.41±1.56 ^{b}
Total P	42.06±4.70 ^{a}	48.12±2.85 ^{a}	51.52±2.57 ^{a}	63.66±3.50 ^{a}	47.13±4.48 ^{b}

Different letters indicate significant differences (one-way ANOVA; $p< 0.05$; Tukey post hoc test, n=4).

6.1.4 Nutrient Retention

Results of protein, lipids, and energy retention of fish fed with different diets are shown in Table 48.

Fish fed new formulated diets did not present statistical differences in Fat and Energy retention ($p>0.05$). However, fish PAP and PAP- diet presented lower protein retention when compared to control diet.

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Table 47. Protein, lipids and energy retention (mean ± SD) of fish fed with five different dietary groups: i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy driven formula algae without processed animal protein, iv) NOPAP+: a diet similar to NoPAP but with a higher protein content, and last, v) PAP-: a diet similar to PAP but with a lower protein content.

Retention (%intake/ feed)	CTRL	NoPAP	NoPAP+	PAP	PAP-
Protein	48.13±2.64^{a}	43.67±0.73^{ab}	46.46±1.26^{ab}	42.78±1.06^{b}	43.07±3.11^{b}
Lipid	75.56±6.67	65.64±6.24	71.11±5.78	69.76±1.43	68.57±3.07
Energy	47.17±2.70	44.14±2.30	47.77±2.18	47.50±1.05	42.96±1.16

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test, n=4).

6.1.5 Fatty acid Retention

Fatty acid retention (expressed in percentage of feed intake) for trout fed various dietary treatments is shown on table 49. Fish fed novel formulated diets had lower DHA retention when compared to control. For 18:3n-3 the retention was higher in NoPAP when compared to fish fed PAP diets.

Table 48: Fatty acid retention of trout fed different dietary treatments. Values are means and standard deviation (n=4).

Fatty acid	CTRL	PAP	NO PAP	NO PAP +	PAP -
Total saturated	94.60±5.55	85.27±2.84	86.50±8.53	92.00±6.52	83.18±4.06
Total monounsaturated	77.58±4.33	73.43±0.34	77.93±6.39	77.40±4.70	75.58±2.40
20:5n-3	29.45±1.31	26.42±0.76	*	27.67±1.63	27.31±0.75
22:6n-3	102.99±7.76^{a}	76.55±4.55^{b}	71.27±9.09^{b}	76.30±4.06^{b}	72.73±6.68^{b}
n-3 HUFA (DHA+EPA)	51.18±1.77	52.21±2.59	*	53.63±2.85	50.74±3.47
Total n-3 PUFA	45.27±16.74	54.16±1.53	56.57±4.56	56.29±2.62	54.22±3.84
Total n-6 PUFA	70.90±24.77	76.71±1.00	83.61±5.95	78.18±4.84	79.26±1.56
Total PUFA	70.45±1.89	68.37±1.40	73.40±5.57	69.05±3.65	69.83±2.63
20:4(ω-6)	84.22±1.03	75.96±9.25	*	59.39±0.61	82.89±10.10
C18:2n-6	*	*	*	*	*
C18:3n-3	56.63±3.51^{ab}	50.51±0.48^{a}	57.90±4.69^{b}	56.84±3.65^{ab}	52.63±1.64^{ab}

Different letters means statistical differences among groups. * Value not detected.

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6.2 Bream2

6.2.1 Growth performance parameters

Data on growth performance from the feeding period are reported in Table 50. After 34 days of feeding, the condition factor was significantly lower ($P < 0.001$) in NoPAP SANA fish than in CTRL fish. The same trend was found in the relative growth rates (RGR) which varied from 2.33 in CTRL fish to 2.27 in NoPAP SANA fish. Although no statistical difference can be calculated due to having only one tank per group, the feed intake appears slightly higher in CTRL fish than in NoPAP SANA fish. The opposite was seen in the FCR values, which were a little higher in NoPAP SANA fish (0.97) in comparison with CTRL fish (0.93). HSI, MFI and ILI were not significantly altered by dietary treatment. However, the IWI was significantly higher in NoPAP SANA fish (4.65%) when compared with CTRL fish (4.15%).

Table 49: Effects of dietary treatment on growth performance of gilthead seabream juveniles. Fish were fed a fixed ration of 2.40 to 2.77% according to the temperature (20-22ºC) and the fish body weight that was estimated twice a week with an approximate feed conversion of 1. The trial lasted from May to June (1 month). Data body weight, body length, condition factor, and specific growth rate are the mean ± SEM of 160 fish from each diet. Data on feed intake and feed conversion ratio are the mean of the whole tank. Data on liver and mesenteric fat and intestine are the mean ± SEM of 9 fish per diet. Different letters indicate significant differences between treatments (T-test $p<0.05$).

Parameters	CTRL	NoPAP SANA
Initial body weight (g)	21.30 ± 0.28	21.32 ± 0.30
Final body weight (g)	43.92 ± 0.53	43.02 ± 0.50
Final standard length (cm)	118.07 ± 0.48	118.01 ± 0.47
Final condition factor	2.66 ± 0.02 [b]	2.61 ± 0.01 [a]
Feed intake (g DM/fish)[1]	22.76	21.71
FCR[2]	0.93	0.97
RGR (%)[3]	2.33 ± 0.02 [b]	2.27 ± 0.02 [a]
Liver (g)	0.62 ± 0.02	0.61 ± 0.02
Mesenteric fat (g)	0.68 ± 0.08	0.62 ± 0.06
Intestine weight (g)	1.81 ± 0.09	2.00 ± 0.09
Intestine length (cm)	10.85 ± 0.45	11.26 ± 0.55
HSI (%)[4]	1.44 ± 0.04	1.44 ± 0.07
MFI (%)[5]	1.54 ± 0.15	1.43 ± 0.13
IWI (%)[6]	4.15 ± 0.08 [a]	4.65 ± 0.11 [b]
ILI (%)[7]	92.65 ± 3.78	94.40 ± 4.47

[1]Feed intake = dry feed weight/fish
[2]Feed conversion ratio = dry feed intake/wet weight gain
[3]relative growth rate = 100 × (e^g-1); with g= (ln final body weight – ln initial body weight) / days
[4]Hepatosomatic index = (100 × liver weight)/fish weight
[5]Mesenteric fat index = (100 × mesenteric fat weight)/fish weight
[6]Intestinal weight index = (100 × intestine weight)/fish weight
[7]Intestinal lenght index = (100 × intestine length)/fish length

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6.2.2 Whole body composition

Whole-body composition of fish fed NoPAP SANA presented a higher value for ash, while had less fat and less dry matter in fish composition when compared to control (P<0.05) (table 51).

Table 50: Whole body composition percentage of dry matter (DM), Ash, Protein, Fat, Energy, and Phosphorous (P) for gilthead seabream fed with two dietary groups (n=2).

%DM	CTRL	NoPAP SANA
DM	31.01±0.71 [a]	29.46±0.20 [b]
Ash	12.00±0.05 [b]	12.87±0.12 [a]
Protein	5 9.01±0.10	59.19±1.07
Fat	33.85±0.77 [a]	31.06±0.10 [b]
Energy	23.91±0.70	23.44±0.45
P	2.30±0.02	2.39±0.07

Initial fish: DM 27.73%; ash 13.34%; protein 63.35%; fat 24.62%; energy 22.84kJ/g; P2.36%.
Different letters means statistical differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

6.2.3 Apparent digestibility

Protein, fat, energy and total phosphorous apparent digestibility are shown in Table 52. Fish fed NoPAP SANA diet presented higher digestibility values for protein and total phosphorous (P) when compared to the control (CTRL) diet.

Table 51: Protein, energy and phosphorus apparent digestibility of gilthead seabream fed with two different dietary groups (n=2).

ADC, %	CTRL	NoPAP SANA
Protein	73.55±0.38 [a]	76.91±0.11 [b]
Energy	62.35±1.27	65.25±0.11
Total P	57.96±0.12 [a]	67.58±0.016 [b]

Different letters means statistical differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

6.2.4 Nutrient retention

Nutrient retentions for protein, fat, energy and total phosphorous (Total P) are shown on Table 53. Fish fed NoPAP SANA diet presented lower values for fat retention when compared to control (CTRL) diet.

Table 52: Percentage of protein, lipids, energy, and phosphorous retention of fish

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fed with two different dietary groups (n=2).

Retention (% intake)	CTRL	NoPAP SANA
Protein	37.05 ± 1.03	34.23 ± 0.44
Fat	65.83 ± 3.28 [b]	52.59 ± 0.22 [a]
Energy	34.57 ± 2.01	30.21 ± 0.90
Total P	46.54 ± 1.69	38.54 ± 1.60

Different letters means statistical differences (one-way ANOVA; p< 0.05; Tukey post hoc test).

6.2.5 Fatty acid retention

Fatty acid retention for seabream fed different treatments are shown on Table 54. No statistical differences could be detected, but there seems tdo be a trend for lower PUFA retention with NoPAP SANA diet.

Table 53: Fatty acid retention in seabream fed different dietary treatments. . Values are means and standard deviation (n=4).

Fatty acid	CTRL	NoPAP SANA
Total saturated	45.59±4.95	38.61±6.09
Total monounsaturated	43.47±2.66	36.81±1.45
20:5n-3	15.94±11.11	9.99±6.66
22:6n-3	36.24±28.70	14.48±11.94
n-3 HUFA (DHA+EPA)	23.11±17.32	12.22±9.28
Total n-3 PUFA	22.62±15.38	11.79±9.02
Total n-6 PUFA	30.00±7.64	18.06±7.10
Total PUFA	26.24±11.39	14.17±7.84
20:4(ω-6)	23.88±13.14	10.72±8.96
C18:2n-6 %,	14.03±6.95	7.14±2.73
C18:3n-3 %,	17.79±7.91	9.11±6.03

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6.3 Salmon 2

6.3.1 Growth performance parameters

Growth performance details after 73 days of experimental feeding are displayed in Table 55. The highest final body weight was verified on fish fed with the NOPAP + diet, followed by CTRL and the lowest in PAP diet (Figure 48). Fish from group control are significantly heavier than fish from groups PAP-, NOPAP and PAP. Group fed with the NOPAP + diet shows a trend to relatively bigger fish compared to group PAP. Regarding the indicator Weight Gain and RGR displayed in Figures 4 and 5, the highest value was verified on fish fed with the NOPAP + diet, followed by CTRL and the lowest in PAP diet. When compared to the control diet it is possible to see significant differences comparing to the PAP diet. FCR shows a highest value in fish fed with the PAP diet, followed by PAP- and the lowest on NOPAP+ diet. When compared to the control diet it is possible to see significant differences between this and all the other diets except when compared to the NOPAP+. Protein efficiency ratio seems to show the opposite results of FCR.Voluntary feed Intakedid not present significant differences among diets.

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Table 54. Growth performance of Atlantic salmon fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

	CTRL	NOPAP	PAP	NOPAP+	PAP-
IBW (g/fish)	1242.±15.1	1262.4±9.9	1238.8±7.4	1230.0±26.4	1246.9±14.8
FBW (g/fish)	2820.3± 105.7^{ac}	2629.5± 69.4^{ab}	2511.3± 38.8^{b}	2935.8± 3.8^{ac}	2582.1±18.9^{b}
Weight gain (g/fish)	1577.8± 90.6^{a}	1367.1± 60.9^{b}	1272.52 ±37.9^{b}	1705.80± 30.2^{a}	1335.2±4.1^{b}
RGR (% BW/day)	1.13±0.04	1.01±0.03	0.97±0.02	1.20±0.03	1.00±0.01
VFI (%/day)	1.24±0.04	1.190.04±	1.19±0.03	1.23±0.05	1.22±0.00
PER	2.13± 0.03^{a}	1.90 ±0.04$^{\pm b}$	1.91± 0.03^{b}	2.07± 0.05^{a}	1.86±0.01^{b}
FCR	1.10± 0.00^{a}	1.18±0.01^{b}	1.22± 0.04^{b}	1.03± 0.01^{a}	1.22 ±0.01^{b}
Survival (%)	100.00±0.00	97.94±1.89	98.77±1.23	100.00±0.00	97.53±0.00

Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test)

6.3.2 Whole-body composition

Data on the whole-body composition of fish at the end of the trial is presented in Table 56. Dietary treatments did not affect the whole-body composition of fish (P>0.05).

Table 55. Whole-body composition of Atlantic salmon fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

	CTRL	NOPAP	PAP	NOPAP+	PAP-
DM, %	37.50± 0.27^{a}	36.81± 0.21^{ab}	36.91± 0.17^{ab}	36.04± 0.91^{ab}	35.93± 0.45^{b}
Ash, %	4.50±0.11	4.79±0.18	4.63±0.20	4.96±0.94	4.80±0.64
Protein, %	46.26± 0.20^{a}	47.81± 0.38^{bc}	47.22± 0.27^{abc}	46.40± 0.53^{ac}	48.02± 0.51^{c}
Lipid, %	48.30±0.47	47.45±0.65	47.69±0.56	47.47±0.00	47.44±0.45
Energy, kJ/g	30.13±0.89	29.97±0.43	29.06±0.61	29.98±0.76	30.13±0.14
Phosphorus, %	0.75± ^{a}	0.81± ^{ab}	0.80± ^{ab}	0.78± ^{ab}	0.84± ^{b}

Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test)

6.3.3 Apparent digestibility

Protein and energy apparent digestibility of Atlantic salmon fed with five different dietary groups are displayed in Table 57. Protein apparent digestibility was affected by the dietary treatments. Data shows that the lowest digestibility was found on fish fed with the PAP diet when compared to the rest of the diets. Regarding energy, apparent digestibility presents no statistical differences.

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Table 56. Apparent Digestibility measures of Atlantic salmon fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

ADC (%)	CTRL	NOPAP	PAP	NOPAP+	PAP-
Protein	82.21± 2.44^{a}	78.07± 3.91^{a}	61.32± 8.11^{b}	84.08± 0.67^{a}	75.58± 1.05^{ab}
Energy	73.04±5.41	73.25±5.62	65.17±6.54	80.43±0.68	75.49±0.39

Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test)

6.3.4 Nutrient retention

Results of protein and energy retention of fish fed with different diets are shown in Table 58.

Table 57. Retention measures of Atlantic salmon fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

Retention (intake %/feed)	CTRL	NOPAP	PAP	NOPAP+	PAP-
Protein	39.20±0.19	37.67±0.54	36.39±0.53	37.28±1.71	35.93±1.84
Energy	48.85±2.34	45.16±0.62	41.47±1.57	47.17±2.82	42.31±0.75

Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test).

6.3.5 Fatty acid retention

Fatty acid retentions are shown on Table 59. Fish fed PAP- diet presented lower retention for total monounsaturated fatty acid as well as total n-6 PUFA and 18:2n-6 fatty acids. Furthermore salmon fed PAP- diet showed lower 18:3n-3 and total saturated fatty acids retention when compared to NoPAP+.

Table 58: Fatty acid retention of Atlantic salmon fed different dietary treatments. Values are means and standard deviation (n=3 for NOPAP and PAP, n=2 for CTRL, NOPAP+ and PAP-).

Fatty acid	CTRL	NO PAP	PAP	NO PAP+	PAP-
Total saturated	71.47±3.08^{ab}	67.69±1.56^{ab}	65.27±12.61^{ab}	88.06±10.01^{a}	45.69±5.86^{b}
Total monounsaturated	67.04±9.43^{a}	69.64±4.15^{a}	67.33±5.03^{a}	76.22±1.93^{a}	47.27±1.29^{b}
20:5n-3	30.78±9.28	28.21±5.01	22.41±0.25	36.52±2.93	21.84±3.31
22:6n-3	51.20±21.20	49.10±9.00	43.40±4.00	57.90±0.60	35.00±8.20
n-3 HUFA (DHA+EPA)	37.00±12.89	39.30±6.96	33.67±2.18	47.71±1.09	28.18±5.65
Total n-3 PUFA	40.17±11.03	40.17±4.99	38.75±2.95	51.55±1.53	30.45±7.26
Total n-6 PUFA	64.82±9.58^{a}	69.29±5.05^{a}	66.97±4.48^{a}	70.07±2.66^{a}	45.94±2.09^{b}
Total PUFA	52.42±10.47	55.22±5.33	52.84±2.92	60.55±2.15	39.70±4.79
20:4(ω-6)	24.68±9.13	28.15±17.92	30.33±4.54	35.47±1.96	35.26±11.00
18:2n-6	65.76±9.60^{a}	70.20±4.76^{a}	67.84±4.51^{a}	70.77±2.68^{a}	46.12±1.94^{b}
18:3n-3	37.05±5.85^{ab}	38.05±2.75^{ab}	42.86±4.32^{ab}	52.76±1.36^{a}	30.51±9.20^{b}

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6.4 Turbot 2

6.4.1 Growth performance parameters

The growth performance results of pre-adult turbot are summarized in Table 60. Fish fed NoPAP 60 and PAP60 presented lower final body weight when compared to other treatments. On the other hand, FCR, biomass gain and RGR were better on control compared to PAP30, NoPAP60 and PAP60 fed fish. NOPAP30 presented a comparable performance to Control.

Table 59: Performance and flesh quality parameters of the turbot fed with five different dietary groups.

	CTRL	NOPAP30	PAP30	NOPAP60	PAP60
Initial BW (g)	303.2 ± 10.4	301.5 ± 3.7	298.4 ± 13.9	298.9 ± 7.9	299.7 ± 7.3
Final BW (g)	511.2 ± 38.0	485.0 ± 21.6	458.2 ± 31.6	458.8 ± 34.9*	446.2 ± 11.4*
Biomass gain (g)	4058 ± 695	3585 ± 132	2957 ± 581*	3051 ± 679*	2990 ± 205**
RGR (%/day)	0.47 ± 0.05	0.42 ± 0.03	0.38 ± 0.03*	0.38 ± 0.05*	0.36 ± 0.03*
VFI (% ABW/day)	0.61 ± 0.04	0.62 ± 0.01	0.63 ± 0.02	0.62 ± 0.03	0.67 ± 0.03
FCR	1.29 ± 0.14	1.46 ± 0.07	1.69 ± 0.19*	1.65 ± 0.27*	1.75 ± 0.04**
Survival (%)	98.9 ± 2.3	98.9 ± 2.3	96.6 ± 6.8	97.7 ± 2.6	97.7 ± 4.5
CF	2.02 ± 0.02	1.97 ± 0.07	2.02 ± 0.03	1.95 ± 0.03	1.94 ± 0.05
HSI (%)	1.54 ± 0.10	1.55 ± 0.24	1.52 ± 0.18	1.47 ± 0.19	1.46 ± 0.20
Dress-out loss (%)	5.3 ± 0.6	5.4 ± 0.3	4.9 ± 0.5	5.4 ± 0.6	5.2 ± 0.3
Fillet yield (%)	40.9 ± 2.9	41.8 ± 2.1	42.7 ± 5.8	40.7 ± 3.0	41.7 ± 2.9

* Indicates significant difference to control group with Multiple comparisons (Holm-Sidak method; $p < 0,050$).

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6.5 Seabass 1

6.5.1 Growth performance parameters

At the end of the trial (83 days) European seabass fed five different diets presented differences in FCR and RGR. Fish fed control diet presented higher RGR when compared fish fed other treatments. On the other hand, FCR was higher in NoPAP fed fish when compared to PAP- fed fish.

Table 60: Performance parameters of seabass fed five experimental diets (n=4).

	CTRL	PAP -	NoPAP	PAP	NoPAP +
IBW (g)	322.1±47.6	334.1±28.7	322.6±22.8	323.5±27.7	334.8±7.8
FBW (g)	496.2±94.3	475.3±26.3	445.6±35.9	448.2±67.0	475.2±24.7
RGR (%/day)	0.51±0.08^{a}	0.42±0.05^{b}	0.38±0.10^{b}	0.38±0.07^{b}	0.42±0.04^{b}
FCR	1.1±0.1	1.1±0.1	1.1±0.3	1.3±0.2	1.2±0.1
Feed Intake (%ABW/day)	0.25±0.01	0.24±0.02	0.24±0.03	0.23±0.03	0.22±0.00
Survival (%)	78.8±1.4	79.4±0.5	79.4±1.0	78.7±2.3	79.7±0.5

Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test).

6.5.2 Whole body composition

Whole body composition results are shown on Table 62. Different composition was observed in fish fed tested diets when compared to control. Seabass fed treated diets presented higher ash and lower fat composition when compared to control diet.

Table 61: Whole-body composition of fish fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

	Control	PAP-	NOPAP	PAP	NoPAP+
DM, %	37.69±0.50^{b}	38.61±1.98^{ab}	36.57±2.69^{a}	37.45±3.42^{ab}	41.49±6.74^{ab}
Ash, %	10.29±1.22^{a}	10.45±0.56^{b}	10.20±0.70^{b}	10.42±0.31^{b}	11.28±0.94^{b}
Protein, %	18.03±0.15	17.83±0.40	18.57±0.21	18.03±0.15	17.75±0.07
Fat, %	16.47±0.72^{b}	15.87±2.20^{a}	16.37±1.10^{a}	15.60±0.70^{a}	15.45±0.35^{a}
Energy, kJ/g	25.40±1.48	25.10±1.44	26.44±1.47	25.11±1.18	26.08±0.79

Initial values: 31.97±0.28% of DM, 13.10±1.50% of ash, 17.17±1.32 of protein, 11.77±0.67 of fat and 26.24±0.17 kJ/g of energy. Values are mean and standard deviation (n=3). Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test).

6.5.2 Nutrient retention

Results for protein, fat and energy retention are shown on table 63. No differences was observed in retention for this trial.

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Table 62: Retention of nutrients of sebass after feeding of proposed formulations : i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

Retention (intake %/feed)	CTRL	PAP	NOPAP	PAP-	NoPAP+
Protein	33.76±2.41	29.21±11.29	33.48±8.68	32.19±10.63	27.36±2.65
Fat	79.26±8.46	71.39±5.60	66.75±3.36	71.70±16.82	72.75±8.80
Energy	64.24±21.81	57.05±32.13	52.39±7.78	51.71±13.60	74.52±52.43

Values are mean and standard deviation (n=3).

7. Additional specific analysis Block 2

7.1 Trout 2

7.1.1 Flesh quality analysis

Marketable Traits

Organs and tissues were dissected from the whole viscera of individual fish and then weighted. Figure 11 shows the raw data on the sum of liver, mesenteric fat, and gastrointestinal tract, that together represent roughly the whole viscera weight.

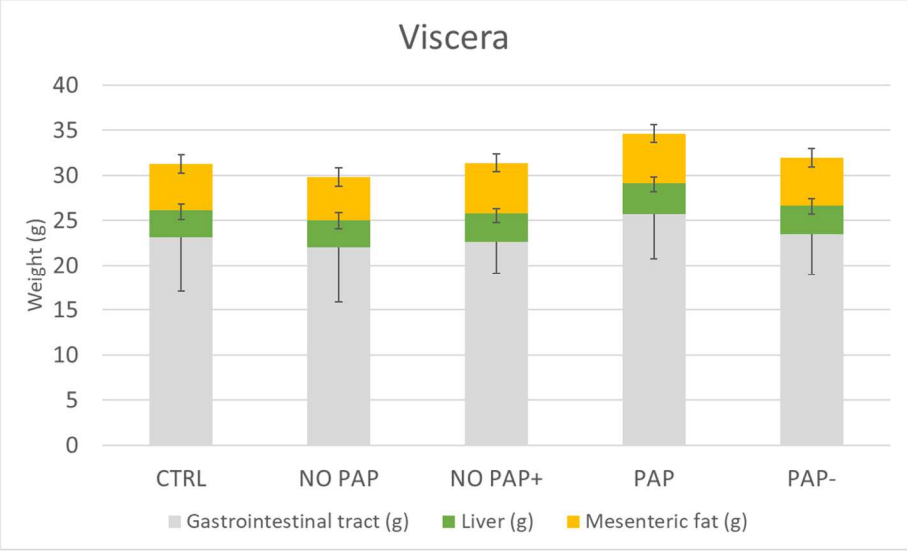

Figure 11: Mean weight of liver, gastrointestinal tract, and mesenteric fat of trout fed with different diets (n=20). The sum of three represent roughly the whole viscera weight

Using Final body weight (FBW) as predictor (319.3 g), an ANCOVA on organs and tissues weight was performed. As shown in Table 64, the mean weight of liver is higher in PAP diet (3.43 ±

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0.64 g) and statistically different from NoPAP, NoPAP+ and CTRL, the last one registered the lower mean weight (2.99 ± 0.71 g). A similar trend is evident in the mean gastrointestinal weight, where the PAP had the highest value (25.72 ± 4.95 g) and was significantly different from NoPAP and NoPAP+ diets, which registered 22.04 g (± 6.18) and 22.65 g (± 3.50) respectively. No statistical differences are reported on the quantity of mesenteric fat between diets.

Table 63: Mean weight of whole body, liver, mesenteric fat and gastrointestinal tract for trout under five different dietary groups i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy driven formula algae without processed animal protein, iv) NOPAP+: a diet similar to NOPAP but with higher protein content, and last, v) PAP-: a diet similar to PAP but with lower protein content.

Diets	FBW (g)	Liver (g)	Mesenteric fat (g)	Gastrointestinal tract (g)
CTRL	325.71 ± 59.09	2.99 ± 0.71 ^{b}	5.15 ± 2.17	23,11 ± 6.02 ^{ab}
NoPAP	303.24 ± 63.82	3.01 ± 0.80 ^{b}	4.74 ± 1.83	22.04 ± 6.18 ^{b}
NoPAP+	328.97 ± 37.27	3.13 ± 0.55 ^{b}	5.59 ± 2.32	22.65 ± 3.50 ^{b}
PAP	330.00 ± 44.72	3.43 ± 0.64 ^{a}	5.46 ± 2.12	25.72 ± 4.95 ^{a}
PAP-	308.61 ± 39.96	3.23 ± 0.70 ^{ab}	5.29 ± 1.62	23.44 ± 4.51 ^{ab}

Different letters refer to statistical differences ($p<0.05$) among diets marked by mean Bonferroni Post Hoc Tests. (n=20; ANCOVA)

From the raw data the main morphological indexes were calculated (Table 65). Morphological differences are detected on trout carcass where carcass yield differ between diets. CTRL and NoPAP+ reached the best yield with 90.49% and 90.47%, followed by NoPAP (90.27%), PAP- (89.68%) and PAP diet (89.55%). Statistical differences are notable between CTRL/NoPAP+ and PAP-/PAP groups by mean ANOVA analysis followed LSD Fisher Post Hoc comparisons. The viscerosomatic index (vsi%) is strictly related to the carcass yield, and represents the complementary part to 100%, so the vsi index is inversely related to cY%. Therefore, PAP and PAP- had the higher vsi (10.45 and 10.32% respectively) against CTRL and NoPAP (9.51% and 9.53%), and the statistical differences between groups are verified. The same goes for hepatosomatic index, where CTRL showed the smallest value (0.908%) against PAP- (1.038%).

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Table 64: Percentage (mean ± SD) of morphological indexes, carcass yield (cy), hepatosomatic index (hsi), viscerosomatic index (vfi) and mesenteric fat index (vsi) for Trout fed under five different dietary groups i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy driven formula algae without processed animal protein, iv) NOPAP+: a diet similar to NOPAP but with higher protein content, and last, v) PAP-: a diet similar to PAP but with lower protein content.

Diets	cY%	hsi%	vfi%	vsi%
CTRL	90.49 ± 1.32 [a]	0.908 ± 0.096 [b]	1.54 ± 0.48	9.51 ± 1.32 [b]
NOPAP	90.27 ± 1.23 [ab]	0.989 ± 0.136 [ab]	1.53 ± 0.37	9.73 ± 1.23 [ab]
NOPAP+	90.47 ± 1.07 [a]	0.948 ± 0.091 [b]	1.69 ± 0.66	9.53 ± 1.07 [b]
PAP	89.55 ± 0.98 [b]	1.037 ± 0.102 [a]	1.64 ± 0.56	10.45 ± 0.98 [a]
PAP-	89.68 ± 1.13 [b]	1.038 ± 0.123 [a]	1.72 ± 0.47	10.32 ± 1.13 [a]

Different letters refer to statistical differences ($p<0.05$) among diets marked by mean Fisher LSD Post Hoc Tests. (n=20; ANOVA).

Texture Profile Analysis (TPA)

Textural features were measured, 24 h after death, on 100 filets by Texture Profile Analysis using a Zwick Roell® 109 texturometer (Ulm, Germany).

Data are presented in table 66. The measured variables (hardness, cohesiveness, gumminess, resilience, and adhesiveness) showing no statistical differences between the experimental diets. The most evident gaps in the batch are concerning the hardness and gumminess measured in fish fed PAP- diet. These parameters are considerably lower in PAP- compared with the other samples, therefore these differences could be felt from consumers in a panel test.

Table 65: Texture profile analysis (mean ± SD) for Trout fed under five different dietary groups i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy driven formula algae without processed animal protein, iv) NOPAP+: a diet similar to NOPAP but with a higher protein content, and last, v) PAP-: a diet similar to PAP but with a lower protein content.

Diets	Hardness (N)	Cohesiveness	Resilience	Gumminess (N)	Adhesiveness (Nmm)
CTRL	6.46 ± 1.79	0.223 ± 0.036	0.036 ± 0.020	1.45 ± 0.51	0.441 ± 0.095
NOPAP	6.93 ± 2.13	0.243 ± 0.046	0.047 ± 0.024	1.66 ± 0.53	0.506 ± 0.529
NOPAP+	6.32 ± 1.47	0.226 ± 0.034	0.041 ± 0.019	1.43 ± 0.41	0.439 ± 0.128
PAP	6.56 ± 2.05	0.224 ± 0.040	0.045 ± 0.024	1.50 ± 0.60	0.362 ± 0.085
PAP-	5.89 ± 1.88	0.212 ± 0.027	0.039 ± 0.021	1.26 ± 0.46	0.328 ± 0.064

Different subscripts refer to statistical differences ($p<0.05$) among diets marked by mean Fisher LSD Post Hoc Tests. (n=20; ANOVA)

Flesh Colour

Colour parameters (CIELab values) are significantly different among dietary treatments (Table 67). The lightness (L*) of CTRL and NoPAP+ is similar and statistically lower than other three

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diets (NoPAP, PAP and PAP-), however it is possible to discriminate NoPAP+ from CTRL by the indexes of red (a*) and yellow (b*), both higher in the first more than in the second one. Moreover, the red index is rather high in PAP- diet too and statistically different from CTRL and NoPAP. In terms of CIE L*C*h colour system, NoPAP+ and PAP- registered the highest Chroma value (or colour saturation), but their Hue differ clearly between them, due to the b* index (0.92 vs -0.68 respectively). This shows us that NoPAP+ diet segregates from PAP- and the other diets as colour, since it is laying in a different quarter of the color space (+b* quarter vs –b* quarter).

Table 66: Colour indexes (CIE L*a*b*) of trout´s flesh after the experiment grouped by diet (mean ± SD).

Diets	L*	a*	b*	Chroma	Hue (rad)
CTRL	43.19 ± 1.93 ^{c}	2.18 ± 1.19 ^{d}	-0.19 ± 1.70 ^{bc}	2.79 ± 1.14 ^{b}	-0.26 ± 0.67 ^{b}
NOPAP	43.90 ± 2.19 ^{b}	2.48 ± 1.22 ^{cd}	-0.013 ± 1.87 ^{b}	3.04 ± 1.35 ^{b}	-0.21 ± 0.61 ^{b}
NOPAP+	42.65 ± 1.49 ^{c}	3.87 ± 1.97 ^{a}	0.92 ± 2.38 ^{a}	4.41 ± 2.44 ^{a}	0.004 ± 0.48 ^{a}
PAP	44.12 ± 1.73 ^{ab}	2.68 ± 1.36 ^{bc}	-0.27 ± 1.49 ^{bc}	3.09 ± 1.33 ^{b}	-0.22 ± 0.52 ^{b}
PAP-	44.55 ± 1.49 ^{a}	3.02 ± 1.43 ^{b}	-0.68 ± 1.41 ^{c}	3.48 ± 1.23 ^{a}	-0.35 ± 0.47 ^{b}

Different letters indicate significant differences (Kruskal-Wallis ANOVA by Ranks; p< 0.05; Multiple Comparison Test, n=900).

Total colour differences between diets were calculated according to the ΔE Lab formula (Mokrzycki & Tatol, 2011), ΔE represents the difference between two colours designated as two points in the Lab colour space. Pairwise comparisons between diets are presented in Table 68.

Table 67: Total colour differences (ΔE Lab) in CIELab colour space presented as pairwise comparisons.

ΔE Lab	CTRL	NoPAP+	NoPAP	PAP	PAP-
CTRL					
NoPAP+	2.094				
NoPAP	0.790	2.093			
PAP	1.060	2.233	0.392		
PAP-	1.679	2.635	1.084	0.693	

A standard observer perceives the differences in colour presented above as follows:

• 0 < ΔE < 1 - observer does not notice the difference
• 1 < ΔE < 2 - only experienced observer can notice the difference
• 2 < ΔE < 3.5 - unexperienced observer also notices the difference
• 3.5 < ΔE < 5 - clear difference in color is noticed
• 5 < ΔE - observer notices two different colors.

As shown in table 56 an unexperienced observed may notice a difference in colour between NoPAP+ diets and the other experimental diets, more difficult to perceive the difference between the Control diet and PAP group (PAP and PAP-), as well as the difference between NoPAP and PAP-. Only the instrumental measurement can discriminate the other matches.
To understand how the colours are different, we can say that a pale grey, pink or brown colour

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is one feature of the flesh of farmed rainbow trout fed without carotenoids. The instrumental data and the main colour indexes suggest that CTRL, PAP and NoPAP diets range close to this target, whereas a mild pink/orange pigmentation of filets has been observed in flesh of trout fed with NoPAP+ diet compared to the other experimental groups. In contrast with the NoPAP+, the lack of yellow (b*) in PAP- diet results in a shift of hue from orange to purple/blue in the eyes of an observer. To conclude, trout fed CTRL, PAP, and NoPAP diet show a colour considered more natural by consumers, and then more advisable. A visual comparison can make between the pictures below (Figure 12).

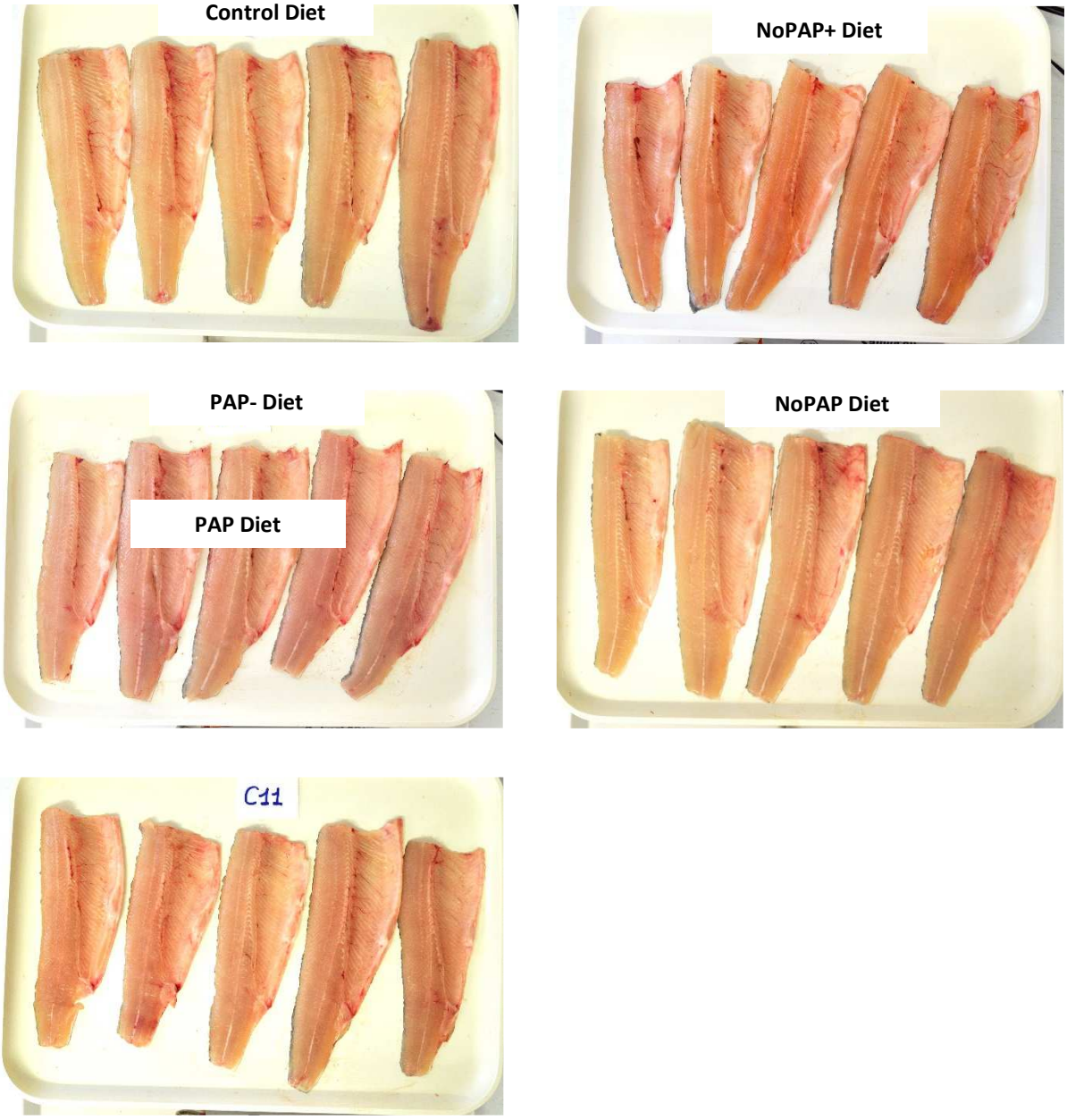

Figure 12: Each picture shows a subsample of fillets analysed by colourimeter and CIE Lab method.

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7.1.2 Sensorial analysis and consumer acceptance

Sensorial analysis revealed few differences resulting in high general acceptance for consumers (Table 69). Consumers exposed fish fed NoPAP diet as being with better texture than fish fed PAP diet, consequently NoPAP fed fish presented higher global acceptance.

Table 68: Sensorial analysis in a panel of 100 consumers appearance, odor, texture, taste; and consumer (global acceptance) for Trout (mean ± SD) fed with three different dietary groups i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy -driven formula algae.

Sensorial Analysis	CTRL	PAP	NoPAP
Appearance	7.8 ± 1.3	8.0 ± 1.0	8.0 ± 1.0
Odor	7.9 ± 1.1	7.9 ± 1.0	8.0 ± 0.9
Texture	7.9 ± 1.2 ^{ab}	7.9 ± 1.1 ^{b}	8.1 ± 0.9 ^{a}
Taste	7.9 ± 1.2	7.9 ± 1.1	8.1 ± 0.9
Global Acceptance	7.9 ± 1.0 ^{ab}	7.9 ± 0.9 ^{b}	8.1 ± 0.7 ^{a}

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test*, n=100*).

7.1.3 Lysozyme analysis

Lysozyme analysis presented different results among treatments (Figure 13). Fish fed NoPAP diet presented a higher concentration (U/ml) of plasma lysozyme (p>0.05) when compared to fish fed Control, NoPAP+, and PAP.

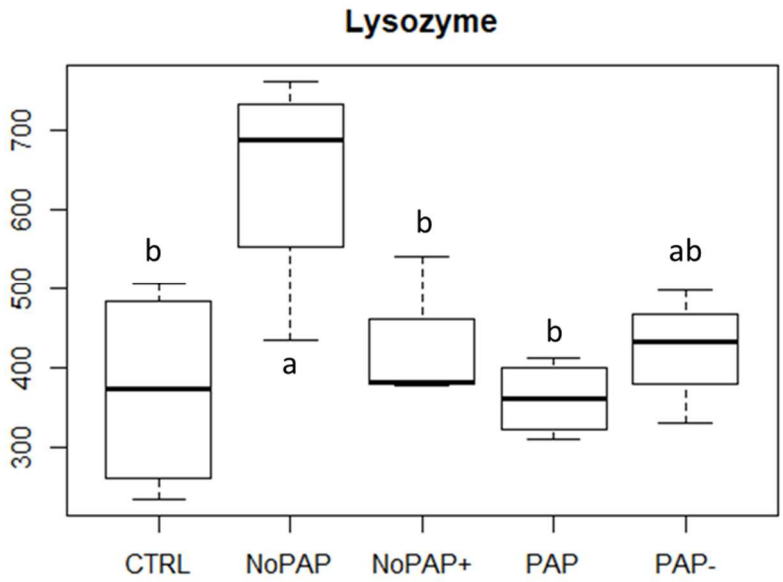

Figure 13: Lysozyme concentration (U/ml) in plasma of fish fed different diets.

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7.2 Bream 2

7.2.1 Gene expression profiling

All genes included in the PCR-array were found at detectable levels in the three tissues analyzed. Results of selected gene expression profiling in the liver, head kidney, and posterior intestine are presented in Figure14. In the liver, 17 out of 44 genes were differentially expressed (DE) in response to NoPAP SANA diet. The expression of markers from GH/IGF system (*igf-i*), lipid metabolism (*elovl6, fads2, scd1a, hl, pla2g6, cyp7a1, pparβ* and *pparγ*), lipid and energy metabolism (*h-fabp, nd5, coxii* and *ucp1*) and antioxidant defense (*gpx4, prdx5, cu-zn-sod/sod1* and *mn-sod/sod2*) was significantly down-regulated in fish fed NoPAP SANA diet. In HK, 2 out of 29 genes were affected by the NoPAP SANA diet. In this case, the expression of the interleukin-8 (*il8*) and toll-like receptor 2 (*tlr2*) genes was up-regulated in fish fed NoPAP SANA diet. In the intestine, 4 out of 44 genes were DE with the experimental diet, all of them showing a down-regulated response. These genes were markers of epithelial integrity (*cdh17*), mucus production (*muc2*), cytokines (*il12β*), and cell markers (*cd8b*) (Figure 1).

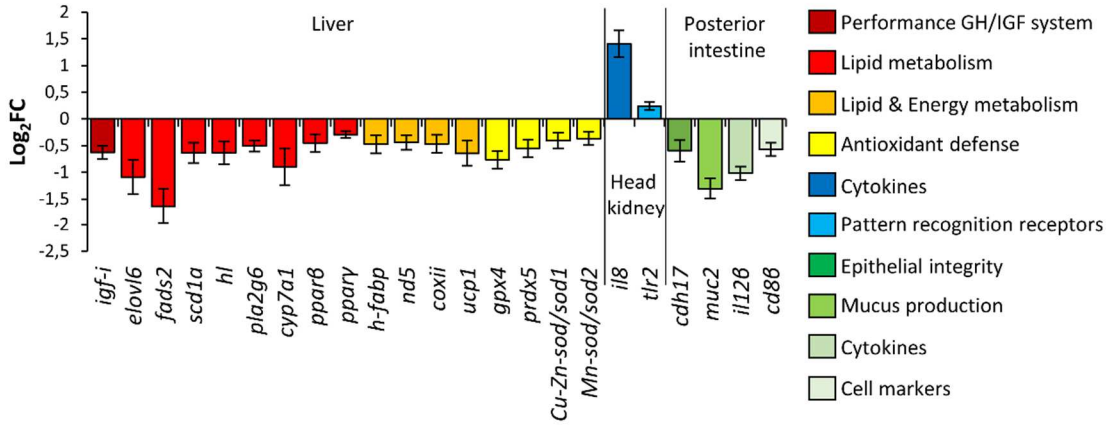

Figure 14: Mean ± SEM of the Log_2 fold changes of the differentially expressed genes from the PCR-array panels run in liver, head kidney and spleen (t-test, $P < 0.05$) when comparing NoPAP SANA vs. CTRL group. Different colors represent different associated functions depicted in the legend on the right.

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7.2.2 Parasite challenge

At the beginning of the experimental infection (0 days post-exposure = 0 dpe) fish fed CTRL and NoPAP SANA diets were selected to not present differences in weight, length, CF and SGR to homogenize the starting point (Figure 15 A-C). At 40 dpe, the prevalence of infection was 45.7% and 66.6% for CTRL and NoPAP SANA fish, respectively. In agreement with the infection status, recipient (R) fish showed significantly lower growth parameters than the control uninfected group (C), but these were not different between diets. At 78 dpe, CTRL and NoPAP SANA groups showed a prevalence of infection of 55.4% and 68.6%, respectively, and differences in the growth parameters followed the same trend as in the intermediate sampling, except for a lower CF in the uninfected group fed NoPAP SANA diet and recovery to control values of the CFs of recipient groups (Figure 15 D). This unexpected increase in CF of recipient fish in the final sampling hinted towards a possible recovery of the infected animals, probably due to the extremely high water temperatures in the last weeks of the challenge (29ºC). This recovery was evident when the intensity of infection between sampling points was compared. The median Ct values are significantly higher after 78 dpe, with no differences between diets (Figure 14 E). In fact, Figure 14 F shows how individual fish from both groups were recovering at the same rate.

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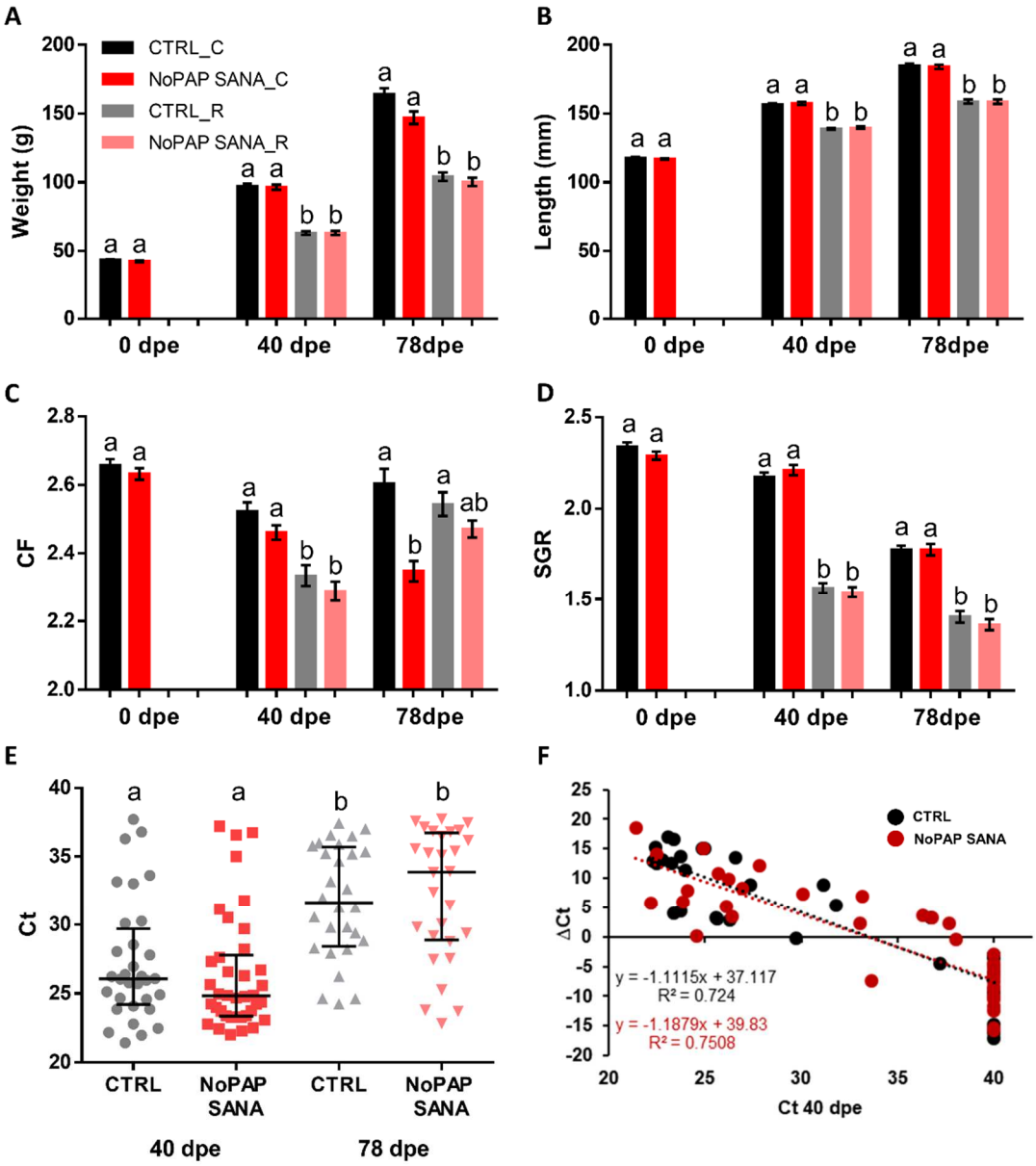

Figure 15: Biometric parameters obtained during the infection challenge of gilthead seabream fed CTRL and NoPAP diets. Weight (A), length (B), condition factor (CF; C), and specific growth rate (SGR; D) of control uninfected (C) and *Enteromyxum leei* recipient (R) fish were measured immediately before infection (0 days post-exposure, dpe) and 40 and 78 dpe. Different letters indicate significant differences within each sampling point (one way ANOVA + Tukey's test, $P < 0.05$). The legend for A-D is located in A. Intensity of infection (E) was evaluated by the Ct values of the diagnostic PCR (lower Ct values = higher parasite load) and is represented as median ± interquartile range. Different letters indicate significant differences among groups (Kruskal-Wallis + Dunn's tests, $P < 0.05$). The recovery trend of each individual fish (F) was determined by plotting the ΔCt (Ct at 78 dpe – Ct at 40 dpe) against the Ct at 40 dpe. The negative slopes showed by the equations indicate a trend to increased Cts at the final sampling point with no

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differences between groups.

7.2.3 Microbial composition

Illumina sequencing of the 27 analysed PI samples yielded 4,068,405 high quality and merged reads, with a mean of 226,022 reads per sample. The reads were assigned to 1,712 OTUs at 97% identity threshold. Up to 66.5% of the OTUs were classified at the level of species and more than 90% at the level of genus (91%), family (96%), order (97%), class (99%) and phylum (99%). Rarefaction analysis showed curves that approximated saturation (horizontal asymptote), thus a good coverage of the bacterial community was achieved and the number of sequences for analysis was considered appropriate.

No significant differences were found in diversity indexes (Shannon and Simpson), but a significantly higher richness (Observed and ACE values, $P < 0.05$) was found in NoPAP SANA fish (Table 59). At the phylum level (Figure 16), as expected for gilthead seabream, Proteobacteria, Firmicutes and Actinobacteria constituted close to 90% of the total bacterial populations with no significant changes between diets. Bacteroidetes, other important phylum in this species, significantly decreased in NoPAP SANA fish (4.3%) when compared to the control group (6.1%). Fusobacteria, usually found in low proportion in the intestine of gilthead seabream (0.1% in control fish) significantly increased to 2.6% in NoPAP SANA group.

Table 69: Richness (Observed and ACE) and diversity (Shannon and Simpson) indexes. Data are represented as the mean for each group ± SEM (n = 9).

	CTRL	NOPAP SANA	*P* value
Observed	207.56 ± 55.98	257.89 ± 52.34	**0.042***
ACE	306.76 ± 128.94	375.06 ± 81.57	**0.017***
Shannon	2.15 ± 0.74	2.42 ± 0.39	0.626
Simpson	0.76 ± 0.24	0.85 ± 0.07	0.758

P-values of Kruskall-Wallis test are indicated, and statistically significant differences are marked in bold font with asterisks (* < 0.05).

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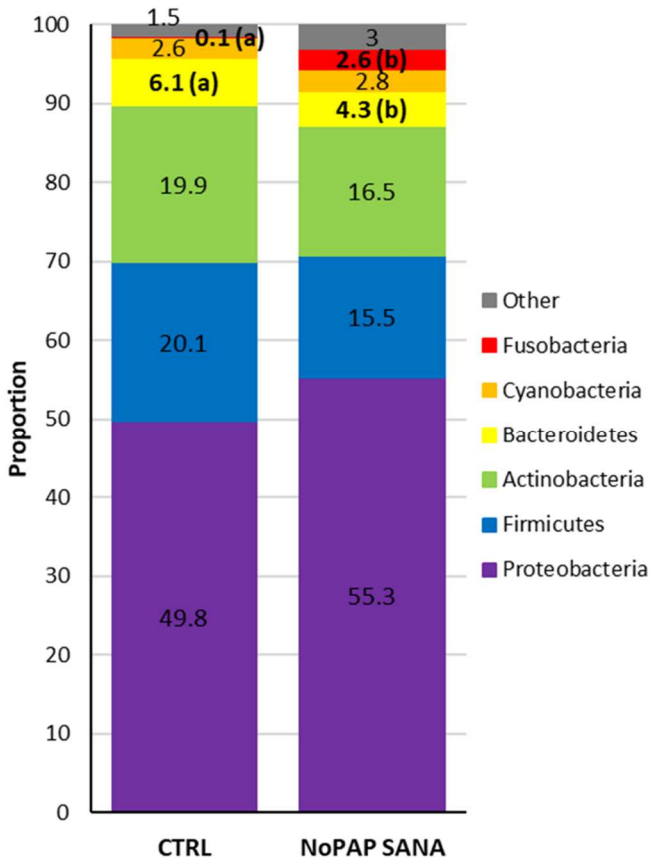

Figure 16: Stacked bar chart representing the relative abundance of bacterial phyla in the two groups. Only the phyla that are present in at least 1% in one of the groups are represented. Different letters indicate significant differences between CTRL and NoPAP SANA groups (Kruskal-Wallis + Holm-Sidak tests, $P < 0.05$).

7.3.4 Microbiota discriminant analysis

PERMANOVA test did not show statistically significant differences in bacterial composition when comparing animals fed different diets ($P = 0.066$, $F = 1.1692$, $R2 = 0.0681$). However, to study and validate and in more detail the microbiota differences among groups, a PLS-DA model ($R2Y = 99\%$, $Q2 = 55\%$) with three components was constructed and statistically validated (Figure 16A). The first two components explained more than 95% of the total variance, clearly separating CTRL fish from NoPAP SANA fish along the x-axis (component 1, 88.79%). To determine which groups of bacteria were driving these separations at a high level of confidence, the minimum VIP value driving the correct separation of groups in the model was determined throughout a heatmap representation (Figure 17B).

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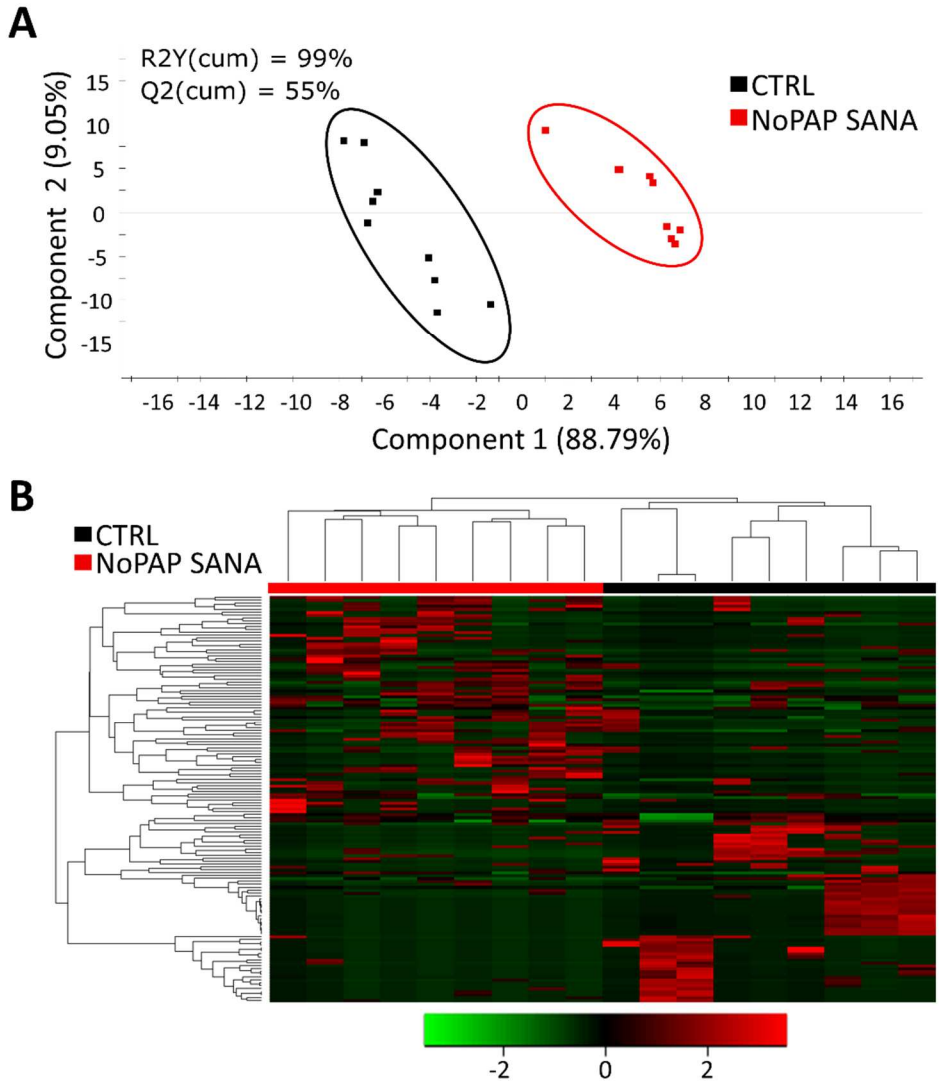

Figure 17: Two-dimensional PLS-DA score plot (A) constructed using the variable diet representing the distribution of the samples between the first two components in the model. The goodness of fit and validation by permutation test can be found in Supplementary Figure 2. The heatmap (B) represents the abundance distribution (Z-score) of the OTUs identified to drive the separation by diet (VIP > 1).

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7.3 Seabass 1

7.3.1 Blood Parameters

Lactate Dehydrogenase

The Lactate Dehydrogenase activity (LDH) in the fish plasma is displayed in Figure 18. No significant difference could be found.

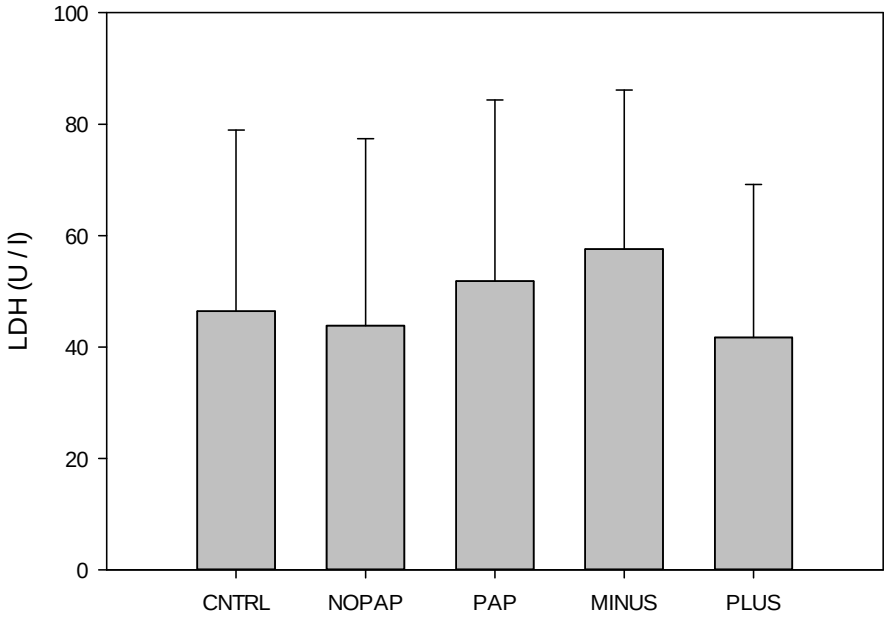

Figure 18 Lactate Dehydrogenase (LDH) in fish exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy - driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the mean counts ± SD (one-way ANOVA; p= 0.478).

Plasma glucose

The Glucose in fish plasma is displayed in Figure 19. No significant difference could be found.

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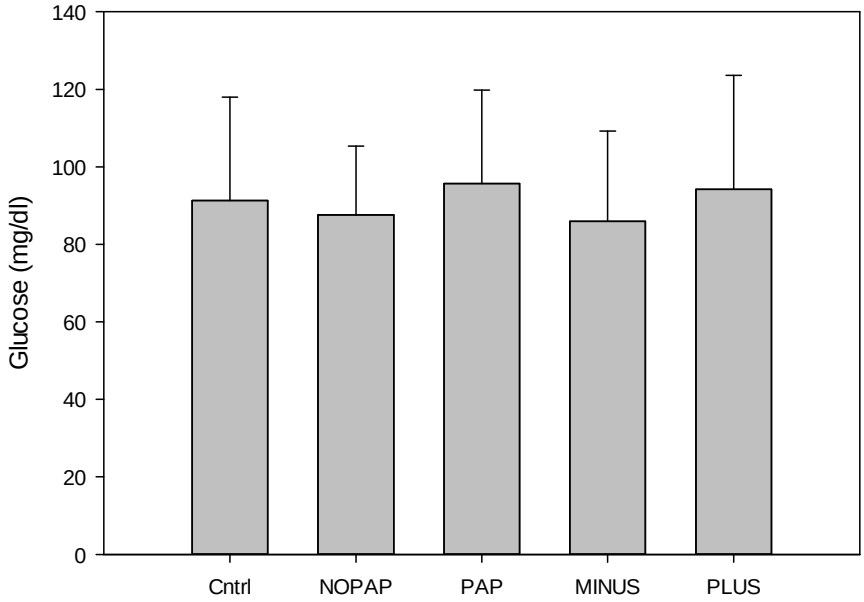

Figure 19: Glucose in fish exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the mean counts ± SD (one-way ANOVA; p= 0.519).

Plasma total Protein

The total protein content in fish plasma is displayed in Figure 20. The fish from the CNTRL group have significantly higher amounts of total protein in the plasma than fish from the PLUS and MINUS groups. The fish from the PLUS group have significantly lower amounts of total protein in the plasma than fish from the PAP and NOPAP groups.

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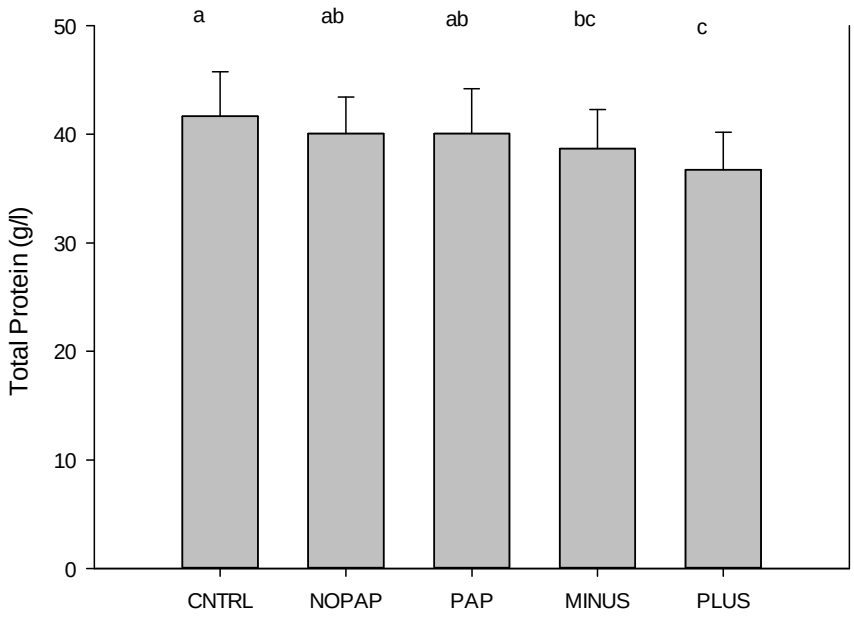

Figure 20: Plasma total protein in fish exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the mean counts ± SD. Different letters indicate significant differences (one-way ANOVA; p= <0,001).

Lysozyme

The lysozyme in fish plasma is displayed in figure 21. No significant differences could be found.

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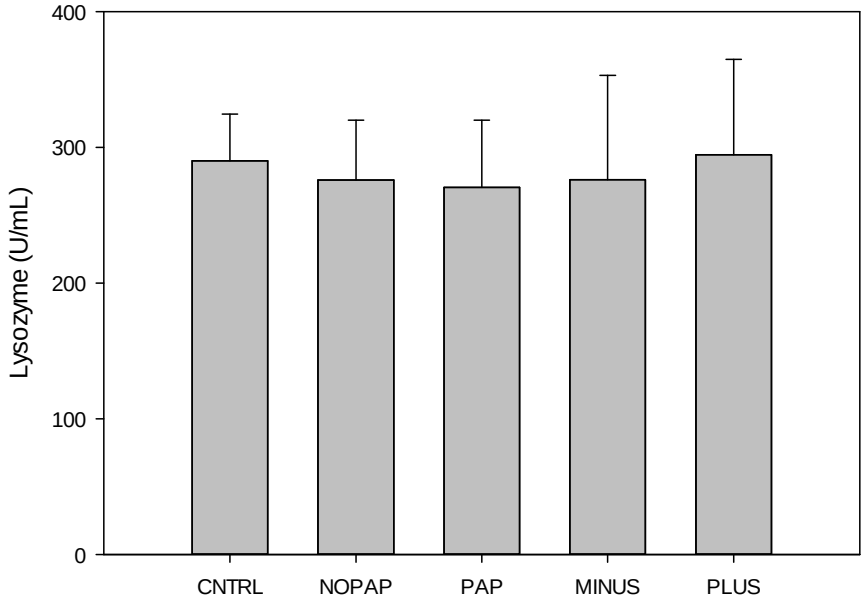

Figure 21*:* Lysozyme in fish exposed to four different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the mean counts ± SD (one-way ANOVA; p= 0,450).

7.3.2 Filet parameters

Characteristics of the frozen filet

The characteristics of the frozen fish filet are displayed in Figure 22. The Control fed fish show a significantly higher proportion of fish filet that is firm and little elastic in the frozen filet than fish from the MINUS group, which is less firm in places.

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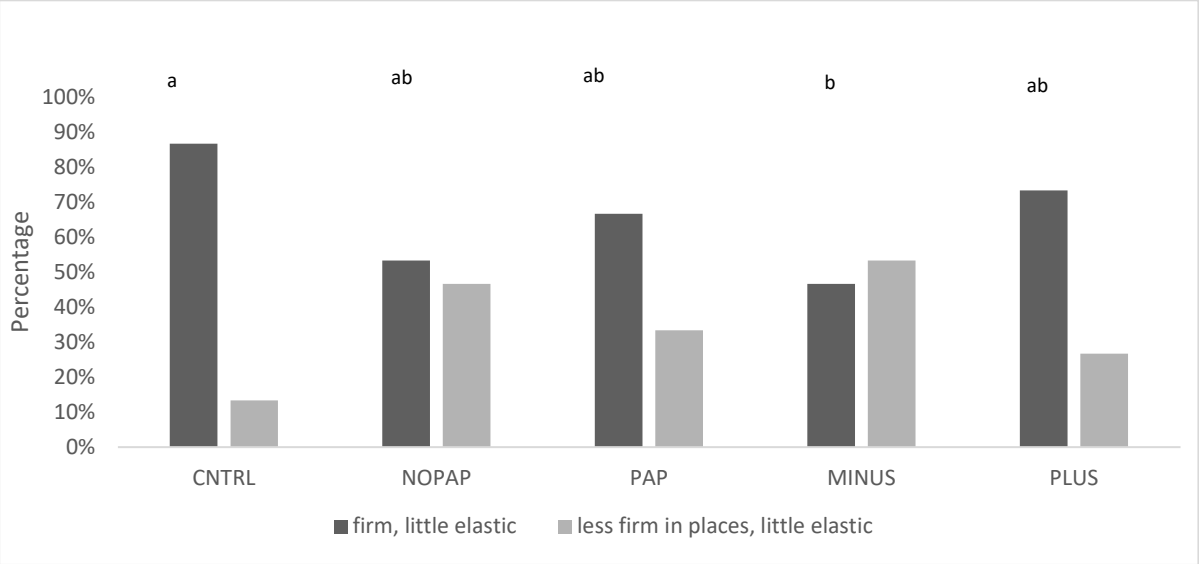

Figure 22*:* Frozen characteristics in fish filet of fish exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) -PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the percentage of filets with a specific characteristic. Different letters indicate significant differences (n=75; ANOVA on ranks; p= 0.157).

Characteristics of the taste

The characteristics of the fish filet taste after cooking are displayed in Figure 23. No significant differences could be found.

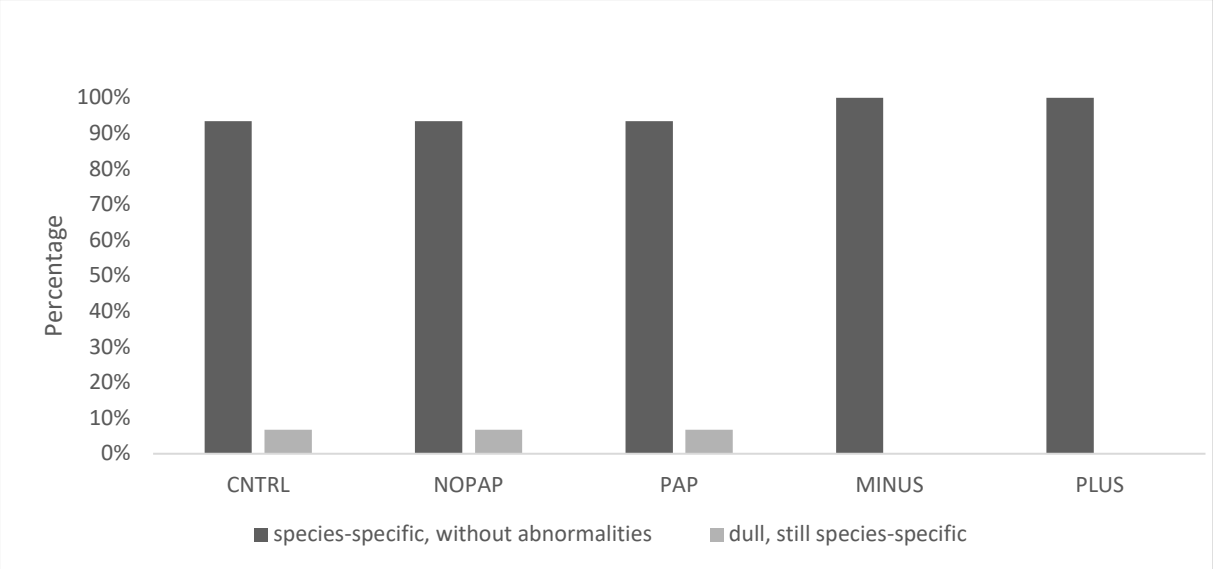

Figure 23*:* Taste in fish filet of fish exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula

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without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the percentage of filets with a specific characteristic. (n=75; ANOVA on ranks; p= 0.726).

Characteristics of consistency

The characteristics of the fish filet consistency after cooking are displayed in Figure 24. The Control group filets are significantly firm to bite after cooking compared to the NOPAP, MINUS and PLUS groups. The fish filets from them MINUS group are significantly more tender and less firm to bite after cooking than the filets from the PAP group. The PLUS fed fish filets are significantly tender after cooking than the filets from the PAP group.

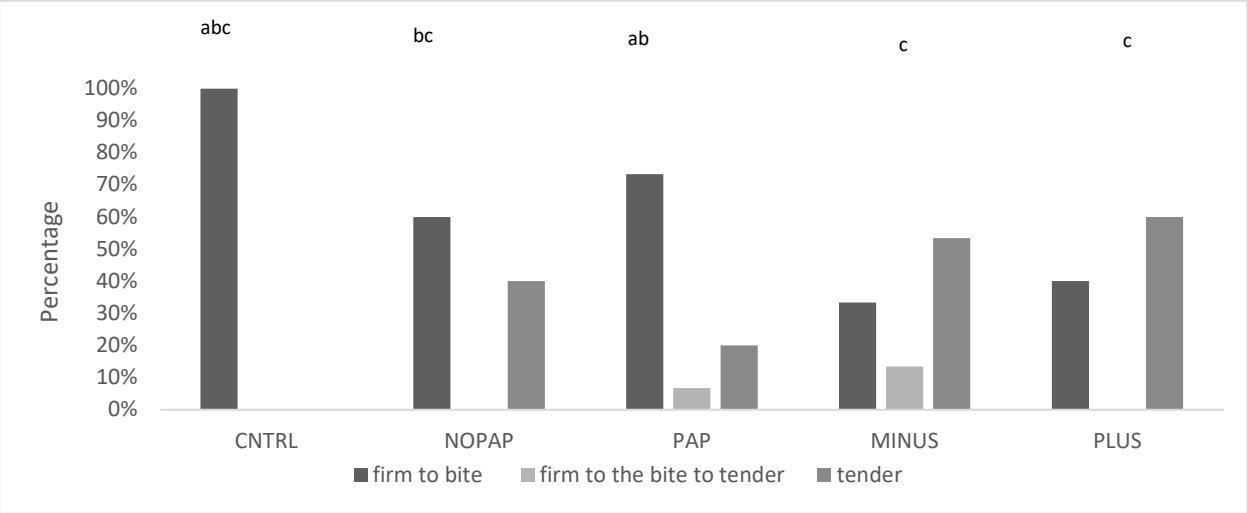

Figure 24*:* Consistency of fish filet after cooking. Fish were exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the percentage of filets with a specific characteristic. Different letters indicate significant differences (n=75; ANOVA on ranks; p= 0.001).

Juice separation

The juice separation of the fish filet after cooking are displayed in Figure 25. No significant differences could be found

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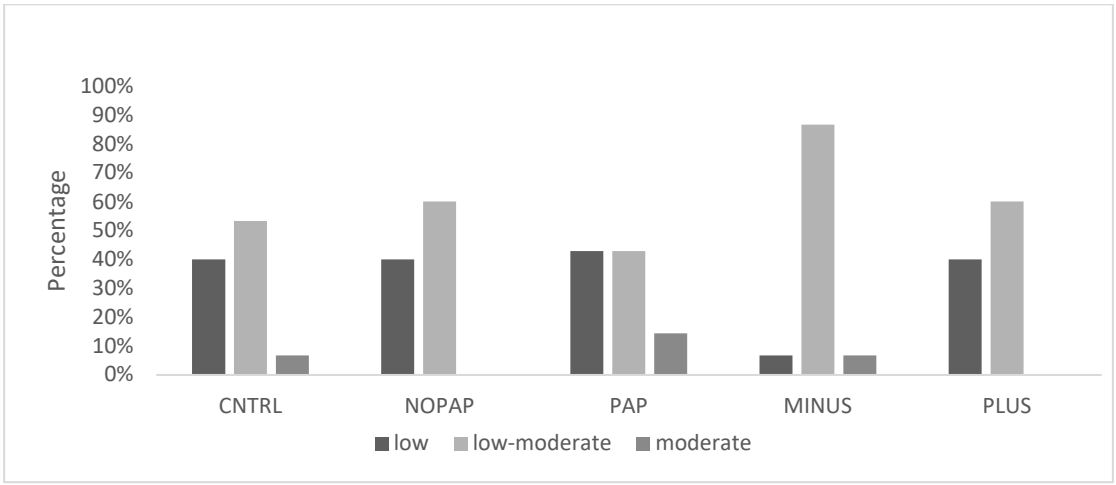

Figure 25*:* Juice separation of fish filet after cooking. Fish were exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the percentage of filets with a specific characteristic. (n=75; ANOVA on ranks; p= 0.240).

Protein precipitation

The protein precipitation of the fish filet after cooking are displayed in Figure 26. No significant differences could be found.

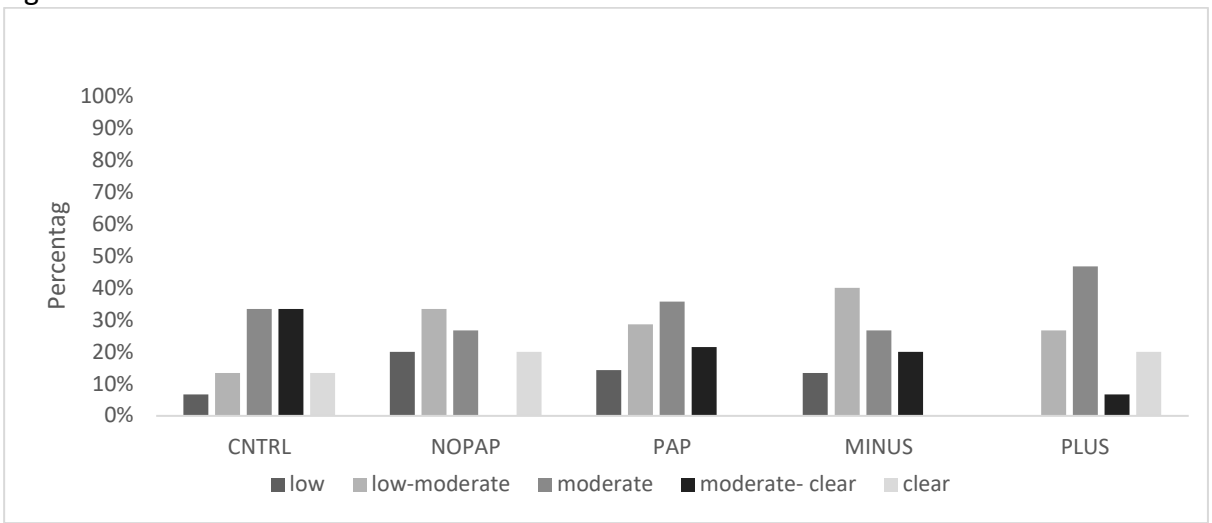

Figure 26*:* Protein precipitation of fish filet after cooking. Fish were exposed to five different experimental groupsi) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the percentage of filets with a specific characteristic. (n=75; ANOVA on ranks; p= 0.182).

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Grease separation

The grease separation of the fish filet after cooking are displayed in Figure 27. No significant differences could be found.

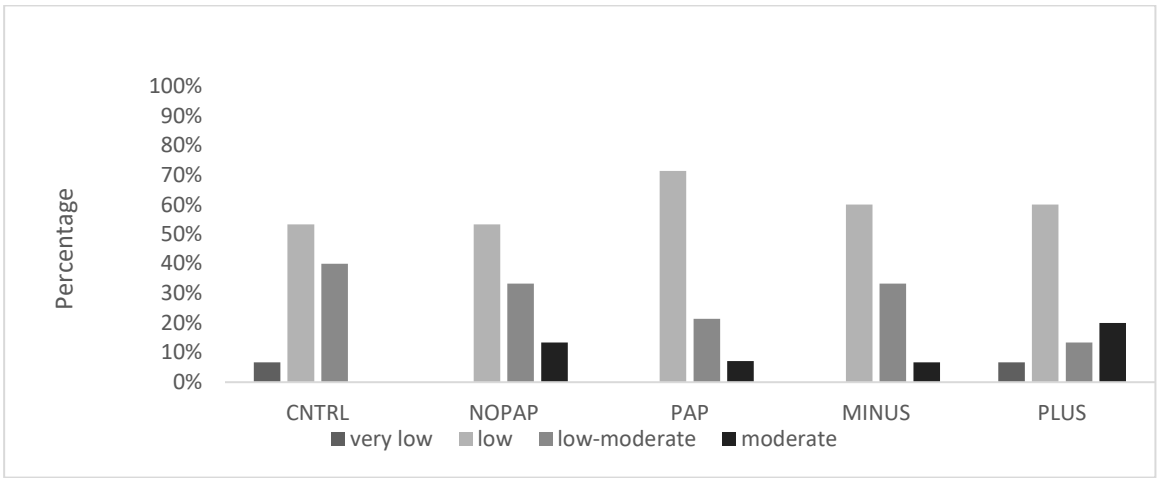

Figure 27*:* Grease separation of fish filet after cooking. Fish were exposed to five different experimental groups: i) Control (n=23) (CNTRL), ii) PAP (n= 25) – Circular economy -driven formula with processed animal protein, iii) NOPAP (n=24) – Circular economy -driven formula without processed animal protein, iv) MINUS (n= 24) - PAP diet with lower protein content, v) PLUS (n=21) -NOPAP diet with an higher protein content. Bars represent the percentage of filets with a specific characteristic. (n=75; ANOVA on ranks; p= 0.886).

7.4 Salmon 2

7.4.1 Humoral Immune parameters

Humoral parameters activity in fish is displayed in Table 71. The highest value was verified in fish fed with the PAP- diet, followed by NOPAP, and the lowest in the control diet. When compared to the control diet it is possible to see significant differences between this diet and the NOPAP and PAP- diets. The highest anti-protease activity value was verified in fish fed with the PAP - diet, followed by NOPAP+ and the lowest on the control diet. When compared to the control diet it is possible to find significant differences between this and the PAP- diet. The immunoglobulin activity parameter had significant differences between diets. The highest value was verified in fish fed with the PAP diet, followed by NOPAP+ and the lower on PAP- diet. When compared to the control diet we can not find significant differences. Moreover, results of bactericidal activity measured in the plasma had no significant differences among diets.

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Table 70. Humoral parameters activity in fish fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

Parameters	CTRL	NOPAP	PAP	NOPAP+	PAP-
Protease (%)	25.54± 2.55^{a}	27.84± 2.29^{b}	27.08± 2.46^{ab}	27.26±2.08^{ab}	28.92± 3.12^{b}
Anti-protease (%)	55.41±16.03^{a}	56.55±16.76^{a}	59.88±14.56^{ab}	63.82±9.67^{ab}	69.65±8.00^{b}
IgM (absorbance)	0.38± 0.18^{ab}	0.43± 0.27^{ab}	0.56± 0.40^{a}	0.48± 0.46^{ab}	0.21± 0.12^{b}
Bactericidal activity (%)	64.21±2.49	58.45±8.96	56.29±17.34	58.38±12.53	56.30±11.06

Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test)

7.4.2 Mucosal Mapping

Results of mucosal mapping are displayed in Table 72. All the mucosal mapping results for foregut show a trend or significant differences between the groups. Fish from group control had the largest mucosal values with mean mucus area of 163um^2, 14% density and a defence activity of 0.84. The lowest mucosal parameters are measured in fish from group PAP having a 119.4 um^2 mucous cell area, 8% density and 0.64 in defence activity. All the mucosal mapping parameters in group control are significantly higher than group PAP.

Table 71. Mucosal mapping data regarding fish fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

	CTRL	NOPAP	PAP	NOPAP	PAP-
Mucous Cell Area (um^2)	161± 34^{a}	142± 16^{ab}	119± 22^{b}	168± 25^{a}	139± 23^{ab}
Mucous Density (%)	0.14± 0.03^{a}	0.10± 0.03^{ab}	0.08± 0.02^{b}	0.103± 0.03^{a}	0.10± 0.03^{ab}
Barrier Status	0.84± 0.08^{a}	0.73± 0.16^{ab}	0.64± 0.15^{b}	0.77± 0.13^{ab}	0.70± 0.11^{ab}

Different letters indicate significant differences (one-way ANOVA; Tukey post hoc test).

7.4.3 Oxidative status

Data on oxidative status biomarkers are displayed in Table 73. This parameter had no significant differences among diets.

Table 72. Oxidative status biomarkers in fish fed with five different dietary groups: i) Control, ii) PAP– Circular economy driven formula with processed animal protein, iii) NOPAP – Circular economy -driven formula algae without processed animal

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protein, iv) NOPAP+- Improved version of the NOPAP diet, v) PAP- – economical version of the PAP diet.

	CTRL	NOPAP	PAP	NOPAP+	PAP-
LPO (nmol/g)	41,93±3,53	47,55±18,40	46,49±13,40	53,93±6,89	44,72±13,25

7.4.4 Sensorial Analysis

Sensorial analysis revealed few differences (Table 74). Consumers exposed fish fed NoPAP diet as being with better odor than fish fed Control diet, consequently NoPAP fed fish presented higher global acceptance.

Table 73: Sensorial analysis (mean ± SD) appearance, odor, texture, taste; and consumer (global acceptance) for Salmon fed with three different dietary groups i) Control, ii) PAP: Circular economy driven formula with processed animal protein, iii) NOPAP: Circular economy -driven formula algae.

Sensorial Analysis	CTRL	NoPAP	PAP
Appearance	7.1 ± 1.5	7.3 ± 1.5	7.3 ± 1.3
Odor	7.2 ± 1.5 ^{b}	7.5 ± 1.4 ^{a}	7.4 ± 1.2 ^{ab}
Texture	7.4 ± 1.4	7.6 ± 1.2	7.4 ± 1.3
Taste	7.3 ± 1.5	7.5 ± 1.5	7.4 ± 1.3
Global Acceptance	7.3 ± 1.3 ^{b}	7.6 ± 1.2 ^{a}	7.4 ± 1.1 ^{ab}

Different letters indicate significant differences (one-way ANOVA; p< 0.05; Tukey post hoc test*, n=100*).

7.4.5 Hepatic gene expression profiling

Data were in reference to the expression level of *gpx* of control fish with an arbitrarily assigned value of 1. Ten out of 40 genes in the array were differentially expressed (DE) at P<0.05, including this set of genes markers of growth performance (igfbp2a, igfr1), energy metabolism (sirt2, ucp2l), antioxidant defense (*gpx1*), immune response (bd3) as well as a wide representation of lipid-related genes (*elovl5*, fads1, fads2, hl). Overall, the gene expression level presented values that put the PAP- group closer to the control group. The NOPAP+ with the NOPAP and the group that shows to be the most different from the control and the PAP group. This was visualized by heat map analysis (Figure 28). Comparing the experimental groups to the control we see an up-regulation of all genes as the fold change graph shows. Regarding the gene igfbp2a we see that the experimental groups present a lower expression when compared to the control one. The gene igfr1 only shows a significant difference with the control on the NOPAP+, in this case with a higher

A

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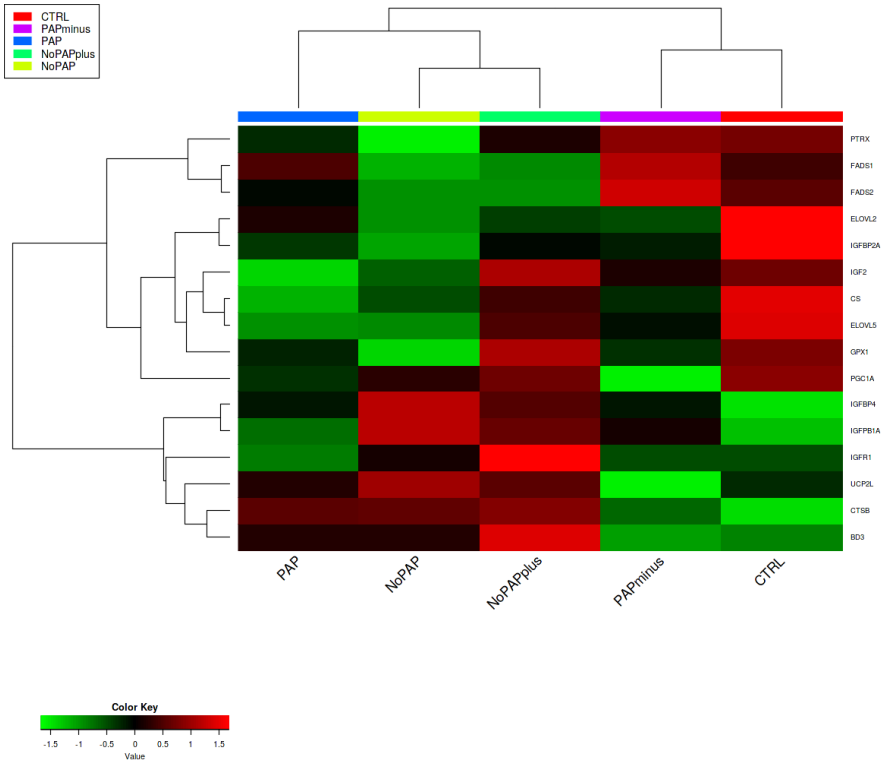

B

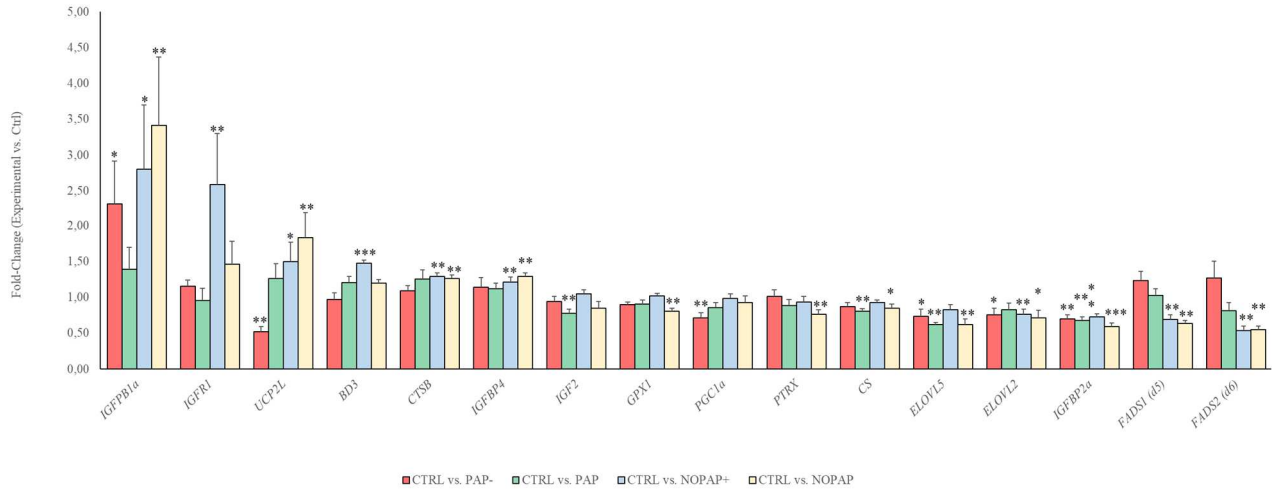

Figure 28. Heat map of liver gene expression profile after filtering for most nutritionally regulated genes (A). Fold changes of changing expressed genes (experimental/control fish) (B). Values are the mean ± SEM of 10-12 fish. Asterisks indicate statistically significant differences by Student-t test (*, P<0.1; **, P<0.05; ***, P<0.001).

expression on fish fed with the NOPAP+ diet. In the gene *elovl5* we see significant differences between the control and NOPAP/PAP groups, in this case with a higher

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expression on fish fed with the control diet. Gene fads1 only shows a significant difference with the control on the NOPAP, in this case with a lower expression on fish fed with the NOPAP diet. The gene bd3 also shows significant differences between the control on the NOPAP+, in this case with a higher expression on fish fed with the NOPAP+ diet.

7.4.6 Head kidney gene expression profiling

Seven out of 29 genes were differentially expressed at $P<0.05$ in the head kidney of salmon fed with control, NOPAP, NOPAP+, PAP, and PAP- diets. These genes include interleukins (il12 and il15), markers of adaptative immunity (*igm* and *igt*), lysozyme (lysc2), and T cell & monocyte/macrophage markers (cd8b and csf1r1). Overall, the gene expression level presented values that put the PAP- group closer to the NOPAP+ group. The NOPAP with the PAP and the control group shows to be in the middle closer to the first cluster. This was visualized by heat map analysis (Figure 29). Comparing the experimental groups to the control we see an up-regulation of all genes as the fold change graph shows. The genes igm, igt, and cd8b only show significant differences with the control on the NOPAP, in the case of the adaptative immunity markers with a higher expression on fish fed with the NOPAP diet and lower on the case of the cd8b gene. Furthermore, gene lysc2 only shows a significantly higher expression on fish fed with the PAP diet compared to the control.

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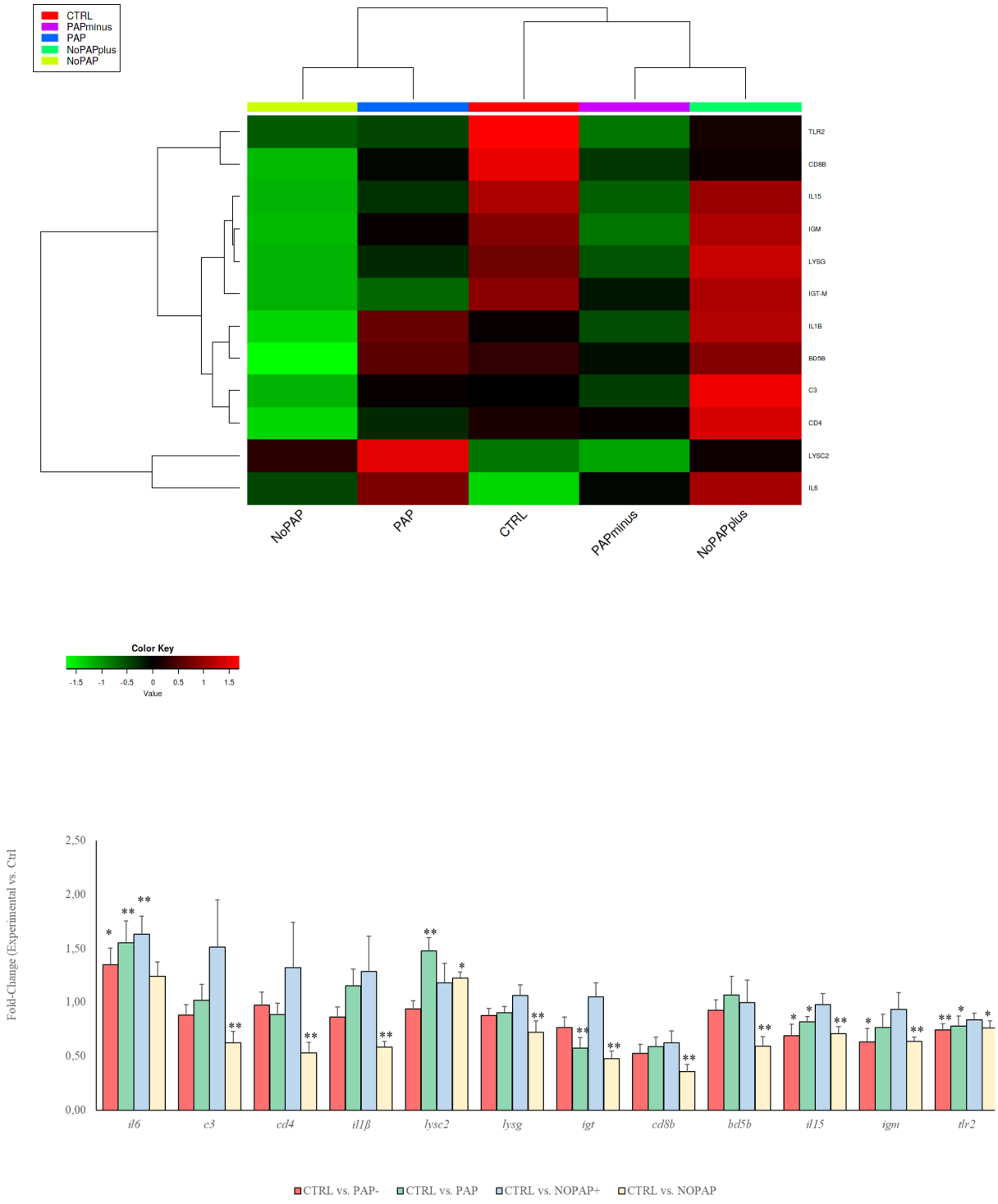

Figure 29. Heat map of head kidney gene expression profile after filtering for most nutritionally regulated genes (A). Fold changes of changing expressed genes (experimental/control fish) (B).Values are the mean ± SEM of 9 fish. Asterisks indicate statistically significant differences by Student-t test (*, P<0.1; **, P<0.05; ***, P< 0.001).

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8. Conclusions

Overall, the growth performances and feed conversions (FCR) in all trials were good to very good for all species and for most feed formulations tested. Moreover, results support that the novel formulations tested are viable options for the target species tested. KPIs other than growth and FCR suggest that the novel formulations affect fish physiology, likely looking for a new allostatic balance, but no major effects on fish health.

The main findings for each species and trial are summarised in Figure 30, and in the text below.

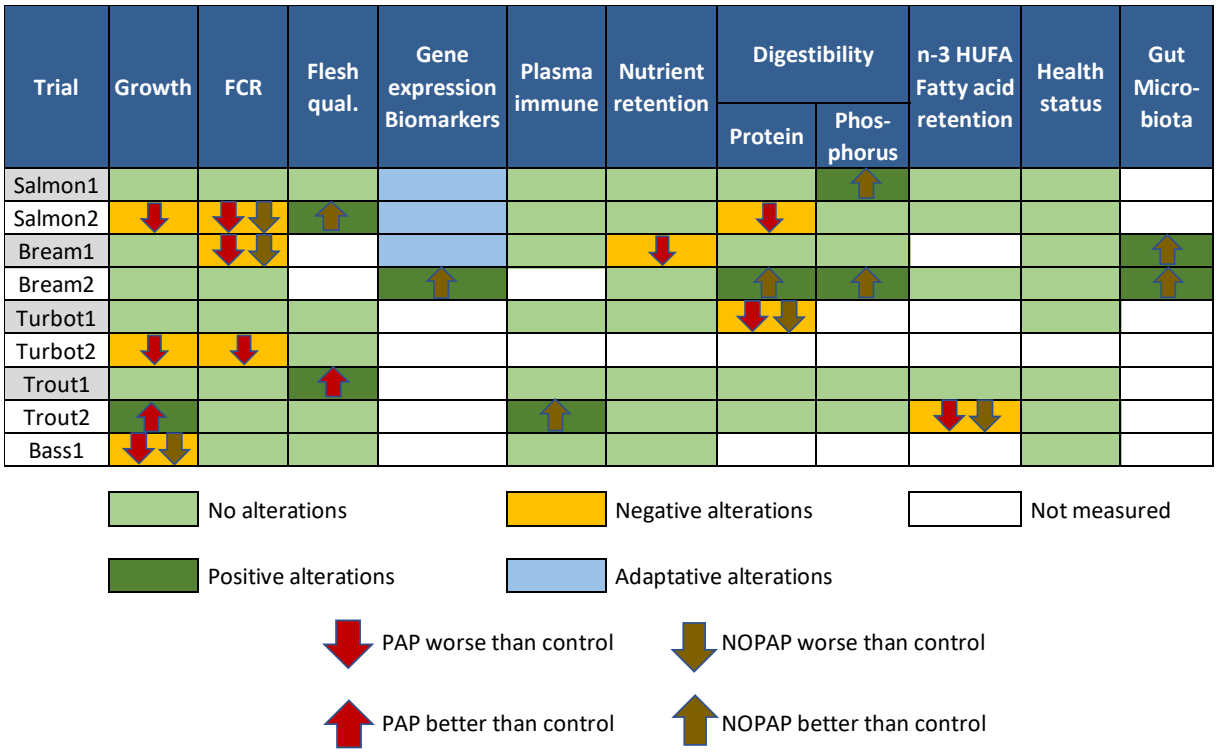

Figure 30. Overview on how the different key performance indicators were affected by the GAIN novel aquafeed formulations in the 9 fish trials conducted.

Atlantic salmon

In trial **Salmon1** growth performance was very similar for the 3 diets tested. All the other parameters analyzed, including health scores and those referring to intestinal mucosa status, plasma innate immune defenses, and oxidative status in the liver did not show significant differences between diets, which suggest that GAIN novel feed formulations lead to fish with good welfare and health. Nonetheless, head kidney gene expression on fish fed with novel feed formulations suggests that there is an adaptation of the inflammatory response profile, which is not a problem in itself. In turn, on the liver gene profile, genes differentially expressed are involved in growth performance, lipid metabolism, and energy metabolism. Moreover, once gene expression was monitored twice; a few weeks after exposure to the diets, and at the end of the trial, it is clear that fish have, and as could be expected, an adaptation period to the novel diets, but seem to reach a new allostatic balance.

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These results were further confirmed in the **Salmon2** trial. Growth performance was good for the 5 diets tested. The growth performance was higher on fish fed with the NOPAP+ diet, followed by CTRL and the lower in the PAP diet. As for the feed conversion ratio, the five diets promoted acceptable results, the best FCR being found on fish fed with CTRL and NOPAP+ diets. It seems that these formulations can promote a better bioavailability and/or increased absorption of key nutrients. No impact of diets could be seen on fish welfare and health status based on the immune parameters measured in the plasma, lipid peroxidation in the liver, and anterior intestine mucosal mapping. However, there was a tendency for worse mucosal status in fish of the PAP diet, but still within normal values for this species. Regarding the gene expression, it followed the trend of the other analyses, with no signs of health and welfare being negatively affected. The head kidney gene expression level presented values that put the PAP- group closer to the NOPAP+ group. The NOPAP with the PAP and the control group show similar gene expression patterns. The liver gene expression level presented values that put the PAP- group closer to the control group. The NOPAP+ with the NOPAP and the group seem to be the most different from the control the PAP group.

In short, feed formulations such as NOPAP and PAP, devoided of fish meal, and containing a basket of alternative protein sources such as microbial biomasses, land-animal processed proteins, insect meal, fish protein hydrolysates (from aquaculture by-products) and vegetable protein concentrates; and replacing 50% of the fish oil by a mix of rapeseed, and algae oils; are likely valuable options to support accelerated growth, good health, and good feed conversion ratio in Atlantic salmon. However, good results will depend on a high protein digestibility of the chosen ingredients. Moreover, positive results on consumer perception may arise due to improvements in flesh quality.

Gilthead seabream

Results of trials **Bream1** and **Bream2** suggest that the novel feed formulations, and in particular the NOPAP diet, give a good growth performance in seabream, and are good alternatives to current gilthead seabream feeds. Still, in Bream1 FCR was worse in both PAP and NOPAP diets compared to Control, and this may be related to lower protein retention. Bream 1 trial suggests that fish fed with NOPAP diet show a slight improvement of innate immunity, as shown by higher IgM, bactericidal, and anti-protease activities. Furthermore, mucosal mapping ™ results agree with the plasma innate immune results where the fish fed with NOPAP and MIX diets presented higher values of barrier status compared to the PAP diet. This result is also supported by the expression profile of the head kidney, where fish fed with PAP diet was markedly pro-inflammatory with also a reduced expression of igt-m, which may be indicative of an impaired immune response at the mucosal level. The head kidney expression profile of NOPAP fish was very similar to that found in control fish. MIX fish exhibited an intermediate head kidney expression pattern between PAP and NOPAP fish. The liver expression profile shows modulation of lipid-related genes. The increased expression of scd, fads2, and elovl6 enzymes in NOPAP group is a typical characteristic feature of a reduced supply of n-3 LC-PUFA.

In **Bream2** trial the NoPAP SANA diet modulated the expression of several genes in the liver showing the capacity to reduce lipogenesis, mitochondrial activity, and the risk of oxidative stress and, at the same time, promoting an anti-inflammatory gene expression profile in the head kidney, and posterior intestine. All these changes may be seen as adaptations to the

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novel diets, the fish looking for a new physiological equilibrium. Therefore, from a fish health point of view, no constraints in using novel diets were found for gilthead seabream. In fact, the NoPAP SANA diet may even promote some improved immune competence, and no increased susceptibility against an intestinal parasite challenge could be observed as in previous studies using alternative formulations.

Clearly, a feed formulation such as NOPAP SANA, devoided of fish meal, and containing a basket of alternative protein sources such as microbial biomasses, insect meal, fish protein hydrolysates (from aquaculture by-products) and vegetable protein concentrates; and replacing 50% of the fish oil by a mix of salmon, and algae oils; seems to be a valuable option to support accelerated growth, good health, and a very good feed conversion ratio in gilthead seabream.

Turbot

In trial **Turbot1**, performed with smaller fish, growth performances and feed conversion ratios were very good for the GAIN novel formulations and comparable to the control diet. Still, fish fed with MIX diet show a slight decrease in the nutritional status due to reduced condition factor and hepatosomatic index, while fish fed with PAP and NOPAP diets also show significantly reduced hepatosomatic indices. Moreover, plasma immune parameters and nutrient retention were unaffected in the novel feed formulations, despite protein digestibility being lower in PAP and NOPAP diets compared to control.

The **Turbot2** trial results suggest that pre-adult fish fed with PAP 60 diet had the overall lowest growth and feed conversion performance, followed by PAP 30 and NOPAP 60. The results on condition factors, hepato-somatic index, and the survival rates indicate a good nutritional and health status, with no differences between the diets. Moreover, the diets had no effects on the dressout loss and fillet yield, suggesting no negative effects on flesh quality.

In short, a feed formulation such as NOPAP 30, based on: 28% of a lower quality fish meal (from by-products), and alternative protein sources such as microbial biomasses, insect meal, fish protein hydrolysates (from aquaculture by-products), and vegetable protein concentrates; and replacing 50% of the fish oil by a mix of salmon, algae and rapeseed oils; seems to be a valuable formulation for turbot in the grow-out phase resulting in good growth, feed performance and health.

Rainbow trout

In trial **Trout1** growth performances in rainbow trout were very good and similar between the control and the novel GAIN feed formulations. Moreover, fish were healthy throughout the trial and no difference in plasma lysozyme could be seen, which supports the suitability of the GAIN formulation concepts for eco-efficient farming of healthy trout. However, flesh quality results suggest trout fed with NoPAP and MIX diets presented a yellowish pigmentation when compared to control. It might suggest some pigments from microalgae and seaweed used in the formulation may have an impact on consumer preferences.

The growth performance and feed conversion in trial **Trout2** were also very good in the 5 diets tested. The best FCR was found in fish fed the NO PAP+ diet, while the highest FCR was in the PAP- diet. Protein and energy retentions were also very good in the 5 diets tested, with somewhat lower protein retention for the PAP and PAP- diets. However, DHA retention

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was lower in the 4 GAIN alternative formulations compared to the control. This leads to suggest that another selection of oils should be tested in future trout trials.

Clearly, feed formulations such as NOPAP and PAP, devoided of fish meal, and containing a basket of alternative protein sources such as microbial biomasses, land-animal processed proteins, insect meal, fish protein hydrolysates (from aquaculture by-products) and vegetable protein concentrates are valuable options to support accelerated growth, good health, and very good feed conversion ratio in rainbow trout. Moreover, consumer perception in terms of flesh quality will be good.

European seabass

Only one trial was performed in seabass. It suggests that NOPAP and PAP diets lead to a slightly lower growth performance compared to seabass-fed commercial-type diet. Moreover, results showed slightly decreased health parameters for the PAP and MINUS groups, while sensory evaluation was not significantly affected by any of the diets tested, except for consistency after cooking.

Overall, these results seem to support the hypothesis that the NOPAP diet and PLUS diets are viable options for seabass, but further analyses are needed to investigate if fish physiology is affected by the different diets.

In general terms, it seems trout was the species that accepted the best new formulations, and turbot the one that accepts them worst. Overall NoPAP diets seem to present better results for all fish species tested during this project. However, the PAP concept seems to be also valid and the less positive results in some species are likely to have to do more with the batch quality of one or more of the ingredients used, namely in terms of protein digestibility, than the PAP concept itself. Moreover, results on sensory evaluation for salmon, trout, and seabass suggest that the novel formulations tested would be well accepted by the consumer. Still, formulation costs tended to be higher in alternative diets, and sustainability evaluation was not favorable (results from WP4, not shown in the present Deliverable).

The 9 trials on fish novel feeds performed during the GAIN project confirmed that it is possible to produce fish using formulation concepts and ingredient baskets that fit into a circular economy framework, which was a main objective of the project. We demonstrated that fish production can be achieved using eco-efficient feeds. For trout, the new formulations even increased production in a cost-effective manner, which may improve the competitiveness of the industry, especially if an eco-efficient label can be added to the product. Furthermore, the very good acceptance of fish fillets after sensorial analysis in salmon and trout reinforces the idea that consumer acceptance for alternative formulations and ingredients will not be a problem. Still, this required that the industry communicates well the pros and cons of eco-intensification, including the circular economy-driven benefits and food safety, of using aquafeed formulations using an alternative ingredient basket including lower quality fish meal (from by-products), microbial biomasses, insect meals, fish protein hydrolysates (from aquaculture by-products), vegetable protein concentrates, macroalgae, microalgae, salmon oil, algae oils, and rapeseed oil.

These GAIN trials on fish novel feeds also demonstrated that fish protein hydrolysates (FPH)

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arising from aquaculture side-streams, as well as macroalgae and microalgae, can be used as effective aquafeed ingredients. FPH are valuable to stimulate feed intake due to their high content in free amino acids, while containing peptides with putative bioactivities that may explain at least in part the positive effect on fish immunity observed in some fish trials. Micro and macro -algae were also successfully used as a source of minerals, in particular Selenium. These algae also pigments, phenolic, polysaccharide, and other compounds with putative bioactivities, which may also explain at least in part the positive effect on fish immunity observed in some fish trials.

In short, GAIN feed formulations, including ingredients using aquaculture and fisheries side-streams, and other emerging ingredients adhering to circular economy principles, are viable options for eco-efficient European fish farming, especially once costs of emerging ingredients become price-competitive, and renewable energies are used to produce them.

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ANNEX 1

1. Detailed diet composition Block1

1.1 Trout1

Table 74: Dietary amino acid content for Trout. Values are mean and standard deviation of duplicates analysis (n=2).

Amino acids (%DM)	CTRL		NoPAP		PAP		MIX	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arginine (Arg)	2.92	0.18	2.80	0.15	2.74	0.07	2.82	0.03
Histidine (His)	1.17	0.09	1.05	0.03	0.99	0.03	1.12	0.10
Isoleucine (Ile)	1.81	0.01	1.80	0.09	1.73	0.09	1.74	0.05
Leucine (Leu)	3.14	0.04	3.14	0.21	3.18	0.10	3.19	0.14
Lysine (Lys)	2.83	0.04	2.97	0.05	2.80	0.22	2.92	0.10
Threonine (Thr)	1.83	0.02	1.69	0.04	1.61	0.09	1.74	0.03
Tryptophan (Trp)*	0.48	-	0.64	-	0.52	-	0.68	-
Valine (Val)	1.91	0.01	1.85	0.03	2.00	0.09	2.16	0.02
Methionine (Met)	1.13	0.03	1.20	0.02	1.12	0.09	1.18	0.03
Cystine (Cys)	0.33	0.01	0.28	0.00	0.29	0.00	0.31	0.01
Phenylalanine (Phe)	2.43	0.18	2.31	0.03	2.26	0.19	2.15	0.02
Tyrosine (Tyr)	1.84	0.15	1.91	0.05	1.53	0.00	1.80	0.04
Aspartic acid + Asparagine (Asx)	3.41	0.02	2.93	0.05	3.25	0.30	3.02	0.18
Glutamic acid + Glutamine (Glx)	7.78	0.35	7.22	0.23	6.65	0.39	6.78	0.20
Alanine (Ala)	2.03	0.08	1.97	0.12	2.23	0.02	2.31	0.18
Glycine (Gly)	1.78	0.12	1.70	0.12	2.15	0.11	2.05	0.06
Proline (Pro)	2.68	0.01	2.64	0.18	2.89	0.04	2.67	0.20
Serine (Ser)	2.17	0.01	1.82	0.04	1.91	0.06	1.90	0.07
Taurine (Tau)	0.46	0.02	0.71	0.04	0.60	0.04	0.53	0.02

* Value not made in duplicate, thus no standard deviation calculated.

Table 75: Fatty acid content of the diets.

Fatty acids	CTRL	NoPAP		PAP		MIX	
	*	Mean	SD	Mean	SD	Mean	SD
14:0	0.270	0.251	0.004	0.319	0.037	0.272	0.018
15:0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
16:0	1.563	1.587	0.003	1.995	0.059	2.071	0.134
18:0	0.367	0.357	0.001	0.448	0.002	0.489	0.033
20:0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22:0	0.000	0.000	0.000	0.000	0.000	0.000	0.000
24:0	0.027	0.024	0.008	0.021	0.001	0.014	0.020
Total saturated	2.226	2.219	0.007	2.784	0.100	2.846	0.129
16:1	0.446	0.470	0.014	0.583	0.025	0.666	0.047
18:1n-9	6.024	5.001	0.131	5.768	0.046	6.364	0.168
18:1n-7	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20:1	0.232	0.296	0.022	0.349	0.010	0.392	0.022
22:1	0.000	0.000	0.000	0.000	0.000	0.000	0.000

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24:1	0.027	0.030	0.000	0.030	0.001	0.034	0.002
Total monounsaturated	6.730	5.798	0.140	6.730	0.032	7.456	0.239
18:2n-6	2.698	2.439	0.073	2.644	0.039	2.528	0.061
18:3n-6	0.000	0.040	0.009	0.011	0.016	0.036	0.006
20:2n-6	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20:3n-6	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20:4n-6	0.045	0.031	0.001	0.043	0.003	0.044	0.004
22:4n-6	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22:5n-6	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Total n-6 PUFA	2.742	2.510	0.083	2.698	0.026	2.608	0.071
18:3n-3	1.393	1.241	0.108	1.409	0.060	1.594	0.062
18:4n-3	0.087	0.066	0.008	0.077	0.000	0.078	0.007
20:3n-3	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20:4n-3	0.000	0.000	0.000	0.000	0.000	0.000	0.000
20:5n-3	1.011	0.640	0.023	0.720	0.048	0.803	0.096
21:5n-3	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22:4n-3	0.000	0.000	0.000	0.000	0.000	0.000	0.000
22:5n-3	0.105	0.117	0.004	0.145	0.003	0.161	0.007
22:6n-3	0.639	0.579	0.032	0.606	0.010	0.601	0.052
Total n-3 PUFA	3.235	2.644	0.065	2.957	0.001	3.237	0.224
Total PUFA	5.978	5.154	0.018	5.655	0.025	5.845	0.295
Total FA	14.934	13.325	0.076	15.337	0.070	16.330	0.683

* Due to technical problems with the machine control diets are showed without replicate and no standard deviation was calculated.

Table 76: Mineral composition of the diets. Values are mean and standard deviation of technical replicates (n=2).

Minerals	**CTRL**		**NoPAP**		**PAP**		**MIX**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
% DM								
P	1.30	0.01	1.33	0.04	1.11	0.02	1.22	0.00
Ca	1.58	0.01	1.17	0.03	1.38	0.03	1.11	0.00
Na	0.48	0.01	0.48	0.01	0.42	0.01	0.39	0.00
Mg	0.18	0.00	0.16	0.00	<LOQ	-	0.16*	-
K	0.95	0.02	0.53	0.01	0.42	0.01	0.44	0.00
mg/kg								
As	3.99	0.57	1.94	0.10	1.85	0.19	1.71	0.09
Cu	13.54	0.19	25.09	0.59	24.74	0.27	34.21	0.23
Fe	177.04	0.87	329.84	3.48	331.16	2.84	410.69	4.17
Mn	69.15	13.39	104.16	37.60	82.16	2.14	86.04	12.30
Y	167.10	4.55	169.62	5.40	176.01	1.95	172.93	2.47
Zn	187.12	8.28	197.74	24.73	197.79	10.69	197.15	5.20

<LOQ means values were too low for quantification. *Second duplicate was too low for quantification; thus, no standard deviation was calculated.

Table 77: Vitamin content of the diets.

Vitamins	**CTRL**	**NOPAP**	**PAP**	**MIXED**
mg/100g				
Vit E	25.00	25.9	22.5	19.8

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Vit B1	0.244	0.22	0.237	0.219
Vit B2	0.81	0.914	0.939	0.88
Vit B3	5.64	6.6	18.2	14.1
Vit B6	0.491	0.516	0.466	0.533
mg/kg				
Vit B5 mg/kg	62.6	61.1	62.2	60.9
Vit C mg/kg	477	482	467	546
ug/100g				
Vit B9	461	534	546	541
Vit B12	36.9	96.3	96.2	118

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1.2 Bream 1

Table 78. Diet fatty acid contents for seabream trial

Fatty acids (% DM)	CTRL		MIX		PAP		NOPAP	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0,22	0,00	0,10	0,01	0,12	0,00	0,08	0,00
15:0	0,01	0,00	0,00	0,00	0,01	0,00	0,01	0,00
16:0	1,40	0,03	1,76	0,12	1,86	0,03	1,36	0,00
18:0	0,31	0,01	0,26	0,01	0,30	0,00	0,26	0,04
20:0	0,03	0,00	0,03	0,00	0,04	0,01	0,03	0,00
22:0	0,02	0,00	0,02	0,00	0,02	0,00	0,02	0,00
24:0	0,02	0,00	0,06	0,01	0,06	0,00	0,06	0,00
Total saturated	2,02	0,03	2,23	0,12	2,40	0,03	1,82	0,03
16:1	0,34	0,05	0,34	0,05	0,28	0,03	0,15	0,02
18:1n-9	4,33	0,09	3,93	0,40	4,29	0,19	4,82	0,49
20:1	0,14	0,00	0,11	0,00	0,11	0,00	0,14	0,01
24:1	0,02	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Total monounsaturated	4,83	0,05	4,37	0,36	4,68	0,16	5,12	0,51
18:2n-6	2,05	0,02	1,85	0,11	2,09	0,06	2,33	0,13
18:3n-6	0,03	0,00	0,03	0,00	0,01	0,01	0,03	0,01
20:4n-6	0,03	0,00	0,02	0,00	0,03	0,00	0,00	0,00
Total n-6 PUFA	2,11	0,02	1,90	0,10	2,12	0,05	2,36	0,12
18:3n-3	0,60	0,00	0,55	0,02	0,54	0,01	0,66	0,05
18:4n-3	0,10	0,00	0,00	0,00	0,00	0,00	0,00	0,00
20:5n-3	0,69	0,04	0,09	0,00	0,10	0,01	0,08	0,00
22:5n-3	0,06	0,00	0,02	0,00	0,03	0,00	0,02	0,00
22:6n-3	0,58	0,00	0,46	0,02	0,50	0,02	0,42	0,04
Total n-3 PUFA	2,03	0,04	1,12	0,04	1,16	0,02	1,18	0,02
Total PUFA	4,14	0,06	3,02	0,07	3,29	0,07	3,54	0,14

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Table 79. Diet amino acid contents for seabream trial

g Amino acid / Kg DM	CTRL		MIX		PAP		NOPAP	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arginine (Arg)	35.7	1.8	34.6	1.3	33.6	1.5	31.6	0.9
Histidine (His)	10.5	0.8	11.6	0.5	11.2	0.5	10.9	0.2
Lysine (Lys)	29.8	0.8	29.2	1.1	29.4	1.7	22.8	1.8
Threonine (Thr)	18.2	0.6	19.2	0.4	17.3	0.3	18.1	0.5
Isoleucine (Ile)	18.5	0.1	17.2	0.7	16.1	0.3	17.9	0.3
Leucine (Leu)	40.2	0.1	33.8	1.1	34.3	0.5	41.7	1.5
Valine (Val)	19.8	0.3	23.7	0.4	21.5	0.1	20.4	0.1
Methionine (Met)	12.2	0.8	14.4	0.1	13.9	0.5	14.4	0.5
Phenylalanine (Phe)	22.9	0.9	20.8	1.6	19.9	1.1	24.1	0.4
Cystine (Cys)	3.8	0.3	3.8	0.2	3.6	0.2	3.5	0.2
Tyrosine (Tyr)	20.4	0.5	20.3	0.7	17.5	0.1	23.0	1.6
Aspartic acid + Asparagine (Asx)	35.5	2.6	31.5	1.5	31.4	0.6	28.1	1.7
Glutamic acid + Glutamine (Glx)	84.7	1.8	55.5	0.8	59.6	3.4	75.4	2.0
Alanine (Ala)	24.1	0.3	26.6	1.3	26.8	1.3	26.0	0.5
Glycine (Gly)	23.1	0.5	27.0	0.9	28.4	0.9	21.0	0.7
Proline (Pro)	28.2	0.0	24.3	0.5	24.8	1.2	28.7	0.6
Serine (Ser)	24.0	1.2	23.2	0.9	22.2	0.6	22.6	0.3
Taurine (Tau)	1.3	0.0	7.0	0.2	7.3	0.3	6.5	0.0
TOTAL without taurine	451.6	10.7	416.7	2.6	411.5	4.9	430.3	3.7

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Table 80. Diet mineral contents for seabream trial

Minerals in DM	CTRL		MIX		PAP		NOPAP	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arsenic (As) (mg/Kg)	3.38	0.06	1.44	0.06	2.05	0.09	2.23	0.02
Calcium (Ca) (g/100g)	19695.86	268.64	14950.12	170.16	18767.20	287.81	12858.56	67.49
Copper (Cu)	16.27	0.30	27.92	0.50	23.08	0.07	21.76	0.59
Iron (Fe)	281.54	4.46	476.03	5.23	464.05	1.58	379.74	3.72
Potassium (K)	10727.31	159.75	9103.88	72.62	9209.29	16.69	7523.20	232.27
Magnesium (Mg)	2298.90	16.40	2857.69	40.08	2525.36	10.06	2823.25	63.36
Manganese (Mn)	108.58	1.57	147.61	19.39	137.63	13.86	136.48	15.32
Sodium (Na)	4213.16	55.56	4859.88	58.82	4338.40	2.72	5418.77	162.62
Phosphorus (P)	16087.50	86.15	15725.16	95.38	16376.24	201.89	14368.36	255.39
Yttrium (Y)	181.67	1.96	172.81	2.58	174.98	0.92	179.11	3.91
Zinc (Zn)	233.93	34.87	240.24	17.69	221.74	1.23	210.91	3.36

Table 81. Diet vitamin contents for seabream trial

	Ctrl	Mix	PAP	NOPAP
Vitamins in DM				
Vit E (mg/100g)	26.1	17.3	22.1	24.5
Vit B1	0.228	0.4	0.493	0.503
Vit B2	0.739	1.15	1.1	0.945
Vit B3	8.46	17.5	20.7	9.17
Vit B6	0.443	0.595	0.577	0.583
mg/Kg DM				
Vit B5	66.8	74	74.3	67.6
Vit C	514	478	558	519
ug/100g DM				
Vit B9	477	569	442	488
Vit B12	34	119	69.4	89.3

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1.3 Salmon1

Table 82. Diet fatty acid contents for salmon trial

Fatty acids (% DM)	CTRL		PAP		NOPAP	
	Mean	SD	Mean	SD	Mean	SD
14:00	0,36	0,01	0,23	0,02	0,24	0,03
16:00	2,09	0,09	1,97	0,08	2,12	0,01
18:00	0,56	0,10	0,49	0,02	0,49	0,00
Total saturated	3,01	0,19	2,69	0,08	2,85	0,04
16:1	0,47	0,04	0,51	0,02	0,55	0,01
18:1n-9	9,52	0,42	8,44	0,19	8,79	0,08
20:1	0,23	0,04	0,46	0,00	0,44	0,02
Total monounsaturated	10,22	0,43	9,41	0,21	9,79	0,08
18:2n-6	4,08	0,07	3,70	0,17	3,96	0,07
20:4n-6	0,04	0,00	0,03	0,01	0,04	0,00
Total n-6 PUFA	4,13	0,06	3,73	0,17	4,00	0,07
18:3n-3	0,82	0,02	0,98	0,04	1,11	0,01
18:4n-3	0,09	0,01	0,07	0,00	0,08	0,00
20:5n-3	1,08	0,06	0,78	0,04	0,90	0,02
22:5n-3	0,10	0,01	0,15	0,00	0,23	0,08
22:6n-3	0,79	0,04	0,66	0,02	0,75	0,02
Total n-3 PUFA	2,88	0,11	2,65	0,10	3,07	0,02
Total PUFA	7,01	0,17	6,38	0,28	7,07	0,05

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Table 83. Diet amino acid contents for salmon trial

mg Amino acid / g DM	CTRL		PAP		NOPAP	
	Mean	SD	Mean	SD	Mean	SD
Arginine (Arg)	30.86	0.37	28.79	0.15	32.28	0.16
Histidine (His)	16.55	0.64	18.87	1.39	19.67	0.15
Lysine (Lys)	33.61	0.38	33.31	1.63	34.71	0.22
Threonine (Thr)	18.02	0.73	18.12	0.69	15.75	0.13
Isoleucine (Ile)	19.47	0.42	17.45	0.17	19.56	0.12
Leucine (Leu)	32.07	0.48	31.34	0.97	29.86	0.14
Valine (Val)	20.13	0.60	22.39	0.23	21.09	0.10
Methionine (Met)	11.75	0.11	12.07	0.28	12.09	0.15
Phenylalanine (Phe)	21.90	0.09	23.92	0.97	23.53	0.62
Cystine (Cys)	2.99	0.14	2.77	0.24	2.74	0.01
Tyrosine (Tyr)	18.22	0.88	17.96	0.09	19.91	0.17
Aspartic acid + Asparagine (Asx)	37.71	0.27	32.31	0.50	35.43	0.41
Glutamic acid + Glutamine (Glx)	80.33	0.43	75.48	2.49	83.24	0.44
Alanine (Ala)	22.08	0.31	20.45	0.18	20.15	0.11
Glycine (Gly)	19.66	0.11	17.71	0.74	17.61	0.07
Proline (Pro)	25.89	0.81	28.31	0.82	28.86	0.19
Serine (Ser)	22.27	0.51	21.41	0.15	19.06	0.10
Taurine (Tau)	5.67	0.47	4.91	0.06	6.92	0.11

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Table 84. Diet mineral contents for salmon trial

mg Minerals / kg DW	CTRL		PAP		NOPAP	
	Mean	SD	Mean	SD	Mean	SD
Arsenic (As)	5.27	0.31	3.03	0.02	2.82	0.16
Calcium (Ca)	15667.29	103.27	7861.23	230.48	8363.51	76.43
Copper (Cu)	15.15	0.27	24.25	0.27	20.75	0.83
Iron (Fe)	148.19	0.27	345.85	22.15	207.84	28.90
Potassium (K)	6286.92	16.53	4641.42	15.01	4657.63	166.65
Magnesium (Mg)	1596.22					
Manganese (Mn)	95.15	47.80	77.12	6.03	93.40	19.53
Sodium (Na)	12330.70	16.19	10284.97	30.14	10282.17	351.59
Phosphorus (P)	17601.74	61.07	14651.04	62.56	15091.18	411.50
Yttrium (Y)	173.49	0.34	177.43	1.62	167.63	5.72
Zinc (Zn)	182.17	7.13	185.85	12.97	193.33	14.89

Table 85. Diet vitamin contents for salmon trial

	Ctrl	PAP	NOPAP
mg/100g DM			
Vit E	29.5	26.6	31.1
Vit B1	0.237	0.227	0.216
Vit B2	0.875	1.07	1.01
Vit B3	7.77	8.33	8.2
Vit B6	0.41	0.434	0.484
mg/Kg DM			
Vit B5	58.9	64.9	62.9
Vit C	464	437	436
ug/100g DM			
Vit B9	355	536	461
Vit B12	28.7	80.9	72.3

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1.4 Turbot 1

Table 86: Dietary amino acid content of diets for Turbot.

Amino Acids (%DM)	CTRL		NoPAP		PAP		MIX	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Arginine (Arg)	3.71	0.08	3.68	0.18	3.40	0.01	3.60	0.03
Histidine (His)	1.29	0.09	1.23	0.10	1.24	0.01	1.26	0.03
Isoleucine (Ile)	2.16	0.00	2.28	0.06	2.04	0.02	2.18	0.05
Leucine (Leu)	3.41	0.06	3.45	0.05	3.42	0.03	3.44	0.07
Lysine (Lys)	3.27	0.08	3.53	0.01	3.50	0.01	3.27	0.13
Threonine (Thr)	2.18	0.00	2.13	0.02	2.07	0.03	2.23	0.05
Tryptophan (Trp)	0.48	0.07	0.60	0.03	0.59	0.27	0.63	0.10
Valine (Val)	2.18	0.13	2.43	0.05	2.47	0.01	2.61	0.09
Methionine (Met)	1.11	0.00	1.27	0.01	1.20	0.01	1.24	0.04
Cystine (Cys)	0.28	0.01	0.30	0.01	2.46	0.02	0.28	0.01
Phenylalanine (Phe)	2.45	0.17	2.52	0.05	0.27	0.00	2.61	0.00
Tyrosine (Tyr)	2.07	0.10	2.12	0.08	2.07	0.01	2.26	0.05
Aspartic acid + Asparagine (Asx)	4.07	0.06	4.11	0.05	3.95	0.00	4.05	0.01
Glutamic acid + Glutamine (Glx)	8.61	0.15	7.95	0.15	7.63	0.04	7.80	0.15
Alanine (Ala)	2.35	0.01	2.56	0.17	2.71	0.01	2.90	0.12
Glycine (Gly)	2.53	0.10	2.28	0.05	2.65	0.01	2.64	0.01
Proline (Pro)	3.15	0.03	2.71	0.01	2.80	0.02	3.10	0.00
Serine (Ser)	2.38	0.05	2.25	0.04	2.21	0.01	2.10	0.05
Taurine (Tau)	0.88	0.04	0.85	0.05	0.88	0.01	0.85	0.04

Table 87: Fatty acid contents in diets for Turbot

Fatty acids (% DM)	CTRL		NOPAP		PAP		Mixed	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0.37	0.01	0.22	0.01	0.19	0.03	0.15	0.10
16:0	1.93	0.01	1.42	0.09	1.28	0.09	1.52	0.04
18:0	0.33	0.00	0.24	0.01	0.28	0.06	0.28	0.07
Total saturated	**2.64**	**0.01**	**1.87**	**0.09**	**1.76**	**0.07**	**1.95**	**0.01**
16:1	0.58	0.01	0.39	0.02	0.33	0.04	0.47	0.03
18:1n-9	1.14	0.01	2.95	0.08	2.32	0.23	2.37	0.08
18:1n-7	0.44	0.10	0.00	0.00	0.00	0.00	0.00	0.00
20:1	0.15	0.00	0.20	0.00	0.17	0.00	0.11	0.04
Total monounsaturated	**2.31**	**0.08**	**3.54**	**0.11**	**2.81**	**0.27**	**2.96**	**0.09**
18:2n-6	0.94	0.06	1.35	0.03	1.05	0.00	1.15	0.14
18:3n-6	0.02	0.00	0.05	0.00	0.03	0.02	0.05	0.00
20:4n-6	0.04	0.00	0.03	0.00	0.02	0.01	0.03	0.00
Total n-6 PUFA	**1.01**	**0.06**	**1.42**	**0.03**	**1.11**	**0.01**	**1.23**	**0.15**
18:3n-3	0.13	0.00	0.27	0.04	0.18	0.02	0.16	0.01
18:4n-3	0.15	0.00	0.07	0.00	0.05	0.01	0.07	0.00
20:5n-3	1.30	0.01	0.61	0.00	0.50	0.01	0.55	0.05

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22:5n-3	0.10	0.00	0.05	0.00	0.04	0.00	0.05	0.00
22:6n-3	1.10	0.05	0.68	0.03	0.49	0.02	0.59	0.02
Total n-3 PUFA	**2.78**	**0.05**	**1.69**	**0.08**	**1.24**	**0.06**	**1.41**	**0.06**
Total PUFA	**3.79**	**0.11**	**3.11**	**0.10**	**2.35**	**0.05**	**2.64**	**0.21**

Table 88: Mineral composition of the diets for Turbot.

Minerals	CTRL		NoPAP		PAP		MIX	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
% DM								
P	1.04	0.01	1.56	0.03	1.56	0.00	1.53	0.01
Ca	0.84	0.00	2.05	0.07	2.38	0.05	1.96	0.00
Na	0.49	0.00	0.81	0.02	0.73	0.01	0.66	0.01
Mg	0.19	0.00	0.20	0.00	0.18	0.00	0.21	0.00
K	0.42	0.00	0.81	0.02	0.79	0.01	0.83	0.02
mg/Kg								
As	7.11	0.28	5.78	0.11	6.07	0.30	4.33	0.47
Cu	30.60	0.03	20.45	0.30	20.03	0.05	27.14	0.62
Fe	293.68	1.98	342.67	17.12	374.85	4.58	382.58	8.82
Mn	78.35	17.91	74.89	6.60	97.80	14.20	130.59	61.68
Zn	215.21	0.41	199.78	3.72	188.29	14.11	214.02	16.86
I	1.96	0.00	2.01	0.00	2.59	0.00	2.63	0.00

Table 89: Vitamin content of the diets.

Vitamins (%DM)	CTRL	NoPAP	PAP	MIX
mg/100g				
E	58.65	44.06	41.24	40.47
B1	0.97	0.16	0.14	0.17
B2	1.76	0.91	0.95	0.88
B3	14.40	9.43	11.89	11.48
B6	1.11	0.46	0.40	0.48
mg/kg				
B5	98.54	59.36	58.80	61.86
C	389.44	198.81	191.36	207.44
Ug/100g				
B9	633.90	370.55	457.04	416.73
B12	30.51	70.56	76.27	86.03

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ANNEX 2

2. Detailed diet composition Block2

2.1 Trout 2

Table 90: Diet amino acid contents for trout.

Amino acid content (%DM)	CTRL	PAP	NOPAP	NOPAP +	PAP -
Arginine (Arg)	2.59	2.57	2.46	2.71	2.41
Histidine (His)	1.00	1.02	0.94	1.03	0.99
Isoleucine (Ile)	1.85	1.74	1.79	1.93	1.49
Leucine (Leu)	3.22	3.18	3.11	3.34	2.96
Lysine (Lys)	2.70	2.71	2.71	2.77	2.70
Threonine (Thr)	1.60	1.72	1.71	1.83	1.64
Tryptophan (Trp)	0.57	0.58	0.53	0.63	0.53
Valine (Val)	1.96	1.97	1.94	2.03	1.85
Methionine (Met)	0.96	0.74	0.79	0.92	0.73
Cystine (Cys)	0.64	0.62	0.70	0.67	0.59
Phenylalanine (Phe)	2.05	2.11	2.01	2.09	1.92
Tyrosine (Tyr)	1.42	1.39	1.50	1.57	1.16
Aspartic acid + Asparagine (Asx)	3.66	3.45	3.50	3.84	3.11
Glutamic acid + Glutamine (Glx)	9.23	8.84	9.00	9.32	7.80
Alanine (Ala)	1.83	1.84	1.81	1.92	1.77
Glycine (Gly)	1.93	1.98	1.89	2.12	1.78
Proline (Pro)	2.82	3.00	2.70	3.04	2.41
Serine (Ser)	2.06	2.02	1.92	2.19	1.85

Table 91: Diet fatty acid contents for seabream trial

Fatty acid (%DM)	CTRL	PAP	NOPAP	NOPAP +	PAP -
C14:0	0.49	0.35	0.31	0.35	0.32
C16:0	1.94	2.30	1.92	2.18	2.11
C18:0	0.46	0.47	0.39	0.43	0.43
Other Saturated	0.32	0.34	0.28	0.32	0.31
Sum Saturated	**3.22**	**3.46**	**2.90**	**3.27**	**3.16**
C16:1n-7	0.69	0.44	0.38	0.41	0.39
C18:1n-7	0.68	0.68	0.60	0.62	0.63
C18:1n-9	9.82	11.35	9.92	10.18	10.50
C20:1n-9	0.31	0.25	0.27	0.26	0.24
C22:1n-11	0.15	0.02	0.06	0.08	0.02
Other Monounsaturated	0.10	0.07	0.08	0.08	0.05
Sum Monounsaturated	**11.74**	**12.81**	**11.29**	**11.62**	**11.84**
C18:2n-6	3.24	3.76	3.30	3.38	3.46
C18:3n-3	1.07	1.24	1.10	1.14	1.15
C20:4n-6	0.06	0.09	0.00	0.10	0.08
C20:5n-3	1.33	1.09	0.00	1.20	1.00

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C22:5n-3	0.14	0.11	0.10	0.12	0.10
C22:6n-3	0.56	1.16	1.03	1.38	1.06
Other Polyunsaturated	0.23	0.08	1.13	0.18	0.09
Sum Polyunsaturated	**6.63**	**7.52**	**6.65**	**7.50**	**6.94**
Non identified	**0.13**	**0.13**	**0.12**	**0.11**	**0.12**

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2.2 Bream 2

Table 92. Diet fatty acid contents for seabream trial

% FA/Total FA	CTRL	NOPAP
C14:0	3.47	3.87
C16:0	12.10	14.47
C18:0	3.06	2.91
Other Saturated	1.51	3.66
Sum Saturated	**20.14**	**24.91**
C16:1n-7	3.24	4.18
C18:1n-7	2.95	2.47
C18:1n-9	39.87	28.59
C20:1n-9	1.20	2.46
C22:1n-11	0.77	2.13
Other Monounsaturated	0.49	0.84
Sum Monounsaturated	**48.52**	**40.67**
C18:2n-6	14.86	13.61
C18:3n-3	4.15	4.37
C20:4n-6	0.43	0.50
C20:5n-3	5.97	6.01
C22:5n-3	0.84	1.42
C22:6n-3	3.35	6.12
Other Polyunsaturated	0.45	1.52
Sum Polyunsaturated	**30.05**	**33.55**
Non identified	**1.29**	**0.87**

Table 93. Diet amino acid contents for seabream trial

AA (% DM)	CTRL		NoPAP SANA	
	Mean	SD	Mean	SD
Arginine (Arg)	3.6239	5.125	3.6261	5.128
Histidine (His)	1.2567	1.7772	1.2419	1.7563
Isoleucine (Ile)	2.2614	3.1981	2.3587	3.3357
Leucine (Leu)	4.8247	6.8232	4.7479	6.7145
Lysine (Lys)	3.0245	4.2772	3.1734	4.4879
Threonine (Thr)	2.2339	3.1592	2.9823	4.2176
Tryptophan (Trp)	0.5396	0.7631	0.7844	1.1094
Valine (Val)	2.532	3.5808	2.7812	3.9331
Methionine (Met)	1.0765	1.5223	1.3252	1.8741
Cystine (Cys)	0.6358	0.8992	0.5654	0.7996
Phenylalanine (Phe)	2.7516	3.8913	2.7651	3.9105
Tyrosine (Tyr)	2.0291	2.8696	2.2923	3.2417
Aspartic acid + Asparagine (Asx)	4.6685	6.6023	5.1172	7.2369
Glutamic acid + Glutamine (Glx)	10.289	14.551	9.7778	13.828
Alanine (Ala)	3.2548	4.603	3.3417	4.7258
Glycine (Gly)	3.2226	4.5574	2.918	4.1267

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Proline (Pro)	2.5527	3.61	2.571	3.6359
Serine (Ser)	2.8708	4.06	2.7269	3.8564

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2.3 Salmon 2

Table 94. Diet fatty acid contents for salmon trial

g Fatty acids / 100g DM	CTRL	NOPAP	PAP	NOPAP+	PAP-
C14:0	0.51	0.29	0.32	0.35	0.38
C16:0	2.06	2.03	2.20	2.17	2.56
C18:0	0.55	0.47	0.48	0.47	0.57
Other Saturated	0.36	0.34	0.34	0.31	0.39
Sum Saturated	**3.47**	**3.12**	**3.35**	**3.30**	**3.90**
C16:1n-7	0.72	0.35	0.39	0.39	0.50
C18:1n-7	0.80	0.65	0.70	0.70	0.93
C18:1n-9	11.20	10.52	11.32	11.24	15.41
C20:1n-9	0.39	0.26	0.29	0.30	0.39
C22:1n-11	0.18	0.02	0.02	0.06	0.02
Other Monounsaturated	0.15	0.10	0.11	0.12	0.19
Sum Monounsaturated	**13.44**	**11.90**	**12.82**	**12.81**	**17.45**
C18:2n-6	3.60	3.41	3.60	3.70	5.06
C18:3n-3	1.65	1.57	1.66	1.68	2.12
C20:4n-6	0.08	0.08	0.09	0.08	0.09
C20:5n-3	1.42	0.95	1.07	1.08	1.18
C22:5n-3	0.14	0.10	0.11	0.12	0.12
C22:6n-3	0.62	1.08	1.25	1.19	1.09
Other Polyunsaturated	0.25	0.08	0.06	0.17	0.09
Sum Polyunsaturated	**7.78**	**7.27**	**7.83**	**8.01**	**9.75**

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Table 95. Diet amino acid contents for salmon trial

g Amino acid / 100g DM	CTRL	NOPAP	PAP	NOPAP+	PAP-
Arginine (Arg)	2.41	2.01	2.17	2.67	1.94
Histidine (His)	1.53	1.32	1.73	1.42	1.08
Isoleucine (Ile)	1.73	1.49	1.56	1.88	1.40
Leucine (Leu)	3.07	2.90	3.03	3.19	2.54
Lysine (Lys)	2.82	2.49	2.68	2.38	2.15
Threonine (Thr)	1.54	1.30	1.43	1.62	1.26
Tryptophan (Trp)	0.52	0.48	0.71	0.54	0.57
Valine (Val)	2.00	1.76	1.91	1.95	1.74
Methionine (Met)	1.00	0.92	1.05	0.89	0.82
Cystine (Cys)	0.59	0.62	0.48	0.41	0.74
Phenylalanine (Phe)	1.98	1.82	1.84	2.00	1.53
Tyrosine (Tyr)	1.34	1.31	1.30	1.58	1.06
Aspartic acid + Asparagine (Asx)	3.70	3.02	3.16	3.78	2.59
Glutamic acid + Glutamine (Glx)	9.27	8.11	6.76	8.31	5.06
Alanine (Ala)	1.85	1.58	2.03	1.94	1.69
Glycine (Gly)	2.01	1.79	1.98	2.29	2.04
Proline (Pro)	2.89	2.70	2.34	2.70	2.38
Serine (Ser)	2.06	1.98	1.68	2.06	1.99
Taurine (Tau)	2.41	2.01	2.17	2.67	1.94

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Annex 3

The key performance indicators (KPI) are attested on Table XX

Table 96: Key performance indicators for feed trial assessments.

Category	KPI	Tissue/samples	Analysis	Species
Performance	Feed intake	Whole body		All
	Weight gain	Whole body		All
	Condition factor	Whole body		All
	Biomarkers	Liver	PCR array	Seabream, salmon
Resource efficiency	Feed conversion (FCR)	Whole body		All
	Digestibility	Feed, Faeces	Macro and micronutrients	All
	Retention efficiency	Whole body	Macro and micronutrients	all
Health & welfare	Mortality			All
	Enteritis	Intestine	Histology	All
	Parasitic infestation	Skin Intestine	Lepeophtheirus salmonis Enteromixum leei	Salmon Seabream
	Mucosal function	Skin, gills	Histology	All
	Plasma lysozyme	Plasma	Enzimatic activity	All
	Bacterial activity	Plasma		All
	Biomarkers	Head kidney	PCR array	Seabream, salmon
Quality	Dressout loss			All

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Annex 4

Summary of rearing conditions of the trials.

Trial	Treatments (Diets)	Replicates	Tanks/ cages	Water temperature	Feeding regime	Duration (days)
Bream1	4	4	16	22.0 ± 0.3	*ad libitum*, 2 daily meals	77
Salmon1	3	4	12	12.3 ± 1.2	*ad libitum*, 2 daily meals	96
Trout1	4	4	16	12.7 ± 0.2	*ad libitum*, 2 daily meals	97
Turbot1	4	4	16	16.4 ± 0.1	*ad libitum*, 2 daily meals	112
Bass1	5	3	15	20.1 ± 0.9	*ad libitum*, 2 daily meals	83
Bream2	2	1 (BC)* 2 (AC)*	2 (BC) 3 (AC)	Natural conditions (40°5'N; 0°10'E)**	*ad libitum*, 1 daily meals	30 (BC) 77 (AC)
Salmon2	5	2/3¥	10/15	10.8 ± 1.4	*ad libitum*, 2 daily meals	73
Turbot2	5	4	20	17.5 ± 0.1	*ad libitum*, 2 daily meals	112

* For Bream2 trial BC, means before challenge and AC after challenge.

**Water temperature increased from 18°C in May to 24°C in June, and 28°C in August.

¥ Two cages were assigned to diet CTRL, PAP– and NOPAP+, and 3 cages were assigned to diets PAP and NOPAP.

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List of Tables

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

List of Figures

The project has received funding from the European Union's Horizon 2020 Framework Research and Innovation Programme under GA n. 773330

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