Taste of Tomorrow's Fish

The Impact of Novel Sustainable Feed on Muscle Metabolites and Sensorv Attributes in Rainbow Trout (*Oncorhynchus mykiss)*

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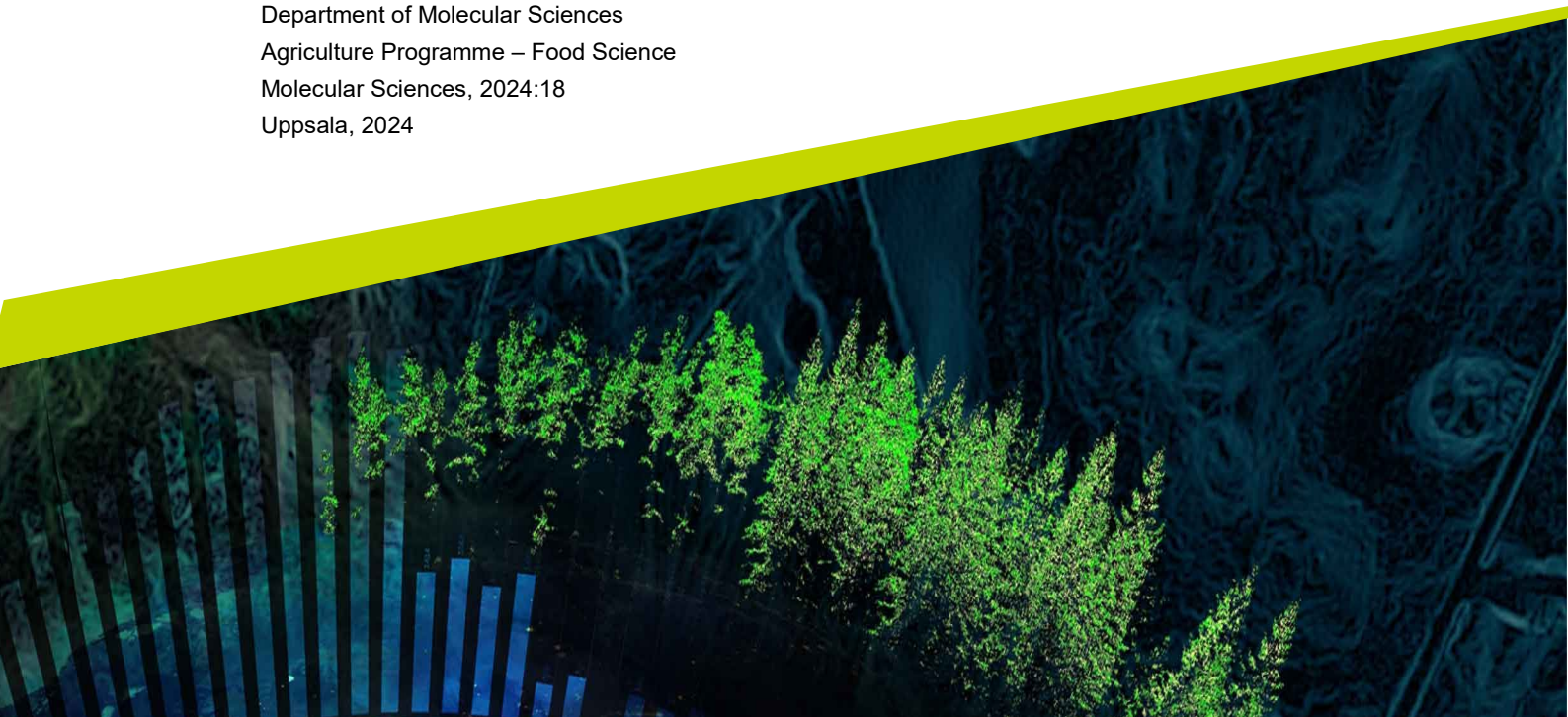

Taste of Tomorrow's Fish – The Impact of Novel Sustainable Feed on Muscle Metabolites and Sensory Attributes in Rainbow Trout (*Oncorhyncus mykiss*)

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Abstract

Aquaculture is a food industry that has grown over the past century, and which still is growing. Development also entails challenges. The largest challenge in sustainable aquaculture development is the feed. Today, raw materials such as fish oil, fish meal, and soybeans are utilized. These are often considered unsustainable, and possible replacements are thus widely investigated. The aim of this thesis was to compare one soy-based control diet (*Glycine max*; Control) and four novel sustainable alternatives based on: Black solider fly Protein (*Hermetia illucens*; BSF prot); Black solider fly oil (*H. illucens*; BSF oil); Seasquirt (*Ciona intestinalis*; Ciona); and a Singe cell fungi protein (*Paecilomyces variotii*; SCP), to evaluate their effect on rainbow trout (*Oncorhynchus mykiss*) muscle metabolite composition and sensorial attributes. Water soluble metabolites were extracted from the white muscle tissue of Rainbow trout, and ^{1}H-NMR was performed to determine the metabolic profile of fishes fed the different feeds. The metabolite data was evaluated statistically by ANOVA, PCA, and sPLS-Da and compared to the sensory data. The results showed that Black Soldier fly protein caused higher tissue concentration of Methionine, and Betaine. The single cell protein diet gave a similar pattern as the control feed. The metabolome of the Seasquirt and Black soldier fly groups were partially overlapping the control, i.e. similar to some extent. The sensorically attributing metabolites did probably affect taste the most. BSF prot was the feed category which indicated the most differences compared to the other treatments. It had lower concentrations of IMP, and higher levels of Hypoxanthine, which indicates that the diet generates a fillet which might lose freshness faster than the other feed categories. AMP acts as a bitterness blocker and is found in higher concentration in the SCP-group. The muscle of the BSF oil group had higher amounts of Lysine which indicates a potentially sweeter taste. Betaine, which was higher in the BSF prot treatment, acts as an osmoregulator in the muscle, and could improve the texture or mouthfeel. The sensory panel did not pinpoint any large differences between the groups, and no clear connections to the metabolite data could be seen. However, sensoric experiences is a complex mixture of several molecules, and it is not possible ta draw a conclusion based on the result from this study.

Keywords: Aquaculture, Metabolites, Sensorics, ^{1}H-NMR, Rainbow Trout, Aquafeed, Black Solider Fly, Ciona, Single Cell Protein, ANOVA, PCA, sPLS-Da

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Abbreviations

AMP	Adenosine monophosphate
ANOVA	One Way Analysis of Variance
BSF	Black Solider Fly (*Hermetia illucens*)
FAO	Food and Agriculture Organisation
IMP	Inosine monophosphate
NMR	Nuclear Magnetic Resonance
PCA	Principle Component Analysis
SCP	Single Cell Protein
SLU	Swedish University of Agricultural Sciences
sPLS-DA	Sparse Partial Least Squares Discrimant Analysis
TMAO	Trimethylamine Oxide

1. Background

1.1 Aquaculture

A world population in continuous growth brings an increased need for food. Fish is an important food source to obtain essential nutrients, such as selenium, iodine, vitamin D, and omega-3 fatty acids (Swedish Food Agency 2024). The Food and Agriculture Organisation (FAO) declare that the global fisheries and aquaculture industry has an important role in providing the world population with quality food and nutrition (FAO 2022). However, increasing problems concerning overfishing and environmental complications such as anoxic seabed zones, threaten the future of many wild stocks and ecosystems (FAO 2022). Myers and Worm have shown that reduced fishing pressure can restore wild living stocks (Myers & Worm 2005). Thus, to be able to meet the need for fish as a food source, while reducing fishing pressure — aquaculture will be needed.

The development and growth of aquaculture has increased significantly during the previous 50 years, while wild capture has stagnated (Naylor et al. 2000, 2021, FAO 2022). FAO predicts a further increase in aquaculture between the year 2022 to 2030 (FAO 2022). Aquaculture involves a variety of organisms including algae, aquatic plants, crustaceans, freshwater fishes, marine fishes, diadromous fishes, and molluscs. Freshwater fish is the most commonly farmed group in the world, while diadromous is the most common in Europe (Naylor et al. 2021). In a local perspective, which is Sweden in this context, rainbow trout (*Oncorhynchus mykiss*) is the most common species (SCB 2022). One important factor in aquaculture is feed. Farmed fish are effective as feed-to-meat converters . However, to obtain an ecological development of aquaculture and reaching the climate goals, it is important to develop it sustainable.

Aquaculture has been criticized regarding several sustainability aspects. One of the aspects is the environmental impact in aquaculture, of which feed could cause more than 90 % (Naylor et al. 2021). Fishes requires certain essential nutrients which need to be included in the feed, such as leucine, lysine, and methionine. Changes in feed composition could interrupt the metabolism of the fish. Another important

characteristic is the physiochemical properties of the feed. The feed pellets must sink at an appropriate pace, and should not dissolve before being consumed by the fish. The optimum goal is for the fish to eat every single pellet to minimize waste. The fish usually has to appreciate the food to eat all of it. Consequently, there are often feed enhancers added to the pellet (Encarnação et al. 2016). However, the issue with primary ingredients arises when we catch wild fish directly consumable by humans to feed the aquaculture, or when we replace this fishmeal with questionable plant proteins. About 70 % of a typical Scandinavian salmonid feed consists of plant ingredients, and the single most used ingredient is soybean protein concentrate (Aas et al. 2019). Soy is problematic as it can be linked to relatively high CO_2-emissions, deforestation, and monoculture issues (Gasparri et al. 2013; Lathuillière et al. 2014). However, it is relatively cheap, and of course fish feed development also has an economic aspect to take into account. Especially as the price of feed ingredients, such as fish meal and fish oil, is rising.

Aquafeed production has increased during the 21st century due to expansion of aquaculture. While fishmeal and fish oil production has decreased (Tacon & Metian 2008). The high prices together with the sustainability aspects accelerate the demand for innovative sustainable feed ingredients. Single cell proteins (SCP), Aquatic filter feeders, insects, and crops from marine and terrestrial ground are being tested. One important aspect in the search for new sustainable feed resources is the source and stream of nutrients in nature. Recirculating local nutrients is one way of finding novel sustainable feed. This can be obtained in different ways by various new protein sources whose benefits are now being researched. Black Soldier fly (*Hermetia illucens*, BSF) is an insect which is widely studied and shows potential to be a new protein source for fish feed. Rainbow trout eat insects in their natural habitat, and BSF has a suitable nutritional profile for the fish (Sealey et al. 2011). The sustainability aspect in this case implies what the insects eat, and the key idea is that BSF can live on food wastes from industries or households and thus convert waste to viable protein. Another possibility would be to use single cell organisms such as different bacteria, algae, or fungi. *Paecilomyces variotii* is one example within the fungi kingdom. These show potential as they can live on residues from the forest industry. It is also possible to use species from the sea which are not consumed to the same extent. Sea vase, or *Ciona intestinalis,* (Ciona) has proven to be a promising species as it can be farmed in a technically simple manner and filters eutrophic coastal waters of nutrients. However, it is not only a matter of price, availability, or circularity, the feed also has a large impact on fish health and metabolism. It is important to maintain accomplish studies to evaluate the novel feed ingredients considering fish health, and sustainability. Metabolomics is one way of studying this.

1.2 Metabolomics

Metabolites are associated with biological systems as either intermediates or end products of metabolism. This includes molecules such as amino acids, nucleotides, fatty acids, and vitamins (Wishart 2008). The composition of metabolites in a fish is changing constantly. Changes in the metabolite composition could depend on both endogenous factors, such as age, sex, reproductive status etc., and exogenous factors such as nutrients, environment, and microbiome (Shearer 1994). Metabolites have been analyzed and evaluated for a long time, and have been noticed as an important part of the development of new feed (Roques et al. 2020).

Metabolites are often examined using Nuclear Magnetic Resonance (NMR) or Mass Spectroscopy (MS) to find specific biomarkers or fingerprints in the form of the metabolomic profile. NMR is used when you want to analyze a sample that should not be destroyed, if you want to quantify, or when many molecules present have the same molecular mass. It is possible to perform NMR on ^{1}H, ^{13}C, ^{15}N, and ^{31}P, of which ^{1}H is the most common (Markley et al. 2017). These atoms have a specific nuclear spin which maintains a magnetic moment. An external magnetic force from the NMR sends pulses that change the spin. The NMR device recognizes changes and converts the information to a data spectrum of signals from each proton in the sample (Bovey et al. 1988). These signals must be handled in a data program through baseline adjustment, alignment, and compound fitting. Further quantitative analysis of the signals could determine the metabolite profile. NMR could be used in samples where both a few and a large number of molecules are to be analyzed. However, it is an appreciated method to analyze biological samples with a complex mixture of several metabolites, such as blood or tissue. NMR does not give the full image of the sample, since it only detects medium and highly abundant metabolites. NMR generates a large amount of data rapidly. At such high amounts of data, statistical multivariable analysis methods are required to rapidly have an overview of the data, and find results (Ren et al. 2015).

Multivariable analysis is used to analyze metabolite data. One of the most common methods to analyze NMR metabolite samples is Principal Component Analysis (PCA). The PCA transforms multivariate data to fewer dimensions, which makes it easier to discover trends, and outliers. The data is expressed as scores along each of the Principal Components (PC). The PC is commonly plotted in 2 or 3 dimensions where one data point symbolizes the localization of one sample. Datapoints which are close together have more similarities than datapoints with a longer distance between them. Another multivariate method is Partial Least Squares Discriminant Analysis (PLS-DA) which is used to find connections between data generating groups. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA)

is a method which combines PLS-DA with sparsity, to find the variables responsible to differences between groups (Ren et al. 2015).

1.3 Sensory

Sensory has a fundamental role in the complex world of food quality (Peri 2006). Fish is a sensitive product which quickly after slaughter can develop undesirable smell and taste. This development is associated with the natural progress in fish, often due to available autolytic enzymes, pH, water activity, and amount of connective tissue (Franceschelli et al. 2021). The scientific descriptive sensory evaluation of food is usually based on appearance, odor, flavor, and texture (Civille & Oftedal 2012). Sensory refers to the perceived characteristics measured by the human sensory organs such as eyes, nose, ear, mouth, and the vestibular system. Appearance and odor are usually what we use first while discovering food. The eyes inspect appearance based on, for example, the color of the meat, whether there is a lot of visible fat, and the lamellae appearance. The nose assesses odor on a slightly more complicated basis, since the odors give a combined impression of, for example, ammonia, butter, fish oil, metallic, sweet, or seawater. When we bring the food to our mouth, the presence of texture and taste occurs. The texture implies how the foodstuff gives a feeling in the mouth, such as firmness, chewability, consistency, or fibrousness. The taste can, for example, be sour, sweet, bitter, or salty.

Odor metabolites often originate from volatile compounds associated with fatty acids (Josephson et al. 1984). Non-volatile compounds, for example free amino acids, nucleotides, and organic acids, are associated with the taste of food items (Kirimura et al. 1969; Duan et al. 2020). Flavor is a variable attribute of meat, and the factors affecting flavor occur both *pre-* and *post mortem*. Both intrinsic factors (age, size, species) and extrinsic factors (nutritional status, water quality, season) affect the flavor *pre mortem*, while slaughter method, hygiene, and storage affect *post mortem* flavor development (Idolo Imafidon & Spanier 1994; Tucker 2000; Duan et al. 2020)

1.4 Aim

The aim of this thesis was to analyze the white muscle of Rainbow trout using ^{1}H-NMR, to detect distinctions in metabolic composition and sensory attributes based on diets. The different diets were composed of novel sustainable feed components which replaced soy- and fish meal protein. The following research questions were used throughout the project:

- Does the feed affect the sensory characteristics of the rainbow trout fillet?
- Does the feed affect the white skeletal muscle metabolome?
- Are there any connections between the metabolite- and sensory results?

2. Materials and methods

2.1 Fish feed trial and sampling

Previously, a group of researchers at SLU conducted a trial concerning sustainable feed to rainbow trout. They problematized the fact that our food does eat our food, and wanted to explore a Swedish sustainable circular feed. The samples from this previous trial was gathered in 2020, and stored in – 80 °C until 2024. Four different feed compositions were studied and compared to a control feed. The four test diets contained Black soldier fly (*Hermetia illucens*) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (*Ciona intestinalis*) (Ciona), or Singe cell protein from *Paecilomyces variotii* (SCP), see table 1. The ingredients were blended with boiling water to get 20 % moisture content. The feed was extruded in a twin-screw extruder (3 mm die, BC-45 model; Clextral, Creusot Loire) at 120-130 °C for 30 seconds. The extruded pellets were dried in 60 °C, and finally the oil ingredients were applied using a vacuum coater (Pegasus PG-10VC; Dinnissen).

Table 1. *The feed ingredient list. Values expressed in % on an as-is basis. All values expressed in their unprocessed form.*

	Diet				
Ingredient	Control	BSF Prot	Ciona	SCP	BSF oil
Fish meal	36	20	24	24	36
Soy protein concentrate	16	15.7	16	13.6	16
Wheat gluten	8	14	12	9.1	8
Wheat meal	18	14	11	9	18
Potato starch	3	5	3	3	3
Fish oil	10	10	10	10	10
Black Soldier fly oil					6
Rapeseed oil	6	6	6	6	
Vitamin mineral premix	1.7	1.7	1.7	1.7	1.7
Paecilomyces variotii				22.2	
Ciona meal (whole)			15		

Black Soldier fly protein meal		12			
DL-methionine	0.14	0.44	0.14	0.24	0.14
Monocalcium phosphate	1	1	1	1	1
Titanium dioxide	0.05	0.05	0.05	0.05	0.05
Astaxanthine	0.01	0.01	0.01	0.01	0.01
Lysinesulphate	0.1	0.1	0.1	0.1	0.1

During the trial in 2020, two white skeletal muscle samples from four fishes per feed treatment group were taken from behind the dorsal fin on both sides(see Figure 1 for illustration). Samples were immediately frozen in liquid nitrogen until storage at -80 °C.

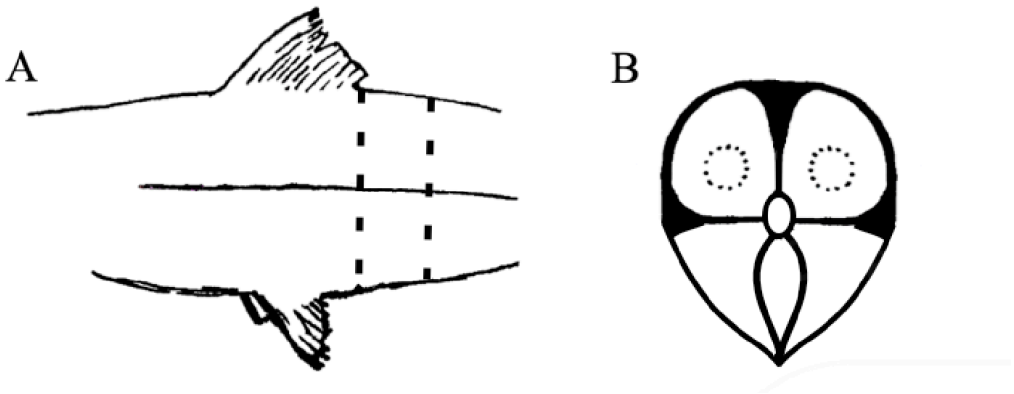

Figure 1. *Description of sampling of the white skeletal muscle. The dotted lines in A marks the cut (Norwegian quality cut) location which creates the cutlet seen in B. The dotted lines in B is the location of sampling site. A Lateral view of the fish. B Transverse view of the fish.*

2.2 Sensory

The sensory assessment of the samples was performed in Torsåker, Upplands Väsby, on the 25th of May 2020. The samples were served on white neutral plates with a three-digit randomized code for each presented sample. The fish was served raw, and all servings were made in four replicates to the panel of 15 chefs. The sensory methods used were Liking and Just-About-Right (JAR). Liking implies that the panel rates various characteristics on a seven-point scale between “dislike very much” to “Like very much”. At JAR, the panel can assess a five-point scale from “Too low” to “Too much”. Here, the scale is assessed to different sensory properties such as umami, tenderness, and juciness.

2.3 Metabolite extraction and NMR

The following steps were performed in all samples. The samples were held on ice during the entire extaction to avoid volatile compounds from evaporating. The muscle samples were cut in 200 +/- 0.02 mg and placed in a two ml precellys lysing kit together with 0.8 ml ice-cold methanol and 0.17 ml ice-cold chloroform. The samples were homogenized in a Precellys 24 homogenizer (Bertin Technologies, France) at 6500 RPM for 2 x 20 seconds with a ten second pause. The homogenising procedure was repeated three times. The homogenate was transferred to a glass test tube, after which 0.8 ml ice-cold chloroform and 0.4 ml ice-cold water was added. Subsequently, the solution was vortexed for one minute. After ten minutes on ice, the samples were centrifuged (HERMLE Larbortechnik Type Z 383K, Germany) at 2000 g, 4 °C for ten minutes. The phases were separated, and 770 µl from the polar phase was dried using a speedvac (Savant, Automatic Environmental SpeedVac System AES2010) overnight at 37 °C. Before transferring it for storage at -80 °C, the remains from the evaporate were dissolved by adding 520 µl sodium phosphate buffer (0,135 mol/L, pH 7.0). Duplicates were made for two random samples.

In preparation for the NMR procedure, 460 µl was transferred to the filters and centrifuged (Eppendorf AG 5424 R, 22339 Hamburg, Germany) at 4 °C and 12000 g for three minutes. The centrifugation was repeated four times. The filtrate was transferred to an Eppendorf tube (Eppendorf Safe – Lock Tubes – Microtube). 50µl D_2O, 30µl internal standard (TSP; sodium-3-(trimethylsilyl)-2,2,3,3-tetradeuteriopropionate) and 160 µl buffer was added. The solution, with a total volume of 560 µl, was transferred to an NMR tube (BRUKER Tupe/Cap 5X4 X0.38S). The ^{1}H-NMR spectroscopy (using a Bruker Avance III; Karlsruhe, Germany) was performed using a zgesgp pulse program at 600 MHz and 25 °C. Each sample was scanned 128 times over a spectral width of 9615.385 Hz using a 1.7 acquisition time with a 4.0 s relaxation delay.

2.4 Data processing and metabolite identification

A Fourier transformation of the ^{1}H-NMR data was made in Bruker Topspin 3.5 pl 7. Thereafter, the spectra were processed using the processor in ChenomX NMR suite 10.1 (ChenomX Inc., Canada). All adjustments were performed manually and included correction of phase, baseline, and shimming as well as a 0.3 Hz line broadening and CSI calibration. The adjusted spectra were exported to ChenomX profiler, where a targeted manual identification of metabolites was made using the ChenomX 600 mHz compound reference library. One peak for each metabolite was

selected as a reference peak to which the height was adjusted to find the peak area of each metabolite. A total of 59 metabolites were identified in all the 42 samples.

2.5 Statistical analysis

The statistical analysis was made in R version 4.3.2 (R core team, 2024) for all 59 metabolites in each sample. Metabolites which had peaks lower than three times the background interference/noise were marked as lower than the limit of detection (LOD). LOD values were replaced with 1/5 of the lowest concentration within the compound. 7 metabolites had missing values, see table 2.

Table 1. *The percent of missing values in the original data.*

Compound	Percent missing values
Acetoacetate	2.4
N.Methylhydantoin	4.8
Propylene_glycol	9.5
Sucrose	7.1
Tyrosine	2.4
AMP	23.9
ATP	21.4

The metabolite data of each sample and the data were normalized by sum, logarithmically transformed with base 10, and mean-centered. A Hoetellings T^2 from the HotellingEllipse-package (v1.1.0; Goueguel 2022) was performed on the values. The 95 % confidence interval showed one outlier, sample 230. The result was plotted in a principal component analysis (PCA) from the FactoMineR package (v2.10; Lê et.al 2008). Therefore, sample 230 was excluded from the following analysis. The data was further controlled through normal probability. Sparse partial least squares discriminant analysis (sPLS-DA) from the mixOmics package (v6.25.1; Rohart et.al 2017) was made with plots of the most influential loadings and biplots to identify patterns of the metabolomes. One-way analysis of variance (ANOVA) was made, followed by Tukey's test, to get a quick overview of metabolite concentration differences between treatment groups. The statistically significant metabolites ($p \leq 0.05$) were plotted as box and whisker diagrams. The p-values were also adjusted using the Benjamini-Hochberg method. All plots were made using the ggplot2 package (v 3.5.0; Wickham 2016)

For the sensory data (Just-About-Right), a linear mixed-effects model was fitted using the lmer-function in the lme4-package (v 1.1.35.1; Bates et al. 2015). The models applied restricted maximum likelihood for each of the six response variables (Tenderness, juiciness, sweetness, acidic, bitterness, and umami) with diet

and the unique three-digit code as fixed effects, as well as assessor assigned as a random effect. Estimated marginal means (emmeans with tukey's method) was used for pairwise post hoc comparisons (package "emmeans" v 1.10.0; Lenth 2024).

3. Results

A total of 59 metabolites were identified in all 42 samples, the mean values of the metabolites can be seen in Appendix 1. The duplicate samples 41 and 42 were similar to the original samples. The duplicate was performed on separate days in comparison to the original samples. They showed the same pattern in the NMR spectra, which indicates that the method was consistent. AMP, Beta-alanine, Betaine, IMP, Inosine, Isoleucine, Leucine, Lysine, Methionine, and N-Methylhydantoin showed significant concentration differences between the diet treatments in the ANOVA analysis, of which Betaine, Lysine, and Methionine differed also after applying Benjamini-Hochberg adjustment.

The sPLS-DA analysis of the first two PLS-components (as well as for component one against three, and two against three) showed that BSF prot is clearly separated from the other treatment groups (Figure 2 & Figure 8). SCP and Ciona are entirely overlapped by the control. BSF oil differs somewhat on component two. The data also shows separation of BSF oil from other tissues in the Component 2 and Component 3 plot (Figure 8). BSF oil overlaps slightly in comparison to BSF prot, Ciona, and SCP, which are completely overlapped by the control.

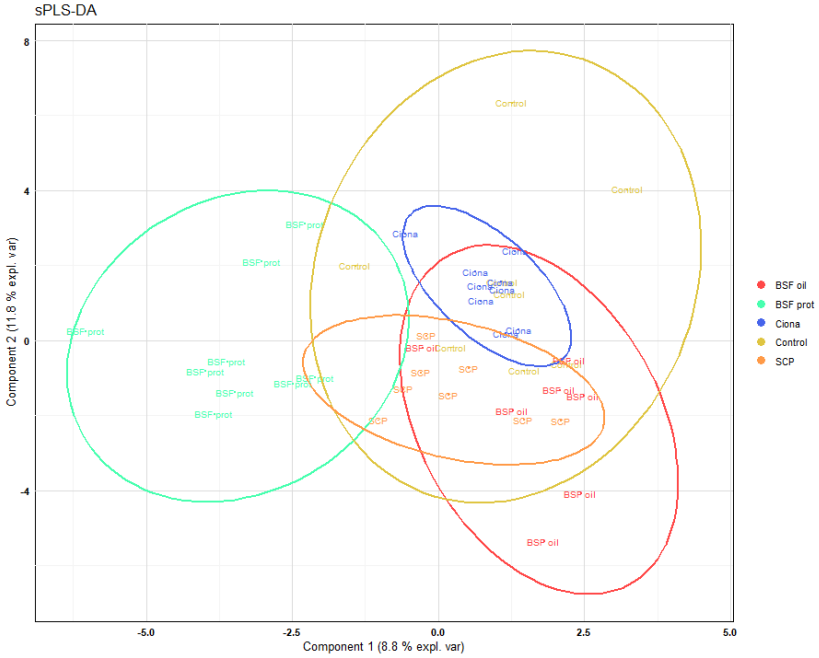

Figure 2. *Confidens interval plot of the sparse least squares discriminate analysis (sPLS-DA) from muscle metabolite samples of five feed groups of rainbow trout (O.mykiss). The five diets contained a control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

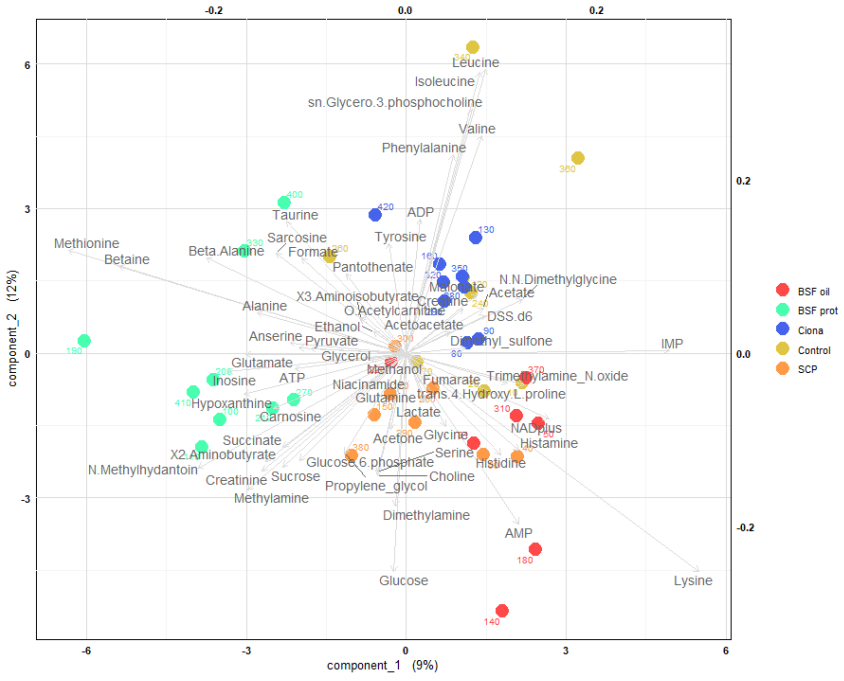

Figure 3 *Biplot of the sparse least squares discriminate analysis (sPLS-DA) from muscle metabolite samples of five feed groups of rainbow trout (O.mykiss). The five diets contained a control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

The sPLS-DA loadings plot (Figure 4) shows that Methionine, IMP, Betaine and Lysine are the four most influential metabolites driving the pattern of the first PLS-component. These metabolites also showed differences in concentration between treatments. The loadings plot indicates that BSF prot stands out regarding the concentration of Methionine (higher), IMP (lower), and Betaine (higher). Lysine levels, however, appeared to be highest in the muscle tissue of BSF oil-fed fish. This is quite clear in the sPLS-DA biplot (Figure 3) as BSF prot is located closer to the area where Methionine and Betaine are located, while it is opposite to the loading direction of IMP. The same applies to Lysine and the correlation with BSF oil. The boxplots of these ANOVA results show that BSF prot has a higher amount of Betaine, Methionine, and N-methydantoin, while IMP is lower in comparison to the other diets. BSF oil had higher Lysine and lower Beta-alanine. SCP was higher in AMP. Both Ciona and Control had higher amounts of Isoleucine, and Leucine. Ciona had similar results as the Control in most metabolites (Figure 5).

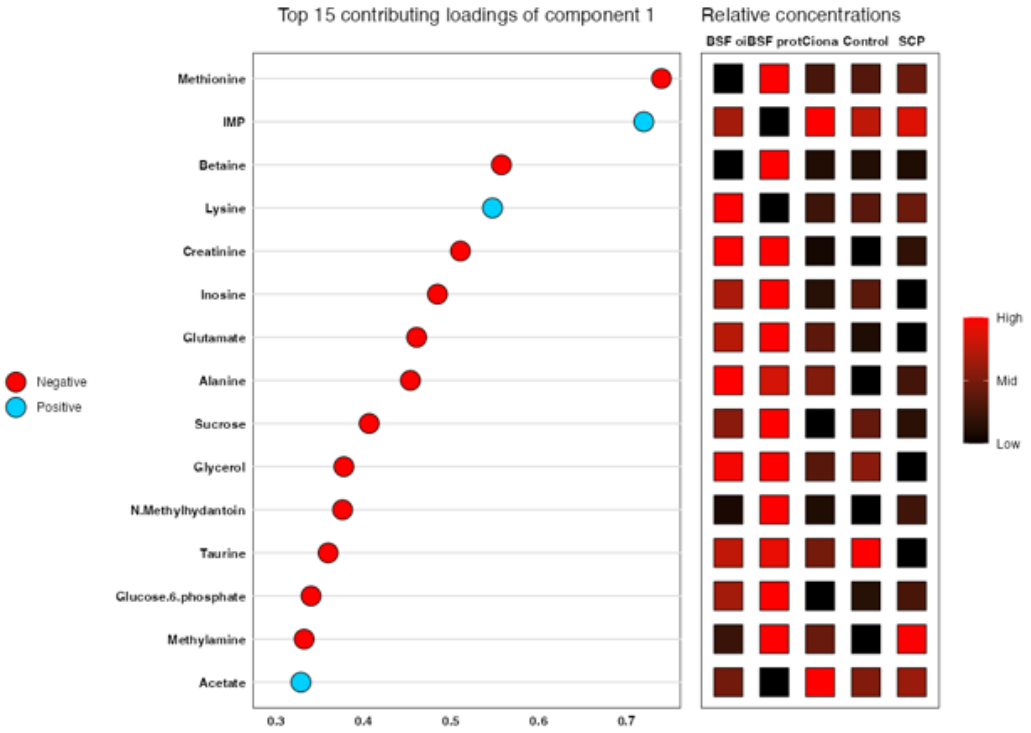

Figure 4. *sPLS-DA loadings plot of white skeletal muscle metabolites in rainbow trout (O. mykiss) under five different diet treatments. Only the top 15 most influencial metabolites for the variance of PLS component 1 are shown. The dots represent the level of negative or postitive contribution, and the colored squares represent the relative concentrations of each metabolite within each diet treatment group. The five diets contained a control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

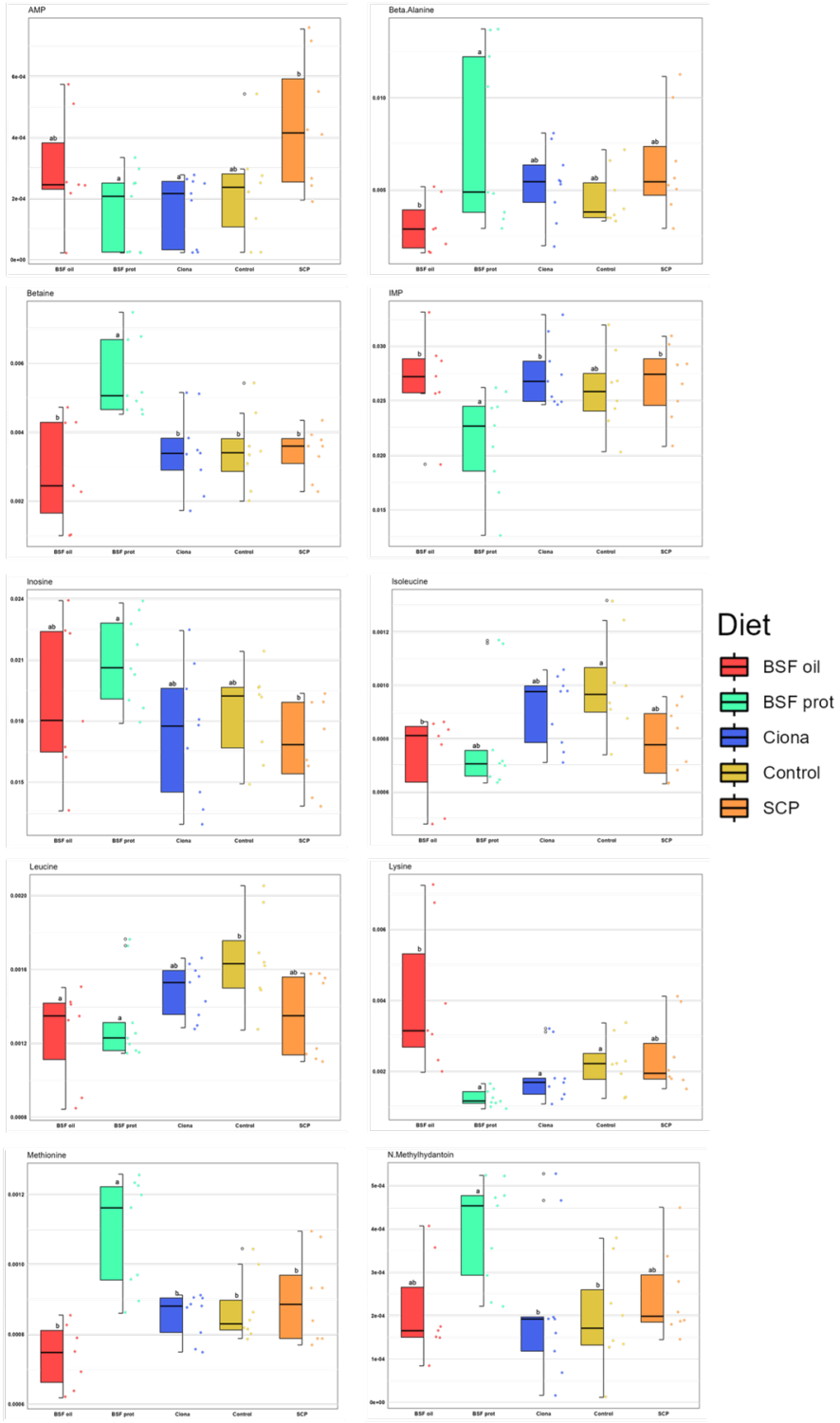

Figure 5. *Relative metabolite composition in muscle from rainbow trout (O.mykiss) fed different diets. Letter markings indicates significant similarities. The five diet categories includes control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

As shown in Figures 6 and 7, the sensory scores revealed no striking differences between the categories. The acidic, bitterness, juiciness, sweetness, tenderness, and umami scores were all close to a Just-About-Right (JAR) rating. The sensory panel rated the control the highest in the liking for taste plot. Ciona was the closest, although all categories had similar results of around four (neither like or dislike). The liking for texture was highest for BSF oil, followed by Ciona. The same pattern could be seen here as all categories were around four. The total liking was positioned around four in the control, Ciona, and both BSF categories, while SCP was slightly lower.

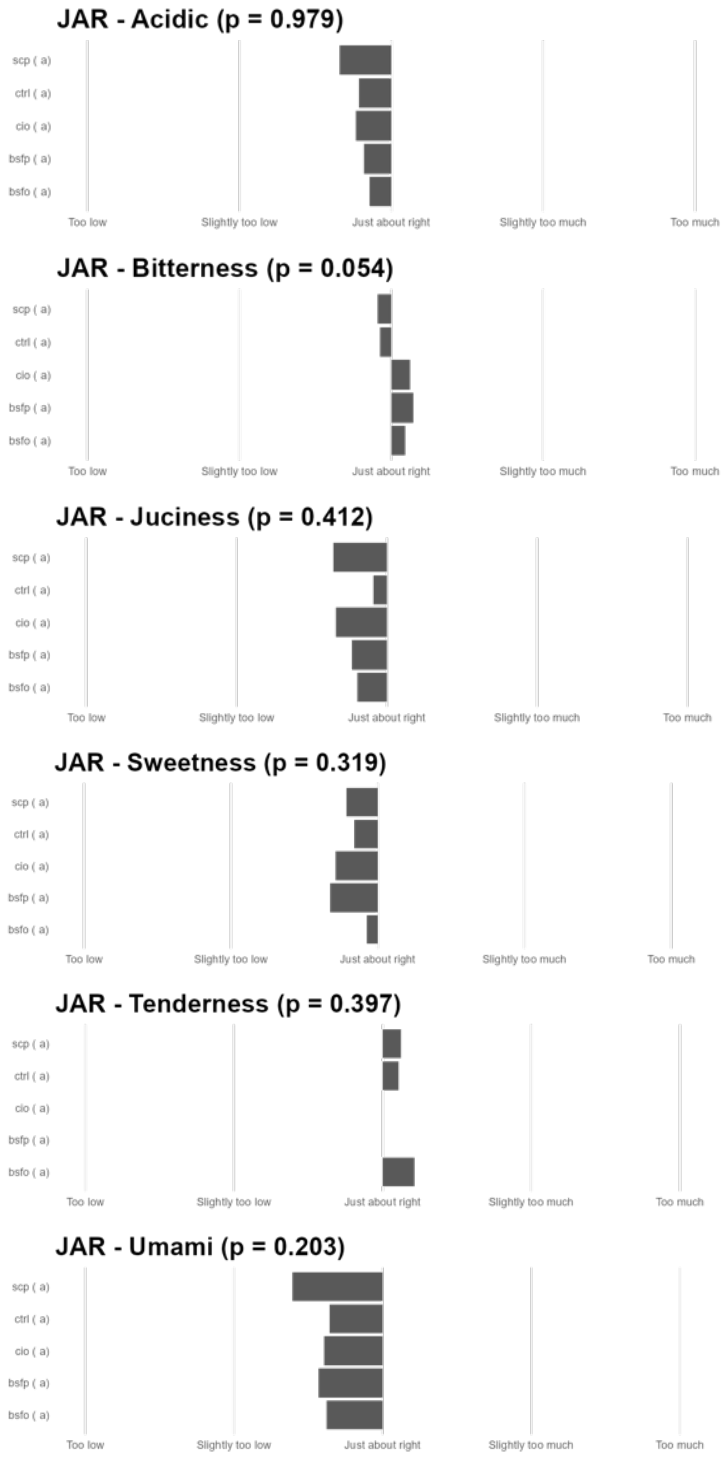

Figure 6. *The results from sensory panel of Just-About-Right (JAR), including Acidic, Bitterness, Juciness, Sweetness, Tenderness, and Umami.. JAR has a five-graded scale from "Too low" to "Too much". The p-values are included, there is no significant difference between the categories. The five diet categories includes control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

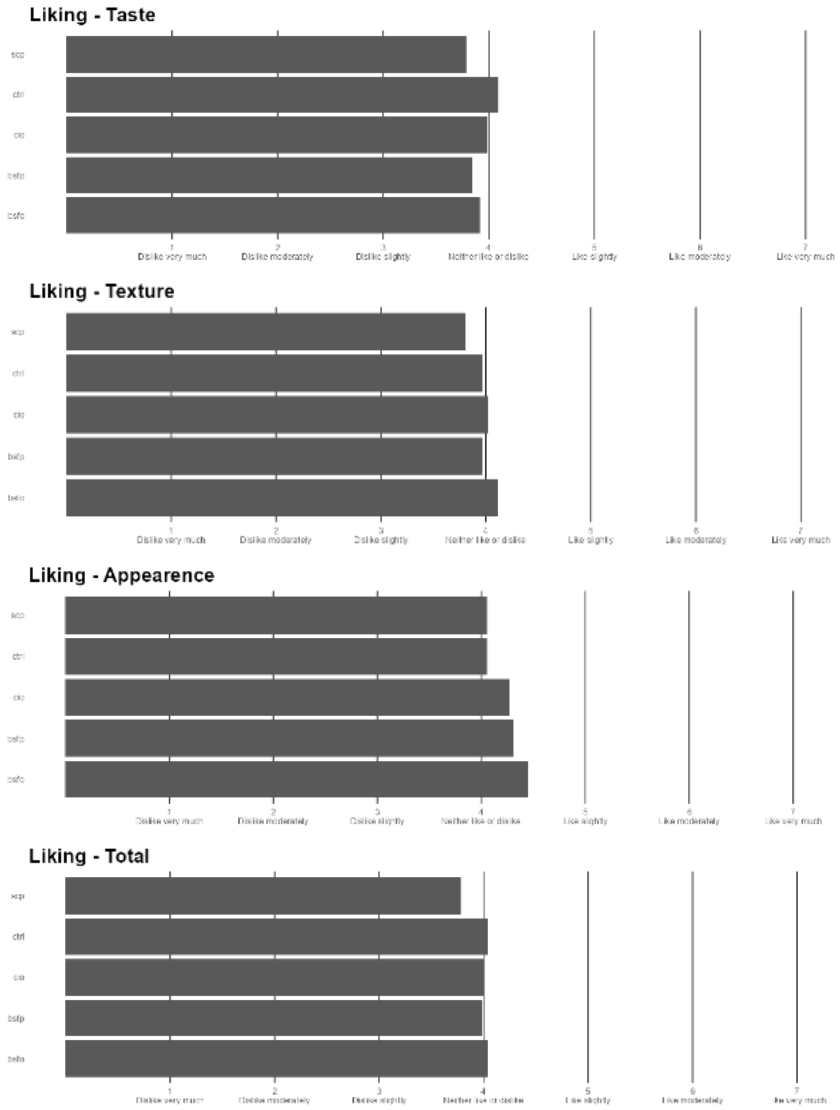

Figure 7. *The results from sensory panel of Liking, including taste, texture, appearance, and total.. Liking has a seven-graded scale from "Dislike very much" to "Like very much". The five diet categories includes control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

4. Discussion

In this study, ^{1}H-NMR metabolomics was used to study how water-soluble metabolites in white rainbow trout muscle vary depending on different novel sustainable components in the feed. This metabolite data was further evaluated to detect potential markers of various sensory attributes in the fish fillets. The analysis of the NMR data presented ten metabolites with differences in concentration, of which three showed significant differences after applying p-value adjustment. The trial did include four fish of each feed category as it was considered the lowest acceptable number to gain relevant results according to the 3R principle. The 3R principle stands for replace, reduce, and refine, and aims to facilitate animal welfare (Russell & Burch 1959). It is relevant to underline that including only four fishes of each feed treatment in a metabolomic study, is prone to give less precise results, while a larger sample group could give a more precise indication of the difference (Troisi et al. 2022). Regarding the odour, it is often related to volatile molecules (Amerine et al. 1965; Turchini et al. 2004). Many of the volatile metabolites could be present in the fat-soluble sample which were not included in this trial. None of the metabolites present in the sample were directly connected to odour. All molecules responsible for sensory attributes are not detectable in an NMR metabolomics study of the water phase. Consequently, this discussion will not be able to give the full image of the sensory profile of the different feed categories. And even though all significant metabolites were evaluated, the results are to be observed with uncertainty.

4.1 Metabolome effects of diet

As can be seen in Figure 2, the BSF prot-group differs distinctly from the other samples. This kind of separation indicates that the BSF prot metabolomic profile differs from the other samples. The sPLS-DA biplot (Figure 3) indicates that Methionine and Betaine could be two of the metabolites which strongly drive the characteristics of the group, and that those metabolites are present to a greater extent in this sample. The sPLS-DA loadings plot (Figure 4) clarifies that both Methionine and Betaine are metabolites with a high concentration for BSF prot. The Loadings plot also indicates that IMP is low in BSF prot, in comparison to other diets.

The Control has a great distribution of the samples (the circle in Figure 2 is large). Ciona and SCP are almost completely overlapped in the control area. This indicates similarities in these feed categories, which is clearly seen in the loadings plot as well (Figure 4). When a feed group gives similar results to the control, it could indicate that this feed works similarly to the control, which is known to be a suitable diet formulation. BSF oil was partially overlapped by the control. Moreover, the loadings plot of Component 1 shows a pattern where BSF prot and BSF oil have higher relative concentrations in most contributing metabolites, aside from IMP and acetate. IMP is high in sPLS-DA contribution, which makes it extra interesting.

4.2 Sensory

The results regarding liking from the sensory panel show minimal differences between the different feed categories (Figure 7). The SCP was lowest in the different liking plots. The panel rated the Control as the most liked tastewise. Taste is an individual estimation which, even if the panel is trained, could differentiate between the panellists.

4.3 Metabolites of particular interest

Both Betaine and Methionine showed significant differences in comparison to the other diets. Methionine is added to all the diets used; thus, we know it comes from the feed, see table 1. It is an essential amino acid involved in several functions in the fish and is one of the most common additives in fish feed. Methionine is a precursor of cystine and taurine due to its sulphur content (Wang et al. 2023). Another important function of methionine is the methionine cycle in the one-carbon metabolism in fish. The methionine cycle intermediate S-adenosyl-methionine (SAM) acts as a methyl donor for lipids, DNA, and amino acids (Wang et al. 2023). Interestingly, Betaine is an intermediate in the one-carbon metabolism as well. Betaine acts as a methyl donor when Methionine is created from Homocysteine (Wang et al. 2023). Betaine is also known to be a feed enhancer to attract fish (Mohseni et al. 2021). A feed enhancer in the feed could entail less feed wasted in the water since the fish tend to eat all the feed. This would give less leakage of nutrients in the water and neighboring environments. The fact that there is a higher amount of these one-carbon cycle intermediates in the sample of BSF prot could be explained by two reasons. The activity of the one-carbon cycle could be increased or decreased. Increased activity of the one-carbon cycle could be a sign of increased metabolism and potentially increased growth in the fish. The hypothesis regarding decrease could indicate that the one-carbon metabolism has been blocked,

consequently an accumulation of the intermediates in the muscle. Moreover, Betaine is an osmoregulator in the cell. This may imply that the sensory texture and juiciness will be better with a higher proportion of betaine due to the water-holding capacity. However, the juiciness from the sensory plot shows higher juiciness in the control while the other categories had comparable lower results. The control is not high in Betaine, so other molecules are more involved in the determination of juiciness.

N-methylantoin is higher in BSF prot in comparison to the other feed groups, however, it is not a significant difference. Another interesting metabolite in BSF prot is IMP, which were found in lower concentrations compared to the other feed categories. IMP is an intermediate in the purine cycle and the breakdown of ATP in fish. ATP immediately starts to degrade to ADP and further to AMP *post mortem*. AMP is further degraded to IMP, which converts to hypoxanthine, according to the following steps:

ATP → ADP → AMP → IMP → Hypoxanthine

(Jones & Murray 1964; Shahidi et al. 1994; Howgate 2005). The significantly lower IMP concentration in the BSF prot samples is followed by a slightly higher mean value of the non-significant hypoxanthine in comparison to the other diets. This can indicate that the conversion from IMP to hypoxanthine occurs faster in BSF prot. SCP has a higher amount of AMP according to the boxplots in Figure 5. AMP has a sweet flavour and contributes to the umami as well. AMP concentration is higher in SCP in comparison to the other feed categories which all are on the approximate same level. However, the umami sensory plots show a contrarywise pattern, where SCP is ranked lower in comparison to the other categories. SCP is intermediate in all significant metabolites, which indicates that there is not only one metabolite influencing the umami. It is rather the combination of molecules.

The flavour of fish is strongly associated with IMP and hypoxanthine. IMP is known to be an umami enhancer which gives a fresh and salty fish taste, and studies have shown that IMP is the most abundant nucleotide in rainbow trout muscle (Duan et al. 2020). As mentioned earlier, IMP is lower in BSF prot in comparison to the other feed categories. Hypoxanthine is not one of the significant metabolites present, although the mean value is a bit higher in the BSF prot group than in the other feed treatments. Hypoxanthine is regarded as having a bitter taste and contributes to off-flavour in fish (Zhou et al. 2024). This could indicate that BSF prot fed fish does not taste as fresh as the others. This may be due to the feed which impacts the metabolites since all samples were treated in a comparable routine.

Fish freshness is also connected to the *post mortem* changes regarding trimethylamine oxide (TMAO). Fish muscles use TMAO as osmoregulators for different pressures in the water (Gillett et al. 1997; Raymond 1998). TMAO gets reduced to dimethylamine and trimethylamine *post mortem*, through endogenous-, and bacterial enzymes (Krzymien & Elias 1990). Both dimethylamine and TMAO were present at the approximate same level in all samples. However, none of them had a deviant pattern caused by the diets.

BSF oil has higher Lysine concentrations in comparison to the other feed groups. Lysine is considered a sweet amino acid together with the amino acids proline, alanine, glycine, serine, and threonine (Masaaki Yoshida & Sachiko Saito 1969; Schiffman & Dackis 1975). The sweetness sensory plot does not show large differences between the groups, although BSF oil is the highest ranked feed group. However, sugar is known to give sweetness. The sugars identified do not show big differences between feed groups. Thus, it is probably the combination of different sweet-tasting molecules which affect this taste. Alanine could also be considered as umami tasting. Another metabolite linked to umami is the glutamate (Bellisle 1999). Glutamate was present in the sample but did not differ significantly between feed groups. Although, the mean value is slightly higher in BSF prot in comparison to the other feed categories.

Both leucine and isoleucine are considered almost tasteless metabolites which lean towards bitterness. These AA rather contribute to the consistency of the meat (Schiffman & Dackis 1975). Fish is not primarily known for its bitter taste. Though, leucine, isoleucine, histidine, arginine, phenylalanine, and methionine are AA contributing to bitterness (Masaaki Yoshida & Sachiko Saito 1969; Schiffman & Dackis 1975). All these metabolites were present in the samples, though only methionine was significantly higher in BSF prot. Bitterness is a flavour which often gets rejected by humans (Meyerhof et al. 2010), and historically, this kind of rejection of food was connected to toxic substances. However, there are no obvious links between bitterness and toxicity (Meyerhof et al. 2010). AMP has the capacity to act as a bitterness blocker in food, by interacting with the taste buds related to bitter taste (Ming et al. 1999). The sensory plot of bitterness indicates a slightly lower average score in SCP and control. This pattern could not be linked to the bitter metabolites in the boxplots.

Ciona has a similar concentration pattern as the control in most metabolites. They have an intermediate appearance in all the metabolite boxplots. Although the sensory plots show some differences which again indicates that it is not one single metabolite which determines the sensory, it is rather the combination of several molecules together with temperature.

4.4 Future perspectives

Future research regarding metabolite composition in combination with sensory assessment should include individual fish data which are combined in the statistical analysis. It could also be connected to instrumental measurements such as the electronic tongue. Furthermore, both the water-, and fat-based metabolite phases should be included. It would be interesting to compare the results with wild-caught fish of the same species to see how they differentiate, and which aqua-cultured feed category that were most similar. Another possibility is to see if there are differences between different aquaculture sites. The study could also be extended regarding the period the fish will consume the feed.

5. Conclusion

This study was performed to evaluate different feed components' effect on the metabolomic profile of white skeletal muscle in rainbow trout. The metabolites were then evaluated to look if there would be any obvious sensoric difference between the ingredients. Ten metabolites had statistically significant differences depending on the feed categories. The metabolite profile indicated that BSF prot was an ingredient which differed from the others regarding Betaine and Methionine, which was higher in BSF prot in comparison to the other feed categories. Lysine was higher in BSF oil. Ciona and the control sample had similar patterns in all metabolites which could indicate that Ciona is the feed which has the same potential as the control feed. The sensoriy segment is hard to give a precise conclusion due to the complexity of all metabolites present. It is rather a combination of metabolites which gives a sensory profile and differences in sensory should be coupled with a sensory assessment. Although, BSF prot had a higher (however not significant) concentration of hypoxanthine. A greater concentration of hypoxanthine might indicate that the feed category is less fresh in comparison to the others – i.e. faster spoilage and potentially shorter shelflife. IMP was lower in BSF prot as well, IMP is connected to the taste of fresh fish. SCP differentiates from the others with a higher AMP, which acts as a bitterness blocker. While BSF oil could be sweeter due to its higher amount of Lysine. Regarding texture, Betaine which has a significantly higher value in BSF prot, could have an improved texture due to Betaine's osmoregulatory properties in the muscle. The sensory panel rated the liking as similar in all. No clear connections could be drawn between the metabolite- and sensoric results.

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Smaken av framtidens fisk – Effekten av nya hållbara foder hos regnbåge (Oncorhyncus mykiss)

Akvakultur är en livsmedelsindustri som ökat under 2000-talet, och fortsätter öka. Med stigande problem relaterat till vildfångad fisk, såsom överfiske och döda havsbottnar, beräknas akvakulturen fortsätta öka kommande år. Men akvakulturen har också sina utmaningar. En av de stora utmaningarna med akvakultur är fiskfodret. Dagens fiskfoder innehåller protein och olja från till exempel fiskmjöl, fiskolja, och sojabönor, men detta anses inte hållbart i längden och man letar efter nya foderkällor. Men när man utvecklar nya foder behöver man kontrollera att fiskarna mår bra, och att fiskfiléerna smakar bra för oss människor. Ett sätt att kolla detta är genom att utföra en NMR av muskelprover, för att granska vilka metaboliter som finns i muskeln. Man kan också utföra sensoriska analyser av fiskens filé. I denna studie har en grupp fiskar fått 5 olika foderblandningar bestående av en kontroll, en svart soldatfluga protein (*Hermetia illucens*) (BSF protein), en svart soldatfluga olja (BSF olja), en sjöpung (Ciona intestinalis) (Ciona), och en med encellsprotein från Paecilomyces variotii (SCP). Försöket började med en sensorisk bedömning av fiskens filé, och sedan extraherades vattenlösliga metaboliter från muskeln. Metaboliterna analyserades i ^{1}H-NMR, och statistiska analyser gjordes på resultatet.

Syftet med denna studie var att analysera muskeln hos regnbåge som ätit olika foder, och undersöka följande forskningsfrågor,

- Påverkar fodret regnbågsfilens sensoriska egenskaper?
- Påverkar fodret metaboliterna i den vita muskeln på fisken?
- Finns det några samband mellan metaboliter, och sensoriska datan?

Resultatet visade att de olika dieterna skilde sig åt gällande metaboliternas mängd. BSF prot skilde sig mest mot de övriga dieterna. Ciona och SCP visade resultat som överlappades av kontrollen. BSF olja skilde sig något mot kontrollen. BSF protein hade högre andel betain och methionin. Båda dessa molekyler är involverade i enkolsmetabolismen. Betaine agerar också som osmoreglerare i cellen och kan påverka texturen i fiskfilén. BSF protein hade också högre andel hypoxanthine och

läge IMP, vilket indikerar att fisken inte är lika färsk som de andra proverna. BSF olja har en ökad andel av metaboliten Lysine, som har en sötare smak. SCP hade en högre andel av AMP, som agerar bitter-blockerare i mat. Den sensoriska panelen bedömde inte några stora skillnader mellan dieterna, men bedömde den totala sensoriska gillandet lägre på SCP. Små skillnader kunde ses på surhet, bitterhet, saftighet, sötma, umami, och mörhet. Sammanfattningsvis kan man säga att sensorik är en komplex blandning av flera molekyler som påverkar varandra, och denna studie involverade inte alla närvarande molekyler. Denna studie kunde inte hitta några tydliga kopplingar mellan sensoriken och metaboloterna.

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Appendix 1

Table 2. *Mean relative values of all identified metabolites from the ^{1}H-NMR. The five diet categories includes control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

Diet	BSF oil	BSF prot	Ciona	Control	SCP
2.Aminobutyrate	0.0054	0.0066	0.0051	0.0045	0.0052
3.Aminoisobutyrate	0.0042	0.0053	0.0057	0.0049	0.0072
Acetate	0.0221	0.0216	0.0240	0.0225	0.0233
Acetoacetate	0.0030	0.0019	0.0018	0.0024	0.0024
Acetone	0.0014	0.0013	0.0012	0.0012	0.0013
ADP	0.0286	0.0289	0.0297	0.0285	0.0254
Alanine	0.0156	0.0155	0.0137	0.0117	0.0131
Anserine	1.2517	1.4095	1.3311	1.2799	1.3578
Betaine	0.0394	0.0738	0.0452	0.0442	0.0441
Carnosine	0.0112	0.0120	0.0106	0.0102	0.0109
Choline	0.0291	0.0303	0.0281	0.0277	0.0298
Creatine	3.3015	3.4357	3.4602	3.3043	3.4008
Creatinine	0.0390	0.0407	0.0327	0.0331	0.0347
Dimethyl_sulfone	0.0105	0.0105	0.0108	0.0101	0.0105
Dimethylamine	0.0084	0.0087	0.0084	0.0081	0.0094
Ethanol	0.0201	0.0245	0.0227	0.0205	0.0219
Formate	0.3523	0.4529	0.4173	0.3607	0.3679
Fumarate	0.0036	0.0034	0.0034	0.0032	0.0031
Glucose	0.0267	0.0256	0.0216	0.0214	0.0286
Glucose.6.phosphate	0.0313	0.0347	0.0265	0.0279	0.0292
Glutamate	0.0461	0.0512	0.0418	0.0401	0.0393
Glutamine	0.0116	0.0113	0.0107	0.0116	0.0130
Glycerol	0.0506	0.0528	0.0473	0.0486	0.0460
Glycine	0.9544	0.8661	0.7846	0.9999	0.8859
Histamine	0.0392	0.0377	0.0336	0.0369	0.0417
Histidine	0.2457	0.2561	0.2031	0.2323	0.2580
Hypoxanthine	0.0774	0.0932	0.0767	0.0798	0.0883
IMP	0.3239	0.2822	0.3508	0.3321	0.3466

Inosine	0.2509	0.2789	0.2249	0.2366	0.2218
Isoleucine	0.0095	0.0104	0.0115	0.0128	0.0104
Lactate	4.1288	4.2622	4.2015	4.1232	4.3231
Leucine	0.0164	0.0175	0.0189	0.0211	0.0178
Lysine	0.0506	0.0166	0.0235	0.0288	0.0316
Malonate	0.0273	0.0166	0.0158	0.0213	0.0178
Methanol	0.0697	0.0761	0.0708	0.0689	0.0742
Methionine	0.0097	0.0144	0.0110	0.0112	0.0118
Methylamine	0.0023	0.0028	0.0024	0.0021	0.0028
N.N.Dimethylglycine	0.0082	0.0085	0.0086	0.0084	0.0086
NADplus	0.0080	0.0071	0.0080	0.0076	0.0083
Niacinamide	0.0191	0.0193	0.0191	0.0193	0.0202
N.Methylhydantoin	0.0026	0.0052	0.0032	0.0028	0.0032
O.Acetylcarnitine	0.0047	0.0048	0.0043	0.0047	0.0045
Pantothenate	0.0043	0.0043	0.0038	0.0044	0.0041
Phenylalanine	0.0058	0.0059	0.0063	0.0061	0.0054
Propylene_glycol	0.0022	0.0020	0.0019	0.0023	0.0022
Pyruvate	0.0042	0.0046	0.0042	0.0039	0.0041
Sarcosine	0.0142	0.0171	0.0150	0.0150	0.0159
Serine	0.1082	0.1082	0.1001	0.0941	0.1104
sn.Glycero.3.phosphocholine	0.1013	0.0844	0.1134	0.1153	0.0866
Succinate	0.0460	0.0521	0.0457	0.0437	0.0481
Sucrose	0.0112	0.0151	0.0078	0.0137	0.0084
Taurine	0.2364	0.2699	0.1934	0.2749	0.1443
trans.4.Hydroxy.L.proline	0.0295	0.0229	0.0284	0.0313	0.0276
Trimethylamine_N.oxide	0.2699	0.2224	0.2708	0.2611	0.2916
Tyrosine	0.0053	0.0057	0.0062	0.0054	0.0052
Valine	0.0227	0.0238	0.0253	0.0265	0.0241
Beta.Alanine	0.0377	0.1042	0.0695	0.0594	0.0803
AMP	0.0041	0.0036	0.0032	0.0036	0.0057
ATP	0.0047	0.0043	0.0045	0.0052	0.0047

Appendix 2

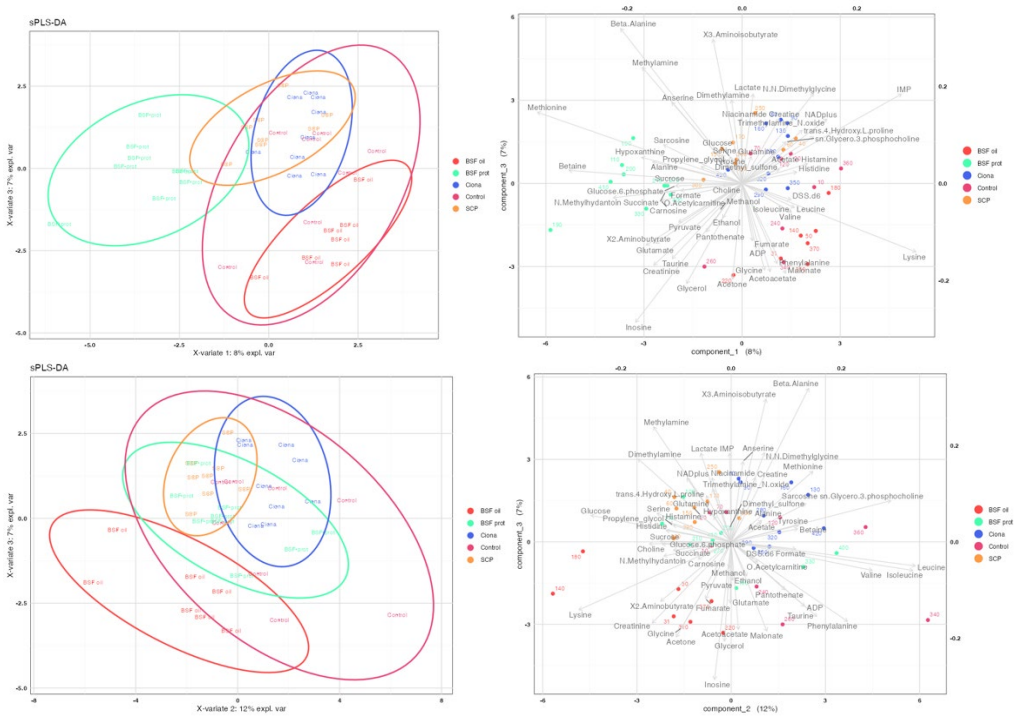

Figure 8. *Confidens interval plot of the sparse least squares discriminate analysis (sPLS-DA) from muscle metabolite samples of five feed groups of rainbow trout (O.mykiss). The five diets contained a control feed with soybean and fishmeal, Black soldier fly (Hermetia illucens) protein (BSF prot), Black soldier fly fat (BSF fat), Ciona (Ciona intestinalis) (Ciona), or Singe cell protein from Paecilomyces variotii (SCP).*

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