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Faculty of Biosciences

Production efficiency, fish welfare and colorimetric characteristics in skin and fillets of juvenile Atlantic salmon (*S. salar*) fed diets supplemented with grass protein (*M. sativa*) in a freshwater RAS-system

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December 15th, 2022.
Richard Taknes

Abstract

The objective of this study was to investigate the effects of serial inclusion (0, 5, 10, 15, and 20%) of alfalfa protein concentrate (APC) in relation to production efficiency, welfare, and colorimetric characteristics of juvenile Atlantic salmon (*Salmo salar* L.) cultivated in a freshwater RAS-system. The feed producers utilized in this study was Aller Aqua and Cefetra Group, of which the former was the financial benefactor of the trials. In relation to growth parameters, we wanted to observe whether APC included feeds was competitive with commercial feed formulations. Regarding welfare several parameters were measured. It was monitored for scale loss, skin bleeding and emaciation state externally by using the collaborative welfare assessment standard "FISHWELL Morphological Operational Welfare Indicators (OWI's) for farmed Atlantic salmon v1.1" outlined by Noble *et al.* (2018). For quantification of internal welfare parameters blood plasma was extracted and analyzed for enzymes associated with salmonid muscle, heart, and mitochondrial function.

It was also of interest to monitor if the experimental feed affected skin and filet color of Atlantic salmon. Color is an important aspect of consumer impression of the product quality. Measurements of potential colorimetric changes were carried out by use of the CIELAB color space outlined by CIE (1977).

Though the experiment was conducted over a relatively short time span, alfalfa infused diets were found to differ signficantly from the control in several parameters. SGR was significantly reduced between control and 20% alfalfa inclusion. FCR was also determined to be significantly higher in all alfalfa dietary treatments. This was also true for final body weight and length as well. Condition factor was significantly higher in the 15% alfalfa treatment compared with control. Liver weight and hepatosomatic indices increased significantly between control and 20% APC inclusion. APC inclusion did not affect fish welfare across all dietary treatments. APC feed led to a significant increase in fillet greenness (negative shift in a*-value) in CIELAB color space, while fish skin colorimetric measurements were inconclusive. No mortalities were recorded in the study.

The experiment commenced on April 14th, 2023, and had a duration of 6.5 weeks (45 days), terminating on June 1st – 2nd where sample extraction for selected analyses was done. Fish were cultivated at Centre for Fish Trials at NMBU (Ås, Norway). Final termination occurred on June 9th, where slaughter of the last specimens took place. An extension was needed to produce enough fecal matter for analysis of growth and faeces scoring.

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1. Introduction

From its inception in the mid-1960's to the present date the salmonid aquaculture industry has seen a shift in choice of feed ingredients (Tilseth *et al.*, 1991). The shift is characterized by a gradual replacement of marine ingredients (i.e., fish meal and oil) as the main dietary protein and lipid sources with plant-based ingredients such as soy protein and rapeseed oil (MOWI, 2022). Fish meal has traditionally been used in the aquafeed due to its complete nutritional profile, inhabiting essential fatty acids (EFA) as well as essential amino acids (EAA) (Daniel, 2018). However, a change was necessitated in salmon feed composition due to overfishing of wild fish stocks, which still is a major issue in the ongoing fisheries today. As a result, the growth of capture fisheries has remained stagnant, settling at 90.3 million tons in 2020 (a decrease from previous years) and maintaining a similar harvest volume from 1985 up to present time. (FAO, 2022). In contrast worldwide aquaculture production has seen substantial growth, experiencing an approximate doubling of production volume from 2000 to 2020 (FAO, 2022).

It is estimated that food fish supply met by aquaculture will range between 60–70% by the year 2030 (Subasinghe *et al.*, 2009). In aquaculture the primary limitation to production is constituted by external inputs such as feed (Tacon & Metian, 2015). Consequently, the availability of the feed itself is determined by the availability of the ingredients required to produce it. This in turn leads to considerable competition for ingredients among the actors in the industry.

The combined factors of limited supply and high demand are making fish meal and fish oil more costly to attain for producers. It is important that aquaculture produces a net amount of more fish for human consumption than it uses for feed production purposes. The aquaculture industry must then look to new avenues of exploitation to meet fish nutrition demands (Hardy, 2010). Most research agrees that based on factors of availability, wild stock conservation and economics that the leading alternative should be plant based. This does however raise the issue of whether plant-based ingredients can compete with the nutritional profile of fishmeal, and if not, whether they affect fish performance and welfare negatively (Daniel, 2018).

Since the decline of fishmeal- and oil availability the leading ingredient in salmon feed has been soybeans. Soy is a readily available protein and lipid source which has been the chief ingredient since ca. 2005-2010 and onwards (NCE Seafood Innovation, 2022). There is however a notable presence of antinutritional factors (ANF) in soy (i.e. lectins, saponins,

phytate etc.) which requires additional processing to be optimized for salmon as a feed ingredient. While the levels and types of ANFs are greatly reduced by processing soybeans into a soy protein concentrate (SPC), there are still problematic ANFs left in SPC after solvent extraction such as fiber and phytate (Gajardo, 2016). Use of full fat soy and solvent-extracted soy protein is therefore limited in feed due to their documented negative effects on salmonid growth and gut health (Krogdahl et al., 2015). Soy is additionally associated with the deforestation of the Amazon rainforest in South America (Pacheco *et al.,* 2021). In addition, the transportation over large distances (i.e., Brazil to Norway) also contributes to making soy a less environmentally sustainable alternative (Josefsen *et al.,* 2023).

In Norwegian salmon farming the feed cost has remained steady at an approximate 50% of the total production costs (MOWI, 2022). It is imperative that such high numbers of investment are reciprocated in the growth and performance of the fish for a successful production volume and economical yield.

In recent years other plant-based protein sources have been considered and researched. Alfalfa protein concentrate (APC) is one of the prospective sources for supplying the protein demand of salmon farming. Alfalfa (*Medicago sativa spp. sativa*) is an important forage crop existing in multiple parts of the world. Forage crops are low trophic plants that constitute grasslands, which a wide variety of animals graze on (Putnam & Orloff, 2014). For categorical purposes alfalfa can essentially be labelled as *grass*. Because of the nature of the protein source APC will for simplicity interchangeably be referred to as grass protein (GP) in this thesis. GP has larger quantities of certain limiting amino acids than SPC, such as methionine, while having competitive amounts of lysine (see table 1). GP is also rich in other desirable components such as omega-3 fatty acids and carotenoids (Samac *et al.,* 2019).

With its complete nutritional profile and lesser amounts of ANFs, GP could be a future ingredient of salmon feeds. However, as with all new ingredients, it is important to monitor how GP substitution affects fish welfare. Welfare is an important aspect of fish husbandry, not only for fish growth and performance, but also to give a worthy life to the cultivated species without unnecessary suffering (Noble *et al.,* 2018). The goal of any new ingredient should therefore be to optimize performance in conjunction with welfare, not at the expense of it.

The present aim of this study was to see how serial inclusion (0, 5, 10, 15, and 20%) of GP would affect the production efficiency parameters, welfare, and colorimetric characteristics of juvenile Atlantic salmon (*S. salar*) cultivated in a freshwater RAS-system.

1.1 Hypothesis formulation

The following hypotheses were formulated:

H_1 = GP inclusion rate will *not* affect production efficiency significantly.

H_2 = GP Inclusion rate will *not* affect fish welfare significantly.

H_3 = GP inclusion rate will *not* affect fish skin/fillet color significantly.

2. Theoretical framework

2.1 Salmonid nutrition

Atlantic salmon (*Salmo salar* L.) is an anadromous species of fish with a carnivorous feeding strategy (Rungruangsak-Torrissen, 2014). This entails a predominant reliance upon the macro compounds of protein and fat to meet their nutritional demands. Atlantic salmon therefore has a reduced capacity for digesting carbohydrates, which is grounded in their low α-amylase and α-glucosidase enzymatic activity (Hemre, 2001). While naturally a carnivore (i.e., piscivore), aquaculture has experimented with protein and lipids from plant-based origins in salmon feeds which is now the established practice (Egerton *et al.*, 2020).

For juvenile salmon it is normal to have a crude protein content of 50-55% in the feed (Storebakken, 2002). Salmon are also incapable of synthesizing omega-3 (n-3) and omega-6 (n-6) fatty acids *in vivo* and are in turn dependent upon attaining these through their diet (Storebakken, 2002). Juvenile salmon requirements for n-3 fatty acids are approximately 1% of dry feed on the condition that the n-3 fatty acid composition primarily contains the long chain marine PUFA's 20:5n-3 (EPA) and 22:6n-3 (DHA). Failure to meet these demands may lead to increased mortalities and stunted growth (Storebakken, 2002). It is important to consider these factors during feed formulation and ensure that the macro compounds are proportional to the requirements.

2.2 Alfalfa Protein Concentrate (APC) - Processing and production

Alfalfa protein concentrate (APC) is a form of leaf protein concentrate (LPC). Leaf protein concentrates are protein-rich byproducts generated from wet fractionation (i.e., mechanical grinding and pressing) of plant leaves (Fiorentini & Galoppini, 1983). The result is a protein rich leaf juice and fibrous press cake (Fiorentini & Galoppini, 1983). APC follows the general manner of production as LPC and green biomass related products (figure 1) (McEniy & O' Kiely, 2014).

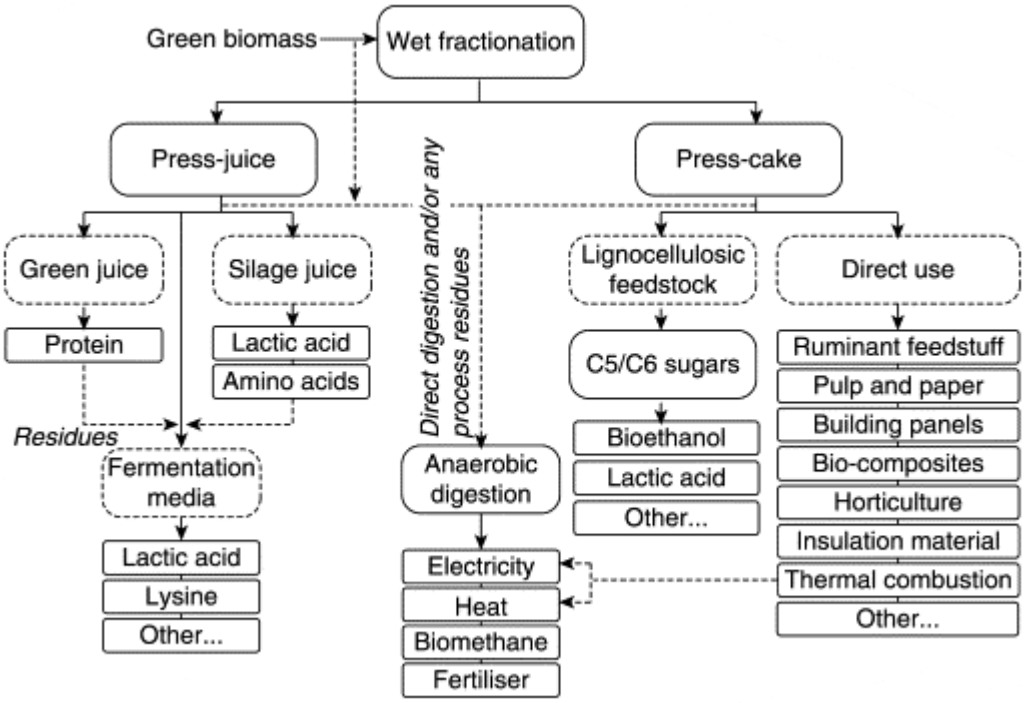


Figure 1: *Chart depicting the general processing pathways of green biomass and its possible utilizations, which alfalfa (M. sativa) protein falls within. GP is produced via the press-juice pathway on the left side of the chart. Source: McEniy & O' Kiely 2014.*

Harvested alfalfa (*M. sativa*) leaves and stems are grinded and further processed into APC by mechanical pressing. Pressing involves passing the grass raw material through several mills to sufficiently break down the cell wall and release intracellular contents (Møller *et al.*, 2021). Most of leaf protein exists in the chloroplasts within the mesophyll of the cell membranes (ca. 80%), followed by the cytosol (ca. 20%), mitochondria (< 5%), and lastly the nucleus (ca. 1-2%) (Fiorentini & Galoppini, 1983). The solid press cake is then pressed again to separate the maximum amount of juice from it (Domokos-Szabolcsy *et al.*, 2023). This is because only 46-53% of the total alfalfa protein is extracted at this stage, the residual protein being bound up in the press cake (Hanna & Ogden, 1980). One can also utilize a twin-screw extruder set up for optimizing pressure and shearing forces to produce a higher juice yield than by using only milling, resulting in up to 60-65% fluid extraction rate from the leaves and a protein content of over 50% (Domokos-Szabolcsy *et al.,* 2023). The residual press cake still contains a considerable amount of protein which is still attainable to an extent and can be re-processed to this end by adding more water mix before pressing. (Knuckles *et al.*, 1972) Pressing done under mildly alkaline conditions (pH 7.0 – 8.0) has been suggested to improve cell wall disruption and protein recovery rate (Santamaría-Fernández & Lübeck, 2020).

Elevating the pH above neutral (pH 7.0) can help accelerate the breakdown of cell walls during pressing and may lead to higher protein yields (Domokos-Szabolcsy et al., 2023). However, it may also lead to premature denaturation of proteins if that limit is exceeded. The same is also true for excessive heating of leaf proteins (Santamaría-Fernández & Lübeck, 2020).

The juice is then heat treated (via steam injection in Cefetra Group APC) to facilitate thermal coagulation and further separate the protein fraction from the fibrous fraction in the liquid (Coburn *et al.,* 2021). Coagulation occurs when the heating causes changes in the conformation of the protein, making it less water soluble (i.e., more extractable) by opening hydrophobic sites in the structure (Bals *et al.,* 2012). While heat coagulation of the juice for protein recovery is the most common there are other methods available. Acid precipitation is one such method which has shown promising total APC yields (Samac *et al.,* 2019). Other means of concentrating the proteins from the juice include membrane filtration and fermentation (Nissen *et al.,* 2022).

After undergoing coagulation, the juice is centrifuged to filtrate the proteins from the fiber-rich liquid. Any residual moisture is removed by way of drying. (Coburn *et al.,* 2021) After the drying process the protein concentrate is compacted into pellets and further stored under positive refrigeration (appendix 7.2). While not palatable to humans and other monogastrics the press cake can in turn be used as ruminant feed, thus improving bio-circularity, and promoting synergy between aqua- and agrifeeds (Coburn *et al.,* 2021). Alfalfa also requires less amounts of inputs such as irrigation and heating in comparison with soy and can be grown in many European regions as well (Li & Brummer, 2012). Additionally, alfalfa also has a reduced need for nitrogen-based fertilizers due to its inherent capability for nitrogen fixation from the surrounding environment (Santamaría-Fernández & Lübeck, 2020). Leaves, including alfalfa forage, constitutes the largest biomass of protein in the world and has a massive potential for utilization in both feed and food (figure 2) (Fiorentini & Galoppini, 1983; Aas *et al.,* 2019).

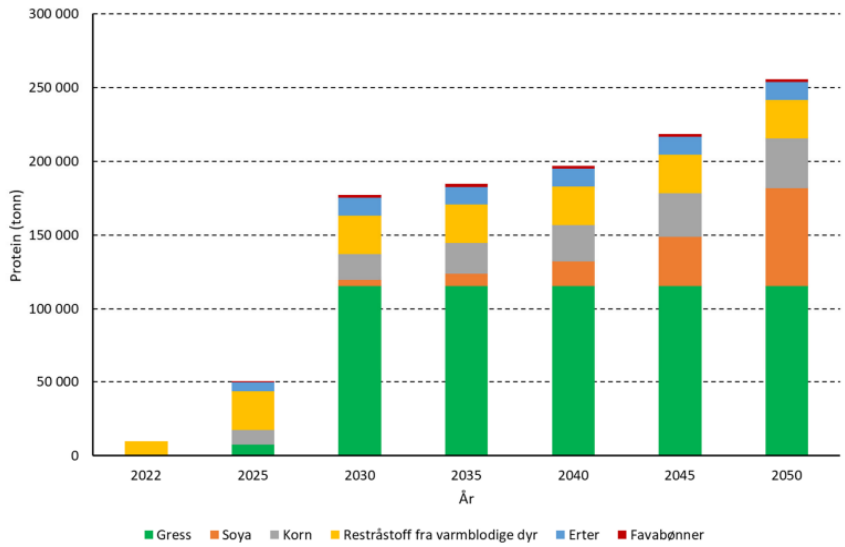


Figure 2: *Bar chart depicting the potential that different terrestrial resources have in supplying the future protein demands of aquaculture. Grass, including alfalfa protein concentrate, inhabits most of the potential in the coming years. Source: Aas et al., 2019*

2.3 Nutritional content of APC

After processing APC protein amounts range between 50-55%. The APC producer (Cefetra Group) utilized in this study estimated a guaranteed 50% crude protein content (see appendix 7.2). While crude protein is not the equivalent of an exact protein content, it gives an estimate on total protein in the APC via the amount of nitrogen present (N × 6.25). It has however been contested that the 6.25 factor is outdated and should be lower for plant proteins (N × 5.36), of which alfalfa falls within (Salo-väänänen & Koivistoinen, 1996). One must also consider that part of the nitrogen is bound up in other structures than proteins referred to as non-protein nitrogen (NPN). This may lead to an overestimation of the protein present in the concentrate (Koschuh *et al.*, 2004).

APC also has high amounts of amino acids often recognized as limiting for salmon. These include amino acids such as lysine, methionine, and threonine (Coburn *et al.*, 2021). If an amino acid is limiting it means that it is a crucial component in protein synthesis and consequently for the growth of the fish. More specifically, a limiting amino acid is an essential (i.e., the organism cannot produce automatically) amino acid that is low in quantity and is therefore exhausted quickly during protein synthesis (Lopez & Mohiuddin, 2023).

For salmonids these scarce amino acids are constituted by methionine (0,7% requirement in diet formulation), threonine (1.4 %), arginine (1.6 - 2.2%) and lysine (2 - 2.2%). Among these the first limiting amino acids are recognized to be methionine and

lysine, which producers must take into consideration during feed formulation (Peterson *et al.*, 2022). These will often have to be supplemented in the feed formulations when using other plant-based protein sources like SBM or SPC (Aas *et al.*, 2019). The micro ingredient portion of salmon feed has seen a steady increase since 1990 and onwards, mainly due to the need for balancing out the AA-composition of the feed. This is done by addition of scarce AAs to the feed, which are supplemented as crystalline-AA compounds (Aas *et al.*, 2019). With APC being composed of higher amounts of salmonid EAA this would potentially result in a lesser micro-ingredient cost. APC is therefore in principle well suited to supply salmonid nutrient requirements. APC also has a fat content of approximately 10% (100g / kg) of which 4% (40g / kg) is omega-3 and exclusively represented by alpha-linolenic acid (18:3 n-3). It is also rich in micronutrients such as antioxidants (i.e., carotene, xanthophyll), minerals and vitamins (Coburn *et al.*, 2021). With regard to minerals, it has very high amounts of potassium, calcium and phosphorus (Domokos-Szabolcsy *et al.*, 2023). In vitamins and associated compounds APC is rich in vitamin E and choline, the latter of which is often associated with vit-B complexes (see table 1) (Britannica, 2023).

Table 1: *Comparative nutritional content of APC, SPC and FM. All data values listed as fed. Note: APC values collected from producer Cefetra Group content sheet (appendix 7.2) as well as feedtables.com where the producer did not explicitly state the given parameter. f = feedtables.com*

*(Feedtables 2023a). All data on SPC and FM collected from feedtables.com (Feedtables 2023b & 2023c). *ND =No data.*

Component	***APC***	***SPC***	***FM***	***Unit***
Dry matter	92	93.3	92.2	%
Crude protein	50	64.7	69	%
Crude fibre	2.3	4.1	0	%
Crude fat	10.5	1	9.1	%
Ash	11	6.1	14	%
Insoluble ash	0.7[f]	0.03	0.4	%
NDF	13.8	9.8	5.3	%
ADF	2.8	5.3	0.3	%
Lignin	1.5[f]	0.2	0.2	%
Water insoluble cell walls	9.5[f]	9	4.6	%
Starch	0.9[f]	7.5	0	%
Total sugars	0.7[f]	0.8	0	%
Gross Energy	4854	4590	4680	kcal/kg
Gross Energy	20.32	19.2	19.6	mj/kg
Vitamins and pigments				
Xantophylls	800	ND*	ND	mg/kg
Vitamin E	400	0	19.6	mg/kg
Carotene	405	ND	ND	mg/kg
Vitamin K	100[f]	0	2.2	mg/kg
Vitamin D	0[f]	0	2	mg/kg
Vitamin A	207[f]	0	0	mg/kg
Vitamin B1 thiamin	2.6[f]	31.6	0.3	mg/kg
Vitamin B2 riboflavin	4.5[f]	14.2	9.2	mg/kg
Vitamin B6 pyridoxine	83.4[f]	13.4	4.9	mg/kg
Niacin	6[f]	71.6	103	mg/kg
Choline	1190[f]	2720	4702	mg/kg
Amino acids				
Lysine	3	4.03	5.2	%
Threonine	2.93	2.36	2.86	%
Methionine	1.02	0.91	1.92	%
Cystine	0.48	1.06	0.58	%
Tryptophan	0.77	0.95	0.71	%
Isoleucine	2.51	2.91	2.9	%
Valine	2.98	3.12	3.43	%
Leucine	4.41	4.99	4.95	%
Phenylalanine	2.96	3.27	2.68	%
Tyrosine	2.12	2.26	2.13	%
Histidine	1.25	0.17	1.64	%
Arginine	2.98	4.73	4.5	%
Aspartic acid	5.01[f]	7.38	6.27	%
Glutamic acid	5.5[f]	11.70	8.75	%
Glycine	2.71[f]	2.66	4.31	%
Serine	2.19[f]	2.75	2.69	%
Proline	2.34[f]	3.19	2.79	%

2.4 Formerly studied effects of APC inclusion in aquafeeds

In studies done by the University of Minnesota (UMN), researchers Samac *et al.,* 2019 found that diets with inclusion of APC were palatable to the species of yellow perch and rainbow trout. While there were no significant differences in growth parameters by inclusion of APC it did not affect feed intake of the fish. (Samac *et al.,* 2019) Given the similarities in anatomy with rainbow trout this indicates that APC-based feed can be palatable to the Atlantic salmon (*S. salar*) as well. While somewhat less comparable to salmon, other studies have experimented with substituting soy-based protein (SBM) with APC in varying degrees in feed for nile tilapia (*O. niloticus*) Abd El-Hakim *et al.,* 2009 discovered that higher inclusion rates (i.e., 50 and 75%) lead to a significant decrease in final fish weight compared to the control group. An inclusion of 25% showed no significant difference from the SBM fed control group in relation to several growth parameters (Abd El-Hakim *et al.,* 2009).

In an earlier study by Olvera-Novoa *et al.,* 1990 on the effects of gradually increasing substitution of fish meal with APC in the diet of tilapia (*O. mossambicus*) found positive results at some percentages. The study concluded that a 35% replacement of the fish meal with APC showed better growth than the control, but anything above this repressed fish growth (Olvera-Novoa *et al.,* 1990). All the above indicates that APC inclusion is a complex but potentially promising prospect for protein substitution in aquafeeds. It must however be tested further and optimized for salmon.

2.5 Colorimetry

2.5.1 CIELAB

Quantification of changes in color in both skin and muscle can be done utilizing the CIELAB color space (CIE, 1977). CIELAB functions as a 3D coordinate system for colors. A given sample will exhibit a certain set of photometric properties (light/dark, red/green etc.) which locates it at a specific color “coordinate” in the system (Ly *et al.,* 2020). CIELAB is a method within the field of measurement that is known as colorimetry. CIE stands for Commission Internationale de l’Eclairage, who standardized the method. L*, a*, and b* each denotes different photometric parameters respectively (Ly *et al.,* 2020). The asterisk behind the letter in each parameter is simply there to distinguish it from other laboratory techniques utilizing the same sign in denotation. (Ly *et al*., 2020)

It is possible to measure color changes in both fish skin and filet using subjective judgment in conjunction with scoring criteria and labeling, yet it is important to note the advantage in accuracy and consequent scientific validity of using quantifiable methods such as CIELAB. In using quantitative measurements, we avoid the bias and potential irregularity of observation linked to subjective scoring. (Ly *et al.*, 2020)

While indeed subjective observation and scoring cards were used in other parts of the experiment (i.e., liver color and feces solidity scoring) it was of importance that a quantitative measurement like CIELAB was utilized to detect any minute changes in skin- and muscle color as a result of the GP inclusion. During the experiment it was desirable to monitor such changes because of the importance it has for seafood consumer acceptance.

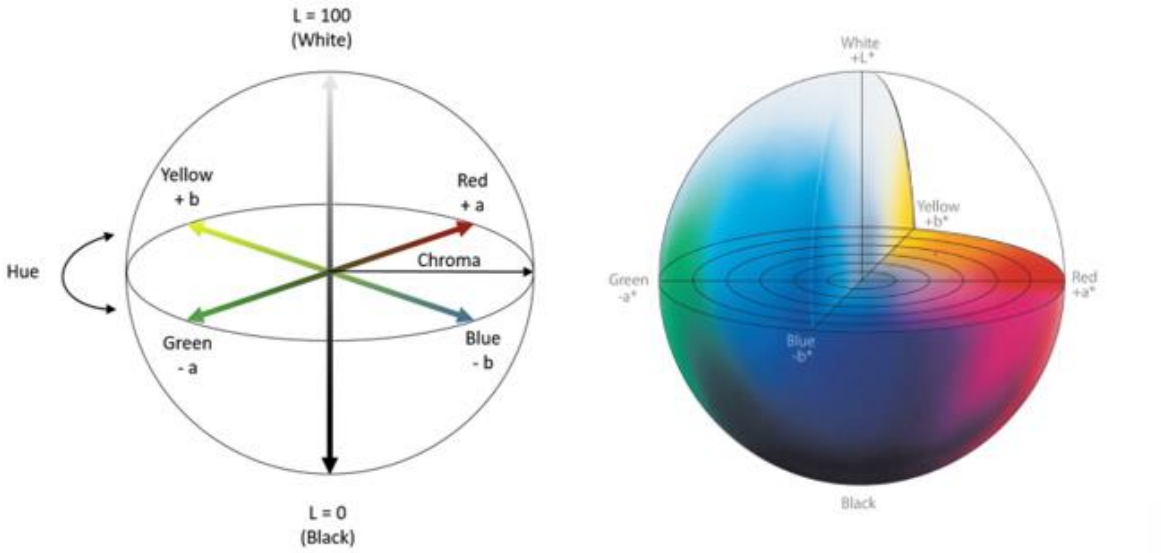


Figure 3: *Three-dimensional axis diagrams of CIELAB color space showing the relationship between L*, a* and b* values with associated ranges, as well as the relation between chrome and hue.*

Source: Ly et al., 2020 & Konica Minolta, 2019.

The L* value represents a point along the L*-axis in the three-dimensional CIELAB color space. This measurement is in grayscale, meaning there is no visible coloration in the different shades along the range of the L*-axis, but that rather it tells us the degree of luminosity (lightness/darkness) of a given sample (Ly *et al.*, 2020). The L*-value therefore correlates with and signifies the degree of pigmentation in the sample or specimen. This ranges from a value of 0 (black) to 100 (white) (Ly *et al.*, 2020). For example, in colorimetric analysis of fish filets one can envision a salmonid fish having a higher degree of

pigmentation and therefore a lower L*-value than a codfish, due to its red muscle being inherently darker than white muscle and consequently less luminous.

The a* value indicates the redness or greenness of an object or specimen. If we observe the a*-axis in figure 2 and 3 we can see that a positive (+) a*-value indicates a certain redness, whereas a negative (-) a*-value is indicative of greenness in the visual properties of the object. It is the same with the b*-axis, albeit that one is then measuring yellowness (+) or blueness (-) (Ünal Şengör *et al.*, 2019).
As one moves in either direction from the colorless intersection of the axes (see figure 2) the degree of saturation increases. (Konica Minolta, 2019b) This can be observed in figure 2, where the colors located towards the center are more "transparent" and less intense than the colors on the outlying a* and b* coordinates. Saturation can then be described as the purity of the color and is dictated by the degree of absence of the achromatic colors of white, gray, and black (Jiménez Guerrero, 2018). As one move towards and along the center L*-axis the degree of purity diminishes as the presence of achromatic colors increase, hence resulting in less saturated color values (Jiménez Guerrero, 2018).

2.5.3 Colorimetric analysis – Minolta™

While the CIELAB color space allows us to map out and categorize the color and luminosity data of a sample in a three-dimensional space, the Minolta™ (KONICA MINOLTA, Japan) chromameter technology is how we obtain that data. Minolta is therefore a colorimetric (i.e., chromatic) measuring device which measures the concentrations of each primary color in a sample, thus determining an exact chromatic signature by using light projection and reflection (Konica Minolta, 2023) At the point of the handheld Minolta device is a light projection tube which is placed in contact with the sample or specimen. It is important that the glass of the tube is placed in close proximity to the object of analysis so that no external light interference occurs, otherwise it might lead to erroneous L*, a*, and b*-values.
Inside the device are two light sources placed identically to each side, emitting light at an angle of 45 degrees relative to both sample and sensor (see figure 4). (Konica Minolta, 2023) The reflected light passes through the trichromatic lens filter. The filter is based upon the three primary colors: red, blue, and green. The reflected light is dispersed into its component colors according to wavelength and captured by the colorimeter device through the filters. The data on color dispersion and luminosity is what determines the sample's chromaticity coordinates in the CIELAB color space. (Ly *et al.*, 2020)

It is important to note that only colors within the framework of the trichromatic lens (i.e., red, blue, and green wavelengths) are captured. This exclusion is essentially the difference between the colorimeter and the spectrophotometer, the latter of which utilizes a monochromatic filter for *all* colors between 360-700 nm (many unobservable to the human eye) (Ly *et al.*, 2020) Colorimetric analysis is based on the tri-stimulus of the primary colors and utilizes those in light filtration due to their corresponding with photoreceptor cells (i.e. cones) in the human eye that facilitate color perception (Teimouri *et al.*, 2013).

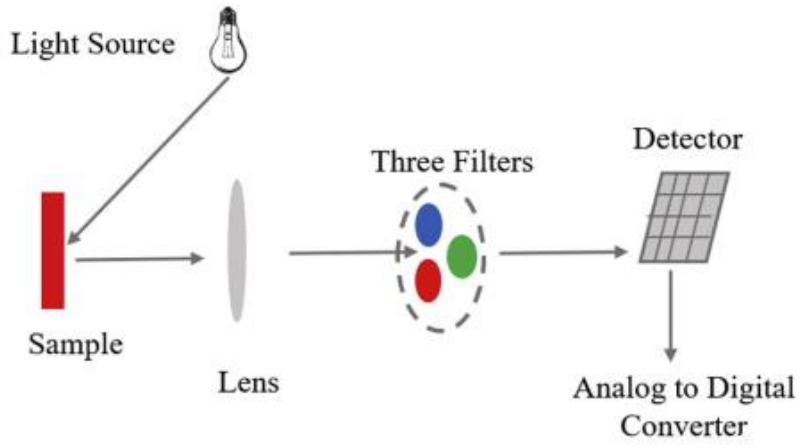


Figure 4: *Working principle of a colorimeter/ chromameter (Ly et al., 2020).*

2.6 Body characteristics and fillet quality

2.6.1 Body shape and composition

Salmonids are naturally fast and durable swimmers with streamlined bodies. Regarding farmed salmon this can be an important feature in the sale of whole fish, but also tells us something about the internal composition of the fish. Our subjective perception of the leanness (or lack thereof) in the fish can be objectively quantified by calculating the relationship between the fish length and weight (see section 3.7). This is otherwise known as the condition factor (K) (Mørkøre, 2023). A high CF in the fish may therefore be indicative of excessive visceral fat accumulation relative to the length at a given life stage. It may also be an indication of vertebral deformity if excessive in early life stages (Hansen *et al.*, 2010). On a more positive view a high CF may also indicate a higher filet volume, implying weight gain in the desired parts of the fish. It is therefore imperative for a high filet yield that the feed formulation facilitates weight gain in muscle and not visceral fat (Mørkøre, 2023). It is

important to note that it is inconsequential whether it is SBM or APC that is used as a plant-based protein source as salmon will still generate *marine protein* in the muscle composition (MOWI, 2022). This is however not the case with lipid deposition, of which the salmon will accumulate the type of lipids that it consumes. Vegetable sources as a substitution for marine protein is therefore less problematic than the use of vegetable oils in place of marine lipids (Mørkøre, 2023).

2.6.2 Skin and fillet color

Color is an important aspect of consumer acceptance of fish as food (Ünal Şengör *et al.*, 2018). Salmon filets should have a pink to red-like color, anything that deviates from this is likely to be looked on with less favorability and reduce product market value. This is an occurring phenomenon in Italian trout farms, where algal spring blooms lead to increased yellowness in the filet and consequently lower market prices (Welker *et al.*, 2001). Rosenau *et al.*, (2022) found a significant linkage between spirulina (*A. platensis*) inclusion and red/yellow fillet color shifts for three salmonid species. This was also the case for Skalli *et al.*, (2020) who experimented with green microalgae *Scenedesmus sp.*, resulting in a yellow color shift in juvenile rainbow trout (*O. mykiss*) fillet (Skalli *et al.*, 2020). Introducing new feed ingredients is therefore not without issue and may thus lead to unwanted effects in the product.

In salmon farming producers utilize dietary carotenoids to maintain the desired coloration of the filet. These are primarily constituted by astaxanthin, as well as canthaxanthin to a lesser extent (Storebakken, 2002). These pigment compounds help give the filet its characteristic color (Teimouri *et al.*, 2013). This process is what is otherwise known as pigment feeding and is an essential practice to maintain salmon product market value (Mørkøre, 2023).

Salmon skin color is affected by the number and type of chromatophores present in the dermis. Chromatophores are specialized cells in the fish skin containing different pigments (Teimouri et al., 2013). Potential quantified color changes in CIELAB may thus be related to the fish gaining more of a specific chromatophore and their related pigmentation. Because of GP and its prominent green color characteristics such changes are not unthinkable upon inclusion in the feed formulation.

2.7 Fish welfare

All vertebrates are protected under the Norwegian Animal Welfare Act (2009) (Noble *et al*., 2018). This includes fish, and therein salmonids, cultivated in either farming or research-based contexts. Suboptimal conditions of cultivation can lead to negative effects on production output via disease outbreaks, excess mortalities, and stunted growth. Fish welfare is therefore not only an important feature of sustainable aquaculture, but of effective production as well (Noble *et al.* 2018). Regardless of the goals of production or research, farmed salmon are still entitled to a life without unnecessary painful conditions (Noble *et al.*, 2018).

2.7.1 Welfare Indicators

In this experiment welfare assessment was done by looking at multiple aspects. When measuring fish welfare, a common method is by using *welfare indicators* (WI). WI can be described on multiple levels to give an estimate of fish welfare (Noble *et al.*, 2018). WI can be direct (i.e., animal based) as when relating directly to the properties or condition of the fish. WI can also be indirect as when related to production environment (i.e., oxygen levels, uneaten feed accumulation etc.) (Noble *et al.*, 2018). Direct and indirect WI can further be elaborated on an either individual level or in the context of a group (Noble *et al.*, 2018). Additionally, some WI are better suited for on-site observation and are therefore referred to as operational WI (OWI). Welfare parameters that are harder to estimate on site often require external analysis in laboratories. This includes parameters related to the internal states of the fish (i.e., molecular imbalances, homeostasis etc.). These are therefore referred to as lab-based WI (LABWI) (Noble *et al.*, 2018).

In this study a conjunction of OWI and LABWI was utilized to indicate fish welfare. It was of interest to observe whether GP included feeds led to detrimental effects in skin condition of the fish. Moreover, it was of interest to see if GP diets had any effect on the CF and whether it would lead to elevated or stunted growth. Among the OWI used were skin related parameters such as skin bleeding (i.e., hemorrhages) and scale loss. Scale loss and damage to skin is hazardous to fish welfare, as the scale and mucosal layer surrounding them is the first line of defense against infections and pathogens (Noble *et al.* 2018). Wounds or bleeding can also be signs of detriment in osmoregulatory function (Noble *et al.*, 2018). Additionally, it was also observed whether the fish were emaciated (i.e., emaciation state) to any extent compared to

fish fed a standard diet composition. This was measured by calculating CF, of which a value of < 0.9 is interpreted as emaciation in the fish. A disproportionately high CF may conversely point to vertebral deformities in the fish (Hansen *et al.,* 2010). It is however important to consider that CF varies with life stage and season and is thus naturally lower at certain times of the year and during developmental stages (Stien *et al.,* 2013).

Other welfare parameters were dependent upon further laboratory-based analysis. This includes organosomatic indices like the hepatosomatic index (HSI), which expresses the relationship between the liver and the total body weight (Noble *et al.,* 2018). Otherwise, the LABWIs were constituted by enzymes which were looked for in the blood plasma of the fish. Among the LABWI measured was creatine kinase (CK), aspartate transaminase (AST) and alanine aminotransferase (ALT).

CK is an enzyme that functions as a catalyst in the reversible reaction occurring between creatine and adenosine triphosphate (ATP) into phosphocreatine (PCr) and adenosine diphosphate (ADP) (Aujla & Patel, 2022). It is therefore an important component in energy demanding chain reactions such as muscle contraction (Aujla & Patel, 2022). CK is most prevalent in skeletal musculature, heart (myocardium) and brain tissue (Cabaniss, 1990). CK is an intracellular enzyme and should not circulate in the body as this may be an indication of disrupted cell membranes in the tissues CK inhabit. The breakdown of cell membranes consequently causes CK to leak into the circulatory system from the cytosol of the cell (Cabaniss, 1990). Presence of CK in blood plasma is therefore associated with injury or detrimental states in heart, skeletal muscle, or brain (Cabaniss, 1990). There are three isoenzymes of CK that have been detected in fish (including Atlantic salmon): CK-M (muscle CK), CK-B (brain CK) and mitochondrial sarcomeric CK (Rojas *et al.,* 2018). This makes it possible to identify the origin of eventual CK leakage and locate potential damage to tissues. Presence of CK in serum (or lack thereof) may thus be used as an indication of fish welfare.

AST is an intracellular enzyme whose primary function is to catalyze the reversible transferal of an amino group between the amino acids of aspartate and glutamate (Aulbach & Amuzie 2017). AST exists in the cytosol of liver cells (i.e., hepatocytes) as well as in skeletal muscle (Aulbach & Amuzie, 2017). When injury occur to these types of cells AST is leaked out into the blood stream, which in turn results in elevated AST levels in plasma. AST presence in the blood plasma will therefore be an indication of damage to either hepatocytes or skeletal muscle structures (Aulbach & Amuzie, 2017).

ALT shares some similarities with AST such as inhabiting the cytosol of liver cells and skeletal musculature. Upon eventual damage to these tissues and consequent leakage, ALT levels are predominantly detected in higher quantities than AST (Aulbach & Amuzie, 2017). This is due to the comparatively longer half-life of ALT and that much of the intracellular AST is bound up in mitochondria (Aulbach & Amuzie, 2017). Both ALT and AST are useful markers for diagnosing liver and kidney imbalances in salmonid fish (Dessen *et al.,* 2020). In this way AST/ALT measurements can also be utilized for assessment of fish welfare.

3. Materials and method

3.1 Study design

Juvenile salmon parr (n = 375, starting weight = 22.4 g) were randomly divided in 15 rearing tanks (n = 25 fish per tank) at the Centre for Fish Trials on NMBU campus. The tanks utilized a freshwater recycling aquaculture system (RAS) technology to filtrate, clean and oxygenate the returning tank water. The tanks utilized were cylindrical (see figure 5) and had an approximate volume of 300 liters.

To monitor how much feed the fish digested and excreted an yttrium-oxide marker was utilized in all diets. Yttrium-oxide (Y_2O_3) is an indigestible compound for salmon and is therefore well suited as a quantifying agent, or inert marker. By observing the amount of yttrium-oxide present in the fecal matter one can backtrack to the total feed intake of the animal (Owens & Hanson 1992). In so doing we can actively see the amount of feed which is taken up in the small intestine of the fish and how much is excreted as feces in the colon, thus giving us an estimate of digestibility of the different feeds. In addition to use of yttrium marker, uneaten feed that accumulated in the steel grating of the tanks was collected for calculations on feed conversion ratio (FCR).

Each tank was delegated a randomized diet of GP-infused feed, with each diet being utilized in a total of three tanks to generate representative results. The experiment commenced on April 24th and terminated on June 2nd, 2023, for a total of 45 days (6.5 weeks). Mean water temperature during experiment period was 14.4 °C.

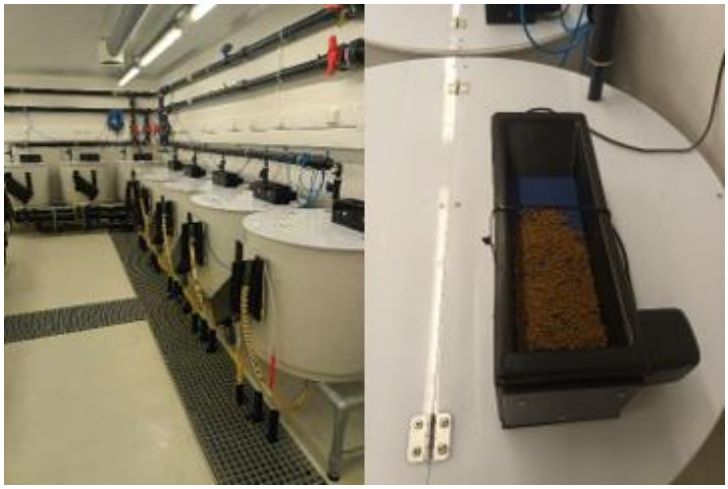


Figure 5: *Setup of tanks and automatic feeding conveyor belt at NMBU fishlab. Uneaten feed and faeces were accumulated in external collector located on the side of the tanks.*

3.2 Feed suppliers

Feed main ingredients supplied by feed producer Aller Aqua affiliate based in Bønes in Vestland (Norway). Grass protein produced by agricultural supplier Cefetra Group (BayWa AG) based in Rotterdam (Netherlands).

Table 2*: Overview of raw material composition of Aller Aqua pre-grower feed Aller Thalassa Ex 2 mm which was used as control feed in the study.*

Aller Thalsassa Ex 2mm

Raw material	***Composition (%***
Fish meal	24.00
Maize gluten	17.50
Soy bean meal	14.68
Wheat	14.31
Sunflower protein concentrate	8.00
Rapeseed oil	4.54
Fish oil	4.50
Shrimp meal	3.00
Soy bean protein concentrate	3.00
Mono-ammonium phosphate	0.66
Vitamin A & D3	0.50
Minerals	0.15
Propyl gallate (E310)	1.00

3.3 Feed production

The grass protein feed (GP) was made between April 12th – 14th, 2023. The control feed utilized in this study was Aller Thalassa 2mm produced. GP feed method of production consisted in grinding the control feed before mixing it with the desired amount of grinded APC pellets in powder form. Both the control feed and the GP infusion feed was ground by employing a Fritsch 19 Pulverisette model cutter mill with a Fritsch 9-p trapezoidal sieve 1 mm screen perforation being utilized. Disc milling cutter rotor with indexable inserts and fixed knives made from hard metal tungsten carbide was used within cutter mill, also from producer Fritsch.

Reprocessing of Aller Thalassa was needed for growth and digestibility quantification purposes (i.e, addition of yttrium-oxide marker). During feed making process the sieve and disc milling cutter rotor had to undergo repetitive cleaning to avoid clogging of the mill canal. Sieve had very fine mesh width which easily clogged after few deposits of feed raw material. If left untreated clogging can lead to excessive friction and consequent overheating that spoils the quality of the feed.

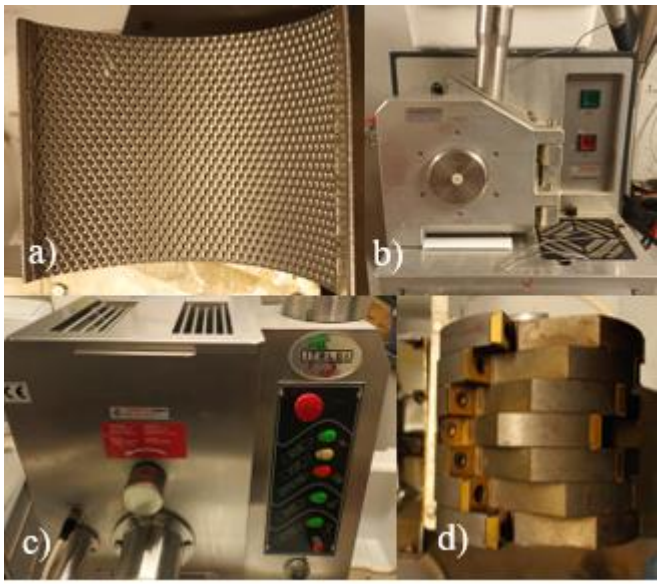


Figure 6: *Feed production equipment from left to right: a) sieve b) Pulverisette cutter mill (Heco AS) c) Italgi cold pelleting machine d) Disc cutter rotor.*

Yttrium marker was mixed manually with 50 grams of GP until satisfactory homogenization was reached. This applied for all GP diets. For control feed the process was done utilizing 50 grams of Aller Thalassa 2mm exclusively where yttrium was mixed in in the same gradual manner. After the initial 50 grams, the residual amount of GP to be added to each respective diet was added in 100 g intervals and manually mixed until homogenization. Followingly, the

GP and yttrium mix were mixed in a Kenwood™ Chef XL Elite kitchen machine for 2 minutes at speed setting 1. A total of 500 g of Aller Thalassa were mixed into the GP diets gradually (50g, 100g, 100g, 100g and 150g) at 2-minute intervals for a total of 10 minutes at speed 1. The yttrium marker mixture was then placed in kitchen machine together with the rest of the grinded feed for each diet and mixed for 10 minutes.

Gelatin was added in powder form (2,5% / 25g per kg feed) to a solution of water (1 L) before being heated up to 60 ºC in a microwave. This was to activate the gelatin and thus enable it to act as a binder in both the control feed as well as the GP diets. Upon activation half of the gelatinous fluid (0.5 L), was added to the feed mixture. After 5 minutes of mixing the residual 0.5 L was added. Feed mixture was then further homogenized in a Prismafood Solutions™ planet mixer until satisfactory degree of homogenization was achieved.

When mixing was complete the homogenized dough was inserted into an Italgi™ cold pelleting machine upon where it was further mixed and eventually pelletized after exiting the die roll and encountering the rotor blades. If the feed pellets were found to be adequately breakage-free it was approved for the study. If there was observed substantial amounts of breakage it was taken to mean an excessive dryness of the pellets, which again could affect floating ability and other physical parameters essential for optimal uptake by the fish in the tanks. When the pellets had achieved the desired texture, they were packed and stored for cooling and drying until the start of feeding.

Figure 7: *Production of grass protein feed. From powder form to dough and finally to pellets.*

Table 3: *Feed composition of diet treatments. Control (0% GP), GP5 (5%), GP10 (10%), GP15 (15%) and GP20 (20%). Analysis carried out by LabTek at NMBU (Ås, Norway). Values for crude protein and crude fat calculated from Aller Thalassa and Cefetra APC content sheets respectively.*

Component	***Control***	***GP5***	***GP10***	***GP15***	***GP20***	***Unit***
Dry matter	91.3	92	92.3	93.2	91.2	%
Crude protein	48	48.1	48.2	48.3	48.4	%
Crude Fat	15	14.77	14.55	14.33	14.1	%
Starch	8.4	7.8	7.5	7.4		
Ash	6.4	6.7	7	7.3	7.4	%
Nitrogen (N)	8.2	8.3	8.3	8.5	8.2	%
Carbon (C)	46.2	46.6	46.4	47.3	46.6	%
Sulphur (S)	0.7	0.6	0.6	0.7	0.6	%
Yttrium (Yt)	0.42	0.37	0.37	0.37	0.37	mg/g
Calcium (Ca)	0.87	1.01	1.17	1.27	1.40	%
Sodium (Na)	0.33	0.32	0.31	0.29	0.27	%
Magnesium (Mg)	0.21	0.21	0.21	0.21	0.20	%
Potassium (K)	1.04	1.03	1.02	1	0.98	%
Phosphorus (P)	1.02	1.03	1.01	1	0.99	%
Fatty acid composition						
C6:0	0.05	0.04	0.04	0.2	0.05	g/kg
C8:0	0.06	0.06	0.06	0.23	0.06	g/kg
C10:0	0.06	0.06	0.06	0.25	0.06	g/kg
C12:0	0.05	0.06	0.06	0.28	0.09	g/kg
C14:0	3.2	3.1	2.9	2.7	2.7	g/kg
C14:1n-7	0.03	0.03	0.03	0.13	0.03	g/kg
C15:0	0.3	0.3	0.3	0.3	0.3	g/kg
C16:0	15	13.6	13.6	12.6	13.7	g/kg
C16:1n-7	3.3	3	2.9	2.6	2.7	g/kg
C17:0	0.2	0.2	0.2	0.3	0.2	g/kg
C18:0	3.3	2.8	2.7	2.7	2.8	g/kg
C18:1n-9t	0.1	0.1	0.1	0.2	0.1	g/kg
18:1n-9c	49.9	38.6	36.9	32.6	34.6	g/kg
C18:2n-6c	22.7	20.4	19.9	17.9	19.4	g/kg
C20:0	0.5	0.5	0.5	0.7	0.5	g/kg
C18:3n-6	0.1	0.2	0.2	0.3	0.1	g/kg
C20:1	5.4	4.8	4.6	4.2	4.3	g/kg
C18:3n-3	5.5	5.7	6.3	6.6	7.7	g/kg
C20:2	2.1	1.9	1.8	1.7	1.7	g/kg
C22:0	0.3	0.2	0.2	0.6	0.4	g/kg
C20:3n-6	0.1	0.1	0.1	0.2	0.1	g/kg
C22:1n-11	6	5.3	5.1	4.6	4.9	g/kg
C22:1n-9	0.6	0.5	0.5	0.6	0.,5	g/kg
C20:3n-3	0.2	0.2	0.2	0.5	0.3	g/kg
C20:4n-6	0.3	0.3	0.2	0.3	0.2	g/kg
C24:0	0.1	0.2	0.2	0.2	0.4	g/kg
C20:5n-3	4.3	3.8	3.6	3.3	3.4	g/kg
C24:1	0.4	0.4	0.3	0.5	0.3	g/kg
C22:5n-3	0.6	0.5	0.5	0.4	0.4	g/kg
C22:6n-3	5.5	4.9	4.7	4.3	4.4	g/kg
Total FA	**130.6**	**112**	**109.1**	**102.9**	**106.5**	**g/kg**

3.4 Sampling, analyses, and data collection

3.4.1 CIELAB – Minolta Colorimetric analysis

For this analysis a Minolta CR-300 model chromameter was used. Scans for determining sample location within CIELAB color space were executed at a singular location on the fish, above the lateral line organ, posterior to the dorsal fin. Scans were only done on the left side of the fish.

The device was held at an angle that facilitated complete contact with skin of the fish relative to the curvature of the body. This was to eliminate other light sources interfering with the Minolta device sensor, which would potentially give misrepresentative readings in the CIELAB color space. The fish were scanned up to multiple times to ensure that the connection between device and skin was satisfactory, as well as ensuring uniformity of measured values on specimen. No additional measures were needed to isolate the samples from the room light pollution in the laboratory due to the disclike shape of the chromameter endpiece, whose function is namely to block out external interfering light sources.

Figure 8: *Minolta CR-300 chromameter console and measuring device utilized in the experiment.*

3.4.2 Feces collection

Fish were sedated with a non-lethal Finquel® vet. tricainmesilat solution after being removed from tanks with a net. Feces was collected by individual stripping of the fish to facilitate emptying of the colon contents. Additional fecal matter was collected routinely in the steel wire grating of the tanks, taking care to separate the feces from uneaten feed. The cumulative fecal matter of each tank was stored in separate containers in a freezer. Each day during the experiment period any occurring feces was added to the assigned containers for future analysis.

3.4.3 Scoring of liver

Ten fish (n = 10) from each tank were subjected to evaluation by liver scoring on the final sampling that occurred between 1st – 2nd June 2023. Scoring of liver was done by applying a discrete color scale for determination of fish liver color. As seen in figure 8, a low score such as 1-2 signifies a pale/yellow color and a high score (4-5) signifies a dark brown color. (Mørkøre *et al.*, 2020)

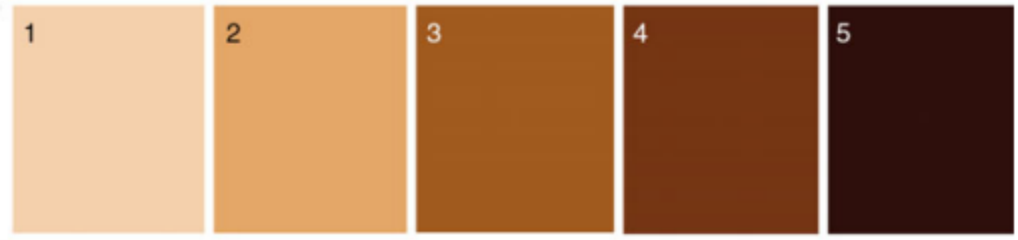


Figure 9: *Liver scoring scale. (Mørkøre et al., 2020)*

3.4.4 Scoring of feces

Ten fish (n = 10) from each tank were subjected to evaluation by faeces scoring on the final sampling that occurred between 1st – 2nd June 2023. Scoring of faeces was done by applying a discrete color scale for determination of faeces score (internal scale, unpublished NMBU). Scale values ranged from 1 to 4. As can be observed in figure 10, a low number in the scoring range implies a poor quality (i.e., lack of solidity) in the fecal material. In turn, a high number (3 or 4) indicates a more solid and consequently better quality of fecal material.

Figure 10: *Feces scoring scale. Source: NMBU unpublished.*

3.4.5 Blood markers

Three fish from each treatment (n = 1 from each tank) was sampled for blood markers. Blood markers CK, AST and ALT were collected via extraction of fish blood on final sampling June 1st – 2nd. Fish were euthanized utilizing a lethal concentration of Finquel® vet. tricainmesilat

(500 mg/L). Followingly, blood was extracted from the caudal vein running along the spinal column of the fish via syringe. Anti-coagulant heparin was added to the samples to avoid potential clotting and undesired interaction between red blood cells and plasma contents. Phase separation of blood cells and plasma was done by centrifugation (4000 g-force for 10 minutes). After achieving phase separation plasma was extracted and pooled together according to tank number. Plasma was stored in designated tubes and kept in a freezer (-25 °C) prior to being delivered to NMBU Central Laboratory (S-Lab) in Ås (Norway), where it was further analyzed using the methodology described by Tietz (1995).

3.5 Welfare assessment methodology

Five fish from each tank (n = 15 per treatment) were examined for external welfare assessment (OWI). Selected specimens were isolated in a neutral background chamber and individually photographed. Assessment of welfare via OWI followed "FISHWELL Morphological Operational Welfare Indicators (OWI's) for farmed Atlantic salmon v1.1" methodology outlined by Noble *et al.*, 2018 (figure 11, see appendix 7.1). All welfare parameters scored on a scale of 0 – 3. Level 0: Little or no evidence of OWI (i.e. normal), level 1: minor, level 2: moderate and Level 3: clear evidence of the OWI (Appendix 7.1) (Noble *et al.*, 2018). Increase in numerical value along the scale thus implies increased severity in relation to fish welfare status. All OWI were measured on final sampling. LABWI including blood plasma analysis was conducted in the posterity of final sampling and termination of experiment.

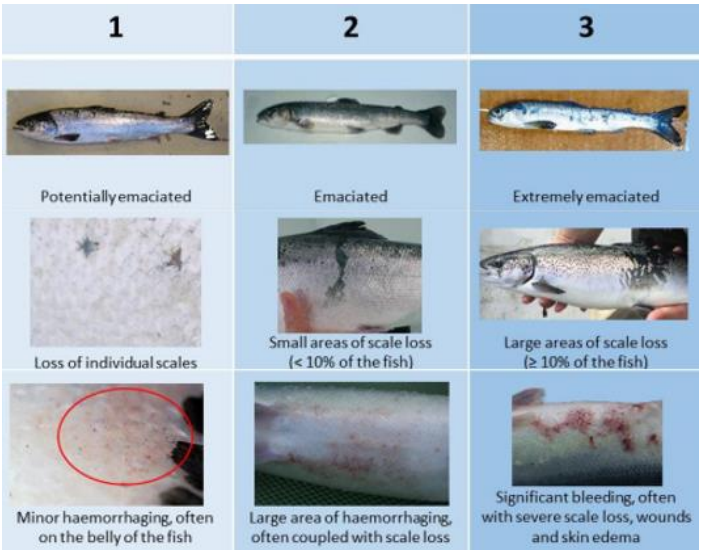


Figure 11: *Excerpt from FISHWELL OWI methodology (appendix 7.1) of the external welfare assessment criteria which were examined in this study. Source: Noble et al., 2018*

3.6 Statistical analysis

Statistical analysis and interpretation of significance was done by utilizing SAS software program (SAS, version 9.4 for Windows, SAS Institute Inc.). All data are listed as mean values with their respective standard errors (SE) unless explicitly stated otherwise. Significance level operated within 5% ($P < 0.05$). One-way ANOVA was used to determine significance between GP inclusion rate (0, 5, 10, 15, 20%) and response parameters. Linear regression was utilized to determine correlation between parameters and GP inclusion rate.

3.7 Calculations

Body weight gain (BWG) is the difference of the fish weigh upon slaughter versus its starting weight. BWG on a tank basis.

$$BWG\ (g) = \ final\ body\ weight\ (g) - \ initial\ body\ weight\ (g)$$

The condition factor (K) expresses the relationship between the fish body weight and length. The CF is therefore an estimate of the voluminousness of the fish.

$$K\ = 100\ \times body\ weight\ \times body\ length\ (cm)^{-3}$$

The specific growth rate (SGR) is a coefficient which expresses the percentage of fish weight gain per day. W0 = initial weight in grams at start of period; Wf = final weight in grams at the end of the period; t(d) = period (number of days) and Ln = the natural logarithm.

$$SGR = \frac{\big(Ln(Wf) - Ln(W0)\big)\ x\ 100}{t[d]}$$

The feed conversion ratio expresses the kilograms of feed material needed to produce one kilogram of fish body weight.

$$FCR = \frac{Feed\ intake\ (g)}{Body\ weight\ gain\ (g)}$$

Final body weight is the weight of the fish at the end of the experiment period and is calculated by adding the BWG to the initial body weight (IBW).

$$FBW = IBW\ (g) + BWG(g)$$

The hepatosomatic index (HSI) expresses how much how the fish body weight is constituted by the liver. The relationship can be expressed as a percentage.

$$HSI = \frac{Liver\ weight\ (g)}{Body\ weight\ (g)}\ x\ 100\%$$

The slaughter yield (SY) expresses how much of the fish results in saleable parts (i.e., after gutting). SY is expressed as a percentage of the body weight.

$$Slaughter\ yield\ = \frac{Gutted\ weight\ (g)}{Body\ weight\ (g)}\ \times 100\%$$

The fillet yield (FY) expresses how much of the body weight the fillet constitutes. FY is expressed as a percentage of the body weight.

$$Filet\ yield\ = \frac{Filet\ weight\ (g)}{Body\ weight\ (g)}\ \times 100\%$$

R^2 (R-squared) is a coefficient of determination, which is a measure of how well a statistical model predicts (i.e., explains) the data occurring within a sample population. R^2 is calculated by dividing the variation between the regression line and the data points (SS_{RES}) over the variation between the mean and the data points (SS_{TOT}), then subtracting this value from 1. R^2 is therefore always a number between 0-1, indicating the explanatory power of the model as a percentage.

$$R^2 = 1 - \frac{SS_{RES}}{SS_{TOT}}$$

4. Results

4.1 Growth and feed conversion ratio

Specific growth rate (SGR) ranged from 2 ± 0.1 to 2.4 ± 0.1, being significantly highest for the Control group and lowest for GP20 (figure 12A). The feed conversion ratio (FCR) showed a more pronounced variation pattern between the dietary treatments, with the Control group (0.75 ± 0.01) being significantly different compared with all other treatments GP5-20 (figure 12B). FCR ranged between 0.75 ± 0.1 to 0.83 ± 0.003, being highest in GP20 and significantly lowest in the Control.

Regression analysis showed a significant negative correlation between GP inclusion rate and SGR (P = .0267) with an R^2 of 86.0%. FCR and GP inclusion rate were not significantly correlated (P = .0663). SGR and FCR showed a significant negative correlation (P = .0197, R^2 = 87.4%).

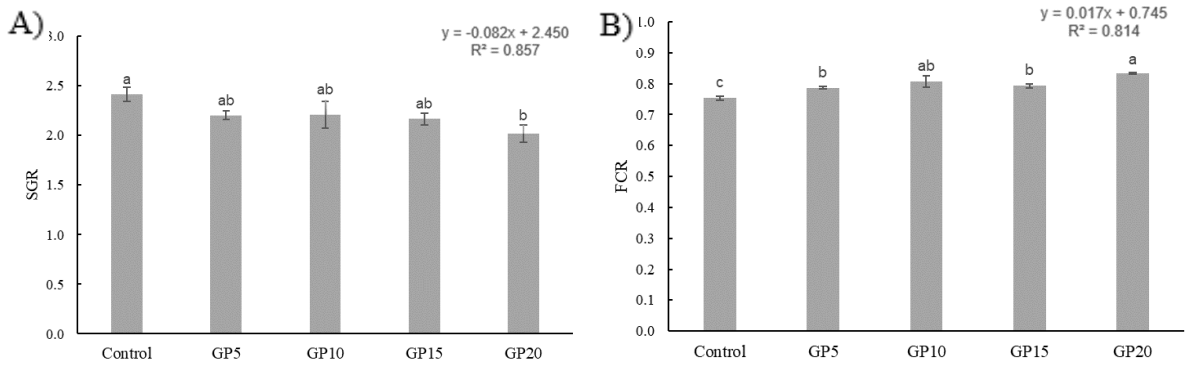


Figure 12: *Bar charts (means ± SE) depicting A) SGR and B) FCR of Atlantic salmon fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Different letters above the error bars indicate significant differences between the dietary groups (P ≤ 0.05).*

4.2 Biometric traits

Final body weight (FBW) ranged from 55.8 ± 0.9g to 66.8 ± 1.3g, being significantly highest for the Control group and lowest for GP20 (figure 13A). The gutted weight (GW) showed a similar, but less pronounced variation pattern between the dietary treatments, with the Control group (53.1 ± 1.6g) being only significantly different compared with the GP20 group (52.1 ± 1.2g) (figure 13B)

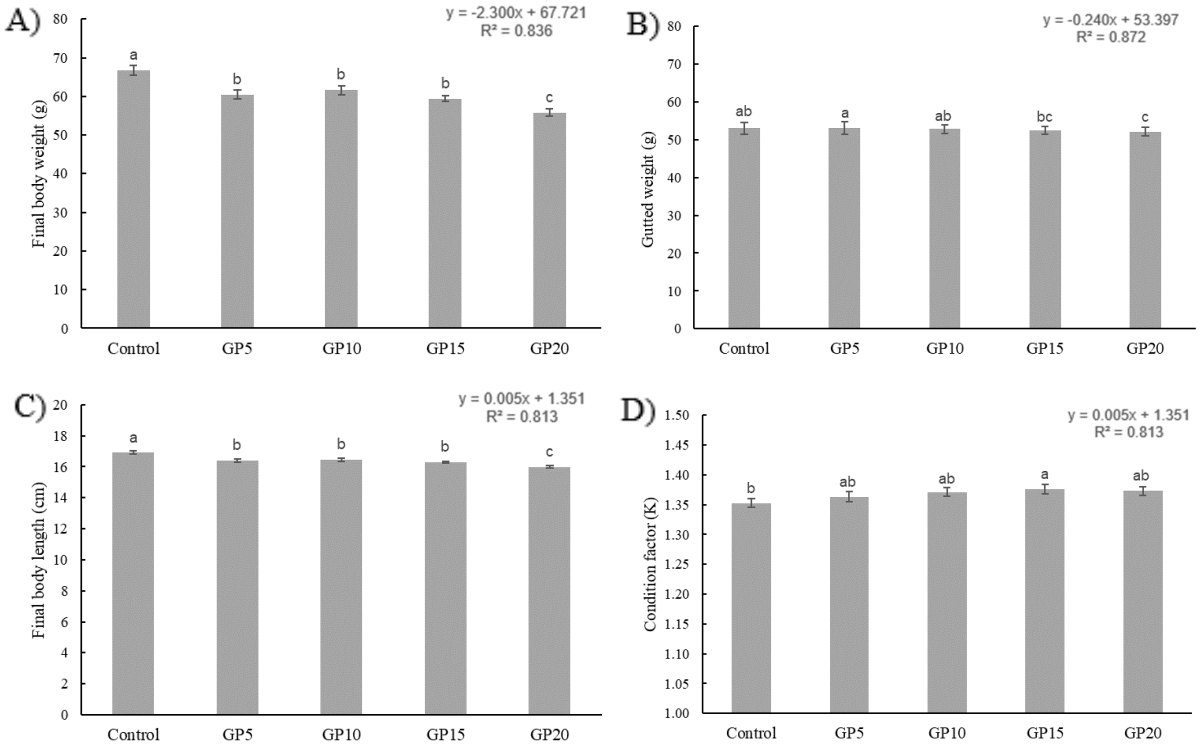
Regression analysis indicated a significant negative correlation between FBW and GP inclusion rate (P = .0292). Regression analysis also showed a significant negative correlation

between GW and GP inclusion rate (P = .0201, R^2 = 87.3%). GW additionally exhibited negative correlations to liver-based parameters HSI (P = .0256, R^2 = 85.1%) and HSI-GW (P = .0447, R^2 = 78.7%).

Final body length (FBL) ranged from 16 ± 0.1 cm to 16.9 ± 0.1 cm, being significantly highest for the Control group and lowest for GP20 (figure 13C). Regression analysis showed a significant negative correlation between FBL and GP inclusion rate (P = .0234) with an R^2 of 86.0%.

Condition factor (CF) showed a less pronounced variation pattern between the dietary treatments, with the Control (1.35 ± 0.01) being only significantly different compared with the GP15 group (1.38 ± 0.01) (figure 13D). Regression analysis indicated no significant correlation between CF and GP inclusion rate (P = .0805).

Slaughter yield ranged from 84 ± 0.5% to 85.8 ± 0.3%, being significantly highest for the GP5 group and lowest for GP20 (figure 13E). GP5 was significantly different from GP15 and GP20 treatments. Regression analysis indicated a significant negative correlation between SY and GP inclusion rate (P = .0217).



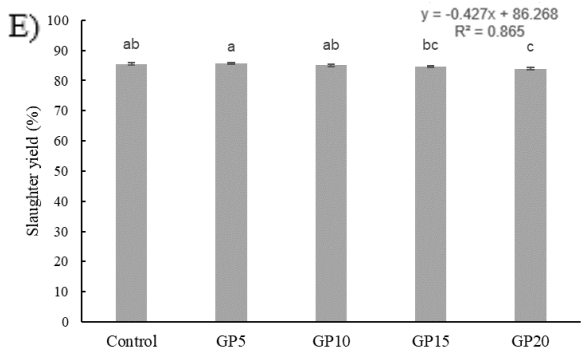


Figure 13: *Bar charts (means ± SE) depicting A) body weight (grams), B) gutted weight (grams), C) body length (cm), D) condition factor and E) slaughter yield (%) of Atlantic salmon fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Different letters above the error bars indicate significant differences between the dietary groups ($P \leq 0.05$).*

4.3 Liver

Liver weight (LW) ranged from 0.65 ± 0.03g to 0.72 ± 0.03g, being significantly highest for the GP20 group and lowest for Control (figure 14A). Regression analysis showed a highly significant positive correlation between LW and GP inclusion rate (P = .003, R^2 = 96.3%). There was also determined a significant negative correlation between FBW and LW (P = .0136, R2 = 90.1%). Additionally, LW and SGR showed a negative correlation (P = .0156, R2= 89.2%). LW and CF exhibited a significant positive correlation (P = .0486, R2 = 77.6%.)

Hepatosomatic index (HSI) ranged from 1.1 ± 0.0 to 1.2 ± 0.0, being significantly highest for the GP20 group and lowest for the Control (figure 14B). HSI calculated based on gutted weight (HSI-GW) showed a similar, but more pronounced variation pattern between the dietary treatments, with the Control group (1.2 ± 0.0) being only significantly different compared with the GP20 group (1.4 ± 0.0) (figure 14C). Regression analysis showed a highly significant positive correlation between HSI and GP inclusion rate (P = .0006) with an R^2 of 98.8%. Regression analysis also determined several highly significant correlations between HSI and biometric and production efficiency traits. HSI showed a significant negative correlation with FBW (P = .0157, R^2 = 89.1%), SGR (P = .0171, R^2 = 88.5%) and SY (P = .0314, R^2 = 83.0%).

Liver color score (LS) showed an inconsistent variation pattern between the dietary treatments, with the GP10 (3.2 ± 0.1g) and GP20 group (3.3 ± 0.1g) being significantly different compared with the GP15 (2.9 ± 0.1g) and GP5 (3 ± 0.0g) groups (figure 14D).

Regression analysis indicated no significant correlation between LS and GP inclusion rate (P = .8893).

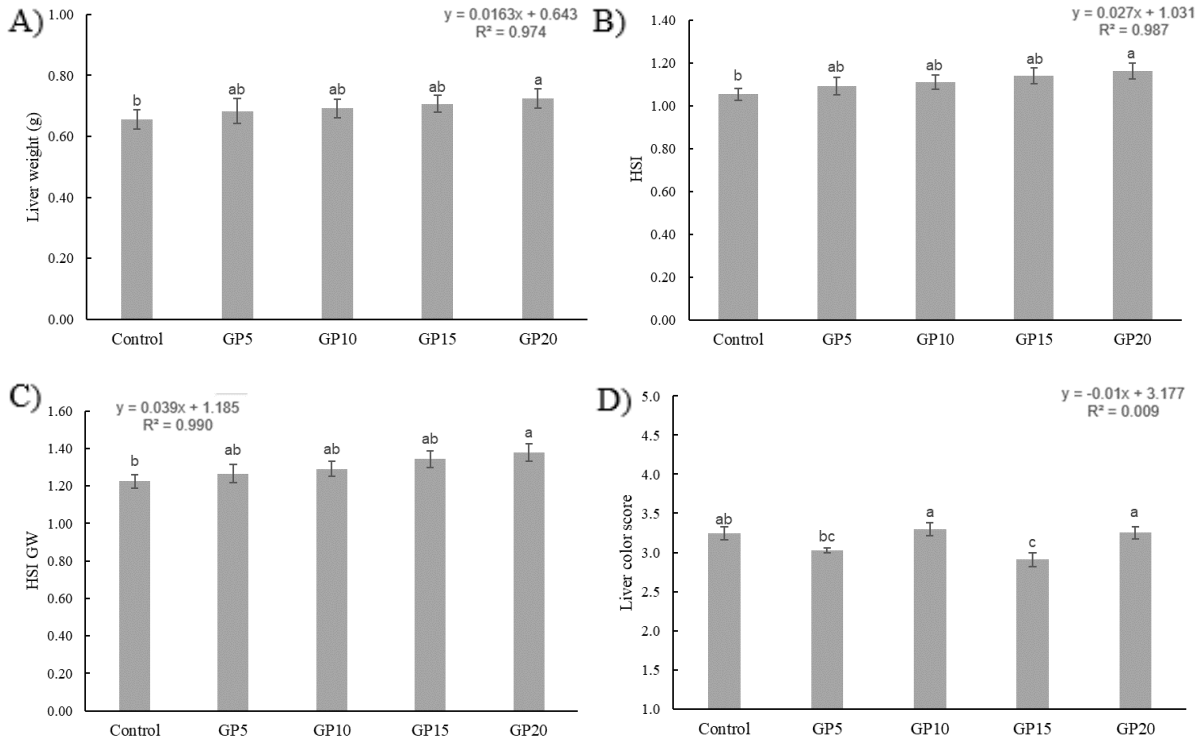


Figure 14: *Bar charts (means ± SE) depicting A) liver weight (grams), B) hepatosomatic index (HIS, % of whole body weight), C) hepatosomatic index gutted weight (HSI-GW, % of gutted weight), and D) liver color score (1 -5) of Atlantic salmon fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Different letters above the error bars indicate significant differences between the dietary groups (P ≤ 0.05).*

4.4 Fish welfare

4.4.1 Blood markers (LABWI)

No significant differences were detected between treatments in relation to CK and AST levels in plasma (P > 0.05) (figure 15 A & B). ALT levels were not listed as ALT plasma values fell below the detection limit (< 7 U/L). CK levels were highest in the Control group (12498.0 ± 3638.5 U/L) and lowest in GP20 (5560.7 ± 1532.9 U/L). AST levels was highest in the Control group (419.0 ± 53.5 U/L) and lowest in GP10 (258.0 ± 97.5 U/L).

Regression analysis indicated no significant correlation between GP inclsuion rate and CK (P = .1414) or AST (P = .4576) levels. There was however detected a significant positive correlation between CK and AST levels (P = .0395) with an R^2 of 80.3%. There was also a significant positive correlation between FBW and CK levels (P = .0398) with an R^2 = 80.2%. In addition, there was also detected a significant positive correlation between CK levels and

FBL (P = .029) with an R^2 of 83.9%. CK levels were also significant when compared with FCR (P = .0471), exhibiting a negative correlation and an R^2 of 78.0%.

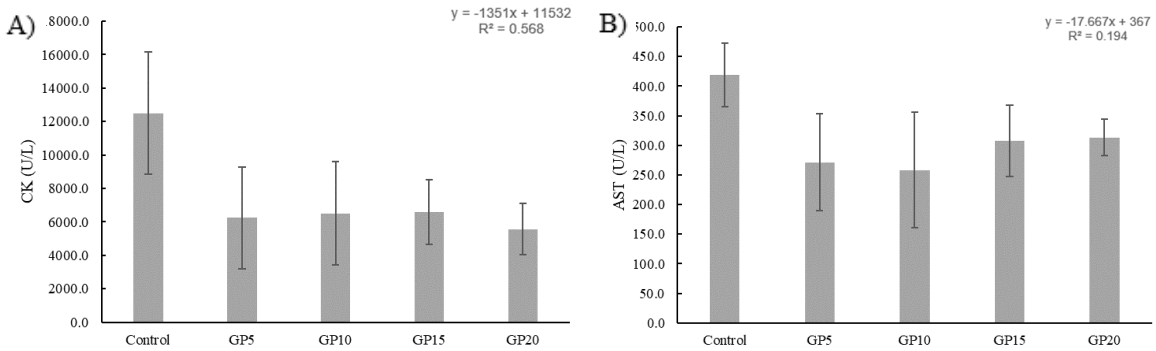


Figure 15: *Bar charts (means ± SE) depicting A) creatine kinase (CK) and B) Aspartate transaminase (AST) in serum of Atlantic salmon fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Both parameters measured in units per liter (U/L). Different letters above the error bars indicate significant differences between the dietary groups (P ≤ 0.05).*

4.4.2 OWI

No significant differences were detected between treatments and OWIs scale loss (SL), skin bleeding (SB) and emaciation state (ES) (P > 0.05). Regression analysis indicated no significant correlations between OWIs and other parameters in the study.

Table 4: *Welfare scoring (means ± SE) according to FISHWELL morphological operational welfare indicators (OWI's) for farmed Atlantic salmon v1.1 (Noble et al., 2018). for Atlantic salmon (S. salar). Fish were fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Different letters above the error bars indicate significant differences between the dietary groups (P ≤ 0.05).*

FISHWELL OWI

Diet	*Scale Loss*	*Skin bleeding*	*Emaciation*
Control	2.23 ± 0.15	0.16 ± 0.09	0.07 ± 0.06
GP5	2.52 ± 0.15	0.01 ± 0.09	0.02 ± 0.06
GP10	2.18 ± 0.15	0.14 ± 0.09	0.09 ± 0.06
GP15	2.34 ± 0.15	0.12 ± 0.09	0.05 ± 0.06
GP20	2.41 ± 0.15	0.17 ± 0.09	0.08 ± 0.06

Figure 16: *Photographs of fish from each treatment, with one representative per tank. 1) Control, 2) 5% GP, 3) 10% GP, 4) 15% GP and 5) 20% GP.*

4.5 Colorimetric analysis

L*-value for skin ranged from 62.2 ± 0.9 to 66.8 ± 1.1, being significantly highest for the Control group and lowest for GP5 (table 5). No significant difference was detected between different GP treatments and skin a*/b*-values.

Table 5: *L*, a* and b*-values (means ± SE) for Atlantic salmon (S. salar) skin measured with Minolta™ CR-300 Chromameter. Fish fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Different superscripted letters indicate significant differences between the dietary groups (P ≤ 0.05).*

	CIELAB Skin		
Diet	***L****	***a****	***b****
Control	66.8 ± 1.1^{a}	-5.5 ± 0.1^{a}	7.5 ± 0.3^{a}
GP5	62.2 ± 0.9^{b}	-5.7 ± 0.2^{a}	8.2 ± 0.5^{a}
GP10	65.1 ± 0.6^{a}	-5.8 ± 0,2^{a}	8.6 ± 0.5^{a}
GP15	62.7 ± 0.7^{b}	-5.6 ± 0,2^{a}	7.9 ± 0.4^{a}
GP20	65.1 ± 0.6^{a}	-5.6 ± 0.2^{a}	8.7 ± 0.5^{a}

L*-value for fillet ranged from 44.5 ± 0.5 to 46.2 ± 0.9, being significantly highest for the GP15 group and lowest for the Control (table 6). Fillet a*-value showed a more pronounced variation pattern between the dietary treatments, with the Control group (2.2 ±

0.2) being significantly different compared with all other treatments (table 6). Fillet b*-value showed a similar, but less pronounced variation pattern between the dietary treatments, with GP10 (7.8 ± 0.4) being significantly different from the Control (5.6 ± 0.3) and the GP5 (6.6 ± 0.3) treatments.

Regression analysis indicated a significant negative correlation between fillet a*-value and GP inclusion rate (P = .0291) with an R^2 of 83.8%. In addition, fillet a*value was determined to have significant positive correlations with parameters FBW (P = .0435, R^2 = 79.1%), FBL (P = .0205, R^2 = 87.1%), SGR (.0282, R^2 = 84.1%) and CK (P = .0425, R^2 = 79.4%).

Fillet a*-values were also determined to have significant negative correlations with CF (P = .086, R^2 = 68.0%), FCR (P = .0082, R^2 = 93.0%). LW (P = .0214, R^2 = 87.0%), HSI (P = .0404, R^2 = 80.0%), HSI-GW (P = .0222, R^2 = 86.4%) and lastly fillet- b*-value (P = .0413, R^2 = 80.0%). No correlation was detected between either fillet L*-value and b*-value in relation to GP inclusion rate.

Table 6: *L*, a* and b*-values (means ± SE) for Atlantic salmon (S. salar) fillet measured with Minolta™ CR-300 Chromameter. Fish were fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Different superscripted letters indicate significant differences between the dietary groups (P ≤ 0.05).*

	CIELAB Fillet		
Diet	***L****	***a****	***b****
Control	44.5 ± 0.5^{b}	2.2 ± 0.2^{a}	5.6 ± 0.3^{c}
GP5	45.1 ± 0.3^{ab}	1.2 ± 0.3^{b}	6.6 ± 0.3^{b}
GP10	45.6 ± 0.4^{ab}	0.4 ± 0.3^{c}	7.8 ± 0.4^{a}
GP15	46.2 ± 0.9^{a}	0.7 ± 0.3^{bc}	7.8 ± 0.4^{a}
GP20	44.9 ± 0.6^{ab}	0.1 ± 0.19^{c}	7.3 ± 0.3^{ab}

4.6 Faeces

Faeces score (FS) ranged from 2.9 ± 0.1 to 3.8 ± 0.1g, being significantly highest for the Control and GP5 treatment, and lowest for GP15 (figure 17). Control (3.8 ± 0.1) and GP5 (3.8 ± 0.1) differed significantly from GP10 (3.2 ± 0.1), GP15 (2.9 ± 0.1) and GP20 (3.1 ± 0.1) treatments. Regression analysis indicated that there was no significant correlation

between FS and GP inclusion rate (P = .0525, R^2 = 76.4%). FS and CF was however determined to be significantly negatively correlated (P = .0182) with an R^2 of 88.0%.

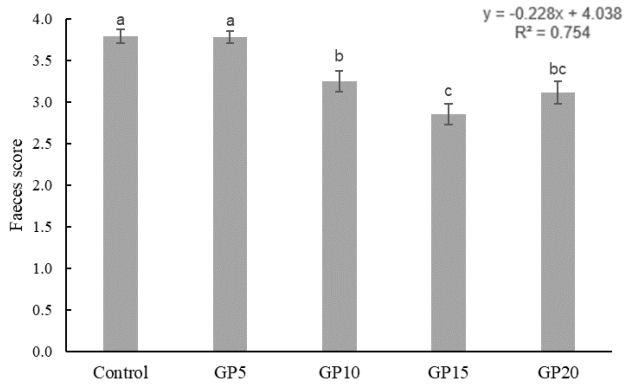


Figure 17: *Bar charts (means ± SE) depicting faeces score of Atlantic salmon fed either a control feed or the same feed with increasing inclusion of grass protein for 6.5 weeks. Different letters above the error bars indicate significant differences between the dietary groups (P ≤ 0.05).*

5. Discussion

5.1 Growth and FCR

Responsible and sustainable aquaculture practices aim to optimize growth while ensuring the overall health and well-being of the fish population. In the present study SGR was used to calculate the growth, being in the expected range for all dietary treatment groups (2-2.4%$^{-1}$) following Austreng *et al.* (1987).

Regression analysis confirmed that a negative correlation exists between the weight gain per day (SGR) and increasing GP inclusion rate. This indicates that higher inclusion rates such as in GP20 lead to poorer growth over time. The duration of the experiment was relatively short, and the observed SGR trend may even out over a longer study. It is important to note that most measurements were close numerically. While ANOVA and regression analyses showed significant difference between dietary groups there was little difference observed subjectively between fish size in each treatment (i.e., little or no runts/ loser fish).

Abd El-Hakim *et al.*, (2009) for instance found that there was no significant difference in SGR between a dietary substitution level of 25% alfalfa grass protein when compared to the soybean meal control for nile tilapia (*O. niloticus*). There is however a considerable difference in digestive anatomy between salmonids and tilapia, which is perhaps why the tilapia tolerated greater levels of GP. Salmon lacks the capacity to digest carbohydrates efficiently due to their low amylase activity (Hemre, 2001). This may indicate

that there are some fibrous compounds left in the GP even after processing. Further processing of GP is probably needed to be suitable as salmon feed commercially. Research conducted by Samac *et al.* (2019) found that diets with 3 – 6% fish meal protein substitution with alfalfa GP did not lead to any significant differences in SGR between control and GP diets for rainbow trout. These findings are perhaps more comparable due to the relatedness of trout and salmon, though fish meal was used as the main protein source and not soy.

While ANOVA established that FCR was significantly higher between diet groups regarding GP inclusion rate, regression analysis showed that this trend was outside the area of significance (P = .0663). This a promising aspect of GP inclusion and may indicate that GP is competitive with SPC as an additional feed ingredient. It is however likely that given a longer duration the data would be fall within significance, given the close relationship between SGR and FCR and their explanatory power (R^2 = 87.4%). This may suggest that even low inclusion rates of GP will lead to higher feed demands to attain the desired growth. This could potentially increase feed costs and prolong production cycles, which can be seen as a potential negative aspect of GP utilization. It is however too early to render a complete judgment on the efficiency of GP and longer trial periods are consequently recommended. Residual fiber mass in the GP concentrate in conjunction with salmonids lack of ability to break down more complex carbohydrates may be the cause of this trend in the results. Further breakdown of the GP before feeding may be required for optimal nutrient uptake by juvenile salmon.

5.2 Biometric traits

5.2.1 Final body weight

All GP treatments (GP5-20) led to significantly lower final bodyweights when compared to the Control group. This trend reached its lowest measurement in the GP20 diet which had the highest GP inclusion rate. This can be inferred from figure 12A, where there is a clear downward inclination in the relationship between GP inclusion rate and final bodyweight. Small standard error ranges relative to the fish weights measured indicate little variance within tank populations and legitimacy of results. Linear regression analysis confirmed this trend and established a negative correlation between FBW and GP inclusion rate. This was also true for FBW and liver-based parameters LW, HSI and HSI-GW, which strengthens the evidence that increasing GP inclusion leads to higher weight gain in liver and lower overall FBW.

While the results indicate that GP inclusion is not optimal for the body weight gain of juvenile salmonids, it may not necessarily be the case in the later stages of their development when the fish has become more robust. In addition, there were no recorded mortalities during the experiment, which can be seen as a promising aspect. The experiment was however conducted over a relatively short time span, and so it will be of interest in future studies to subject the fish to GP diets over longer periods of time. For comparison; Samac *et al.* 2019 did a similar GP experiment for yellow perch (*Perca flavescens*) and rainbow trout (*Oncorhynchus mykiss*) over a 12-week period with promising results. While the perch is not relevant to our target species, the trout is a close relative of the salmon and is therefore more comparable. Samac *et al.* found no significant difference in growth performance or fillet composition in addition to a comparable FCR when fish meal protein was substituted by 3 and 6 % GP in feed formulations for juvenile rainbow trout. This contrasts the current experiment where a 5% inclusion significantly altered final body weight negatively. It would however be interesting to see how the rainbow trout had responded to inclusion rates like 10 and 15%, considering that in our study the final body weight of GP5 (60.5 ± 1.1g), GP10 (61.6 ± 1.2g) and GP15 (59.4 ± 0.8 g) were all measured within approximately 1 – 2 grams of each other respectively. This is also indicative of a lack of runts between the dietary treatments, and that while there are some negative correlations statistically, there is little observed difference in fish size.

5.2.2 Gutted weight

Measurements of GW showed significant differences between 5% inclusion and 15-20% (figure 11B). Most of the data were clustered together in a relatively small range. The highest measurement represented by GP5 (53.1 ± 1.6g) and the lowest by GP20 (52.1 ± 1.2g) were only differentiated by a single gram, with the residual diet groups somewhere in between these measurements. Gutted weight SE across the treatments was relatively small considering the weight range and is implicative of little variation in GW between individual fish subjected to the different treatments. It would be of interest to see whether the GW results would be as closely grouped numerically if subjected to a longer experiment duration.

Despite promising results for low GP% inclusion, linear regression analysis determined a negative correlation between GP inclusion and GW. The lower GW of higher GP inclusion rates may be an indicative that feed of this composition is being converted into weight gain in non-desired parts such as organs and visceral fat. This seems plausible when

considering the significant negative correlation between GW and HSI, which establishes that HSI increases as GW decreases as seen in the higher inclusion treatments.

5.2.3 Final body length

Fish given the control (16.9 ± 0.1 cm) was found to have significantly higher fish body length than all other diet groups with GP20 attaining the significantly lowest fish length (16 ± 0,1 cm) (figure 11C). Fish body length was detected to be significantly lower at a modest 5% inclusion of GP (16,4 ± 0,1 cm), with 10-15% inclusion showing similar results. Very small range of SE implies relatively uniform size distributions within tank populations as regards final body length.

The results of fish body length correspond with those of final body weight and strengthens the evidence for a negative correlation between increasing GP inclusion rate and growth. This was confirmed by linear regression analysis, which showed a significant correlation between increasing GP% and decrease in FBL with approximately 86% of the variance between data points explained by this relation. Much like the preceding biometric traits, FBL showed strong correlation between length and all liver-based parameters, excepting LS which was only significantly correlated with CF.

5.2.4 Condition factor

CF values between treatments were found to differ significantly between Control and GP15 in ANOVA analysis. The lowest CF was represented by the control and the apex by the GP15 diet. While it is qualitatively is hard to observe even minor differences regarding leanness (or fattiness) between fish subjected to the different diet regimes, the results quantify these differences for us. The linear regression model found no significant correlation between CF and GP inclusion rate, though the correlation coefficient indicated a positive relationship between the two. This result is worth considering due to the relatively short time span the fish had to adapt to the diet before eventual slaughter. We can look back to the length parameter and see that even though the fish are getting shorter as GP levels rise, they are however not getting comparatively thinner (i.e., CF and length did not correlate significantly). CF was found to positively correlate with LW significantly, thus indicating that some of the observed increase in CF comes from increased weight gain in liver. CF was however found to correlate negatively with FS in a significant way, indicating that the fattier GP fed fish had the poorer faecal matter.

Like many other parameters in the study, it would be interesting to see how the CF values would be quantified after a longer experiment duration and whether the trend continues in the same manner.

5.2.5 Slaughter yield

ANOVA analysis determined SY to be significantly lower in the highest inclusion group (GP20) when compared to lower inclusion rates like GP5, which attained the significantly highest SY. This reflects the results of the GW and was therefore expected since the most saleable part of the fish is produced after gutting. The Control group and GP10 shared similar features with the GP5 diet, but only differed significantly from the highest GP inclusion rate of 20% (GP20). The differences between treatments indicate that low-GP inclusion treatments produced the most saleable material (i.e., muscle). It is however interesting that there were no significant differences detected between treatments regarding fillet weight. This is a very important observation in the study as the fillet is the most crucial part of the fish as regards production efficiency.

The linear regression model confirmed the observed differences between treatments and established a significant negative correlation between SY and GP inclusion rate. This indicates that increasing GP inclusion rate reduces the saleable material after gutting. No significant correlation detected between fillet weight and GP inclusion rate, though the correlation coefficient indicated a negative trend.

That GW and SY initially showed the GP5 group as significantly highest may indicate a positive growth stimulus response when including small amounts of GP in the feed composition. It does however seem that there is a threshold around 5-10% before GP inclusion starts affecting production efficiency parameters negatively. The negative correlation between SY and HSI is to be expected, as liver counts as cut-off and therefore results in lower amounts of saleable material.

5.3 Liver

5.3.1 Liver weight and hepatosomatic indices

In the present study ANOVA analysis determined a significant difference between the Control and the GP20. While initially perceived as a subtle difference numerically, this represents an increase of approximately 10.8 % in LW between 0 – 20 % inclusion. The middle diets of GP5-15 ranged somewhere in between these extremities and were not

significantly different from each other. The linear regression model revealed a highly significant correlation between LW and GP inclusion rate, indicating that the significance observed in ANOVA analysis is valid and that GP inclusion leads to LW gain. With an R^2 of 96.3% the correlation between LW/GP has a high explanatory power for the observed differences in the data. Regression also revealed a negative correlation between FBW and LW (P = .0136), as well as SGR and LW (P = .0156). This strengthens the evidence for low (or 0%) level inclusion inhabiting the better production efficiency (i.e., desired weight gain) compared to > 5% GP inclusion treatments.

ANOVA analysis determined significant difference in HSI between 0% and 20% GP inclusion in diet, following much the same variation pattern as LW. None of this is surprising as the HSI is merely an index value explaining the fraction that the liver constitutes of the total body weight. HSI-GW also showed the same variation pattern in ANOVA, albeit more pronounced. This is expected as the liver weight will account for more when the weight of the other organ tissues is removed from the calculation. Linear regression model confirmed a highly significant (P = .0006) positive correlation for GP/HSI indicating that increase in GP results in increased HSI over time. The GP/HSI relationship accounts for 98.8% (R^2 = 0.9877) of the variation observed between the data. Predictably, HSI and SY were found to be negatively correlated (P =.0314) and is logical considering higher HSI indicate more organ tissue and thus more cut offs. ANOVA and linear regression analyses thus coincide for HSI related parameters and strengthen the evidence for GP inclusion leading to liver weight gain.

What caused the increased liver weight is subject to debate. Increased liver weight and has previously been associated with high fat diets fed to Atlantic cod (*G. morhua*) but does not translate well to salmon who deposits a greater percentage of lipids in musculature (Kjær et al., 2009). It may be more likely that increased LW and HSI is due to a lack of limiting amino acids such as methionine (Espe *et al.* 2010). In the study conducted by Espe *et al.*, (2010) salmon fed a low-level methionine diet had increased liver size relative to bodyweight as well as increased activity of fatty acid synthase. GP does however have slightly more methionine than SPC (see table 1) and methionine levels should therefore be adequate in the present study.

5.3.2 Liver scoring

Results from liver scoring fluctuated between diet groups making it somewhat difficult to interpret any trend by way of ANOVA. It was however detected a significant difference between GP10 and GP20 compared with the GP5 and GP15 treatments (figure 13D). When

comparing these numbers to the liver scoring scale all values center around the middle score of 3, implying a brownish liver color for all diets on average. This indicates that while there is some numerical fluctuation and statistically significant differences between treatments, their end liver score is largely the same. Liver color is thus qualitatively observed as brown. Linear regression model showed no significant correlations between LS and all other parameters measured in the study. This indicates that GP inclusion has no effect upon LS, an interesting observation considering the tight relationship between GP and liver weight gain.

5.4 Fish welfare

No mortalities occurred during the experiment. While no significant differences were detected between treatments regarding operational welfare indicators in ANOVA analysis, there was still observed a relatively high scoring for scale loss across all treatments. Scale loss was most visible on fish from the GP20 group (see figure 14). Juvenile salmon and scale loss is however a common relation, as the fish starts moving from the parr stage into the smoltification phase and prepares for life in the ocean (Noble *et al.,* 2018). Welfare scoring of skin bleeding and emaciation state revealed very low scores (table 2) and was not prevalent across all treatments. Linear regression revealed no significant correlation between external OWIs and all other parameters tested for in the study. The results indicate that GP inclusion does not significantly affect scale loss, skin bleeding or emaciation state of juvenile Atlantic salmon.

ANOVA determined a lack of significance between treatments CK/AST levels. While CK levels in the Control group was numerically much higher than other groups, large SE ranges made it hard to interpret any definitive trend. Linear regression model determined no significant correlation between GP inclusion rate and CK/AST levels in plasma. CK and AST did however exhibit a positive correlation (P = .0395) with each other, indicating a conjunctive rise in salmon CK/AST plasma levels. This may be because they are located in many of the same tissues (Aulbach & Amuzie, 2017; Rojas *et al.*, 2018). CK/FBW was additionally determined to be significantly positively correlated, as was the case with CK/FBL. This may be because of larger individuals having more units per liter of CK in plasma due to their relative size difference. CK/FCR exhibited a negative correlation (P = .0471), which perhaps strengthens the explanation behind CK/FBW relationship.

5.5 Colorimetry – CIELAB results

5.5.1 L*-value (lightness)

L*-value measurements on fish skin was relatively inconclusive. As can be inferred from table 2, ANOVA analysis showed different treatments alternated in significant difference. This led to the control, GP10 and GP20 being significantly different compared with GP5 and GP15. This makes it difficult to say anything definitive about the effect of the feed on the luminosity of pigmentation of the fish. For instance, 0% GP and 20% GP are found to not differ from each other statistically, and if there was an expected difference to occur it was between these diets. Linear regression model seems to confirm this lack of cohesiveness in results for L*-skin value, showing no significant correlation with any of the measured parameters in the study.

There are two main hypotheses behind the results; 1) inaccuracy in the measurements, or 2) GP has little to no effect on skin luminosity. In relation to 1), other studies such as Rosenau *et al.* (2022) measured two more locations for CIELAB values (anterior to dorsal fin and by the adipose fin) in addition to where it was approximately measured in the present study (posterior to dorsal fin and above lateral line). The same approximate locations were used for fillet colorimetry. The average of three locations is likely to produce more stable values per fish. In the same study spirulina did not however have any significant effect on skin colorimetry of trout. This indicates that muscle pigmentation is more easily affected by novel feed ingredients than skin. The cause of the results in the present study may very well be due to a combination of 1) and 2). It may also be because of the scale loss detected in the OWI welfare assessment, leading to perhaps more silvery skin on some fish than others. In the study we also only measured CIELAB values on the left side of the fish, which may have contributed to the fluctuations in L*-value.

L*-value ANOVA results from salmon fillet was more easily interpreted. The control was found to have the significantly lowest L*-value, indicating the darkest fillet among the treatments. In contrast the significantly highest L*value and consequently the lightest fillet was found in the GP15 diet. Linear regression model showed significant positive correlation between fillet L*-value and CF (P = .0503). This barely qualifies but may indicate that fattier fish with higher CF have lighter fillets. The fish with the highest CF in this study was those fed higher GP inclusion rates, and thus may be indicative that these fish have lighter fillets. L*value for fillet exhibited no other significant correlations with parameters measured in the study.

Rosenau *et al.* 2022 found no detectable difference in luminosity in dietary groups fed spirulina (*A. platensis*), while fillet colorimetric characteristics changed significantly. As suggested before, this may be indicative that it is harder to affect skin pigmentation than fillet color when feeding plant-based ingredients (Schafberg *et al.*, 2020; Teimouri *et al.*, 2013).

5.5.2 a*-value (red/green)

No significant change in skin a*-value detected between treatments in ANOVA analysis. GP inclusion seemingly does not lead to any shift in skin pigmentation in either red (+) or green (-) direction of the CIELAB color space. Measurements averaged -5.6 ± 0.2 across the different treatments, indicating juvenile salmonid skin color is inherently located in the green part of the color space. Linear regression model found no correlation between skin a*-value and GP inclusion rate, confirming what is observed across one-way ANOVA.

Linear regression model showed a negative correlation between increasing GP inclusion rate and fillet a*-value (P = .0291). This coincides with the finds done by ANOVA analysis, wherein the control had the significantly highest a*-value and differed significantly from all other treatments, with the highest inclusion rate being lowest. This is an interesting find as it shows that fillet color is moving from the red part of the CIELAB color space towards the green part by inclusion of GP in the feed. This can be labelled as a non-desired trait of GP utilization in salmon feeds, considering that a large part of salmonid marketability comes from its distinct red/pink fillet color. Similar colorimetric changes were recorded by Rosenau *et al.* (2022) and Skalli *et al.* (2020), albeit with a yellow color shift in fillets for different salmonid species when subjected to dietary treatments containing two different species of green microalgae. This type of colorimetric change can perhaps be expected somewhat due to alfalfa and green microalgae both inhabit chlorophyll which are green pigments.

Fillet a*-value showed positive correlations with production efficiency parameters, which is to be expected since the treatments with the biggest growth exhibited the highest fillet (+) a*-values and are thus located more towards the red part of the CIELAB color space. Fillet a*-values also showed negative correlations with CF, FCR and liver-based parameters, which is predictable due to lower FCR and CF being associated with the lower inclusion diets. Additionally, fillet a*-value and b*-value were negatively correlated. This can be interpreted as greenness and yellowness being correlated, i.e, that low fillet a*-value coincides with higher fillet b*value and vice versa (see table 4).

5.5.3 b*-value (yellow/blue)

Like a*-value measurements, there was not found any significant difference between treatments in ANOVA analysis for b*-skin. Linear regression confirmed that GP inclusion rate and b*-value for salmon skin had no relation.

ANOVA analysis of b*-value in fillet revealed that GP inclusion led to an increase of yellowness. This initially indicates that there is a positive correlation between GP inclusion rate and yellow color deposition (i.e., increase in b*-value) in the salmon fillets. This is similar to the findings of Rosenau *et al.* (2022). Linear regression model rejects this proposed relationship and finds no significant correlation between GP inclusion rate and b*-fillet value. It is not however unlikely that there is some relation between these when considering the results of Skalli *et al.* (2020) and Rosenau *et al.* (2022). Yellow is a color that is often associated with rancidity in fish fillets and particularly in the lipid-rich salmon fillet. It is therefore a good thing that there is no significant connection between GP utilization and yellow coloration.

5.6 Faeces scoring

ANOVA analysis showed showed significant differences between the lowest inclusion rates of the Control and GP5 when compared to the residual treatments (GP10-20) FS. Based on ANOVA results any inclusion degree beyond 5% (GP5) had a detrimental effect on the quality of fish fecal matter. The control and GP5 shared near identical feces scores of approximately 3.8, which is equivalent to a near solid feces or alternatively in the upper range of semi-solid (see figure 14). This is indicative that a low-level GP inclusion such as 5% does not adversely affect fish gut health and faeces formation in the colon. The SE of the GP5 and control groups was relatively low and comparable between the two treatments. This indicates a high degree of uniformity in faeces score between individual fish in the tanks subjected to the control and GP5 diets. At 10% inclusion significant reduction in solidity was observed, dropping an approximate 0.5 point on the faeces scoring scale. Here the SE also had a relatively low range indicating uniformity in the population subjected to 10% GP inclusion. The significant reduction in faeces solidity that occurred between 5-10% inclusion is indicative of a critical threshold existing somewhere between these percentages. If that threshold is exceeded (as in GP10) it is highly probable it will result in adverse effects on gut health and problems with regulating the solidity of faeces in the colon. This is what is observed in consequent treatments and FS (see figure 14), of which solidity of faeces dropped

from semi-solid to more liquid consistency. Reid *et al.* (2024) discovered that time elapsed from feeding affected Atlantic salmon gut microbiota and consequently faecal score. This factor in combination with the adaptation to the grass protein may have contributed to the observed results.

Linear regression determined that FS and GP inclusion rate had a significant negative correlation ($P = .05$, $R^2 = 76.4\%$). This strengthens the observations outlined above and indicates that FS decreases upon increasing GP. FS showed a negative correlation with CF, indicating poorer faecal quality for higher GP inclusion diets which coincidentally had the highest CF as well. It is probable that there is something in GP composition that is harder for salmonids to break down completely without additional processing beforehand. This may be as suggested earlier some type of insoluble fiber or complex carbohydrate. It is well established that soy-based products can lead to detrimental effects on gut health for salmonids (Gajardo, 2016; Krogdahl *et al.*, 2015). It is however noteworthy that lower inclusion (or no inclusion) does not show adverse effects on faecal matter. This may indicate that there is something that irritates the gut in GP diets, in addition to the residual ANFs in the SPC of the feed.

6. Conclusion

The present study found that GP from alfalfa showed promising results in some respects, while highlighting difficulties in others. GP inclusion rate was shown to alter several production efficiency parameters significantly. In some parameters it was adequate with a low-level inclusion of 5% to generate significant differences between groups, while in others it required a high-level inclusion of 20%. Regression analysis confirmed the negative correlation between production efficiency parameters and GP inclusion rate. On the strength of these observations, we reject H_1.

Regarding welfare, no external observations indicated detrimental developments from consuming GP feed. Internal molecules were not detected in imbalanced proportions, indicating that the fish was able to maintain homeostasis when fed GP included feed. Scale loss was normal relative to developmental stage. Linear regression model confirmed the lack of significance between GP inclusion and all welfare parameters. On the strength of these observations, we accept H_2.

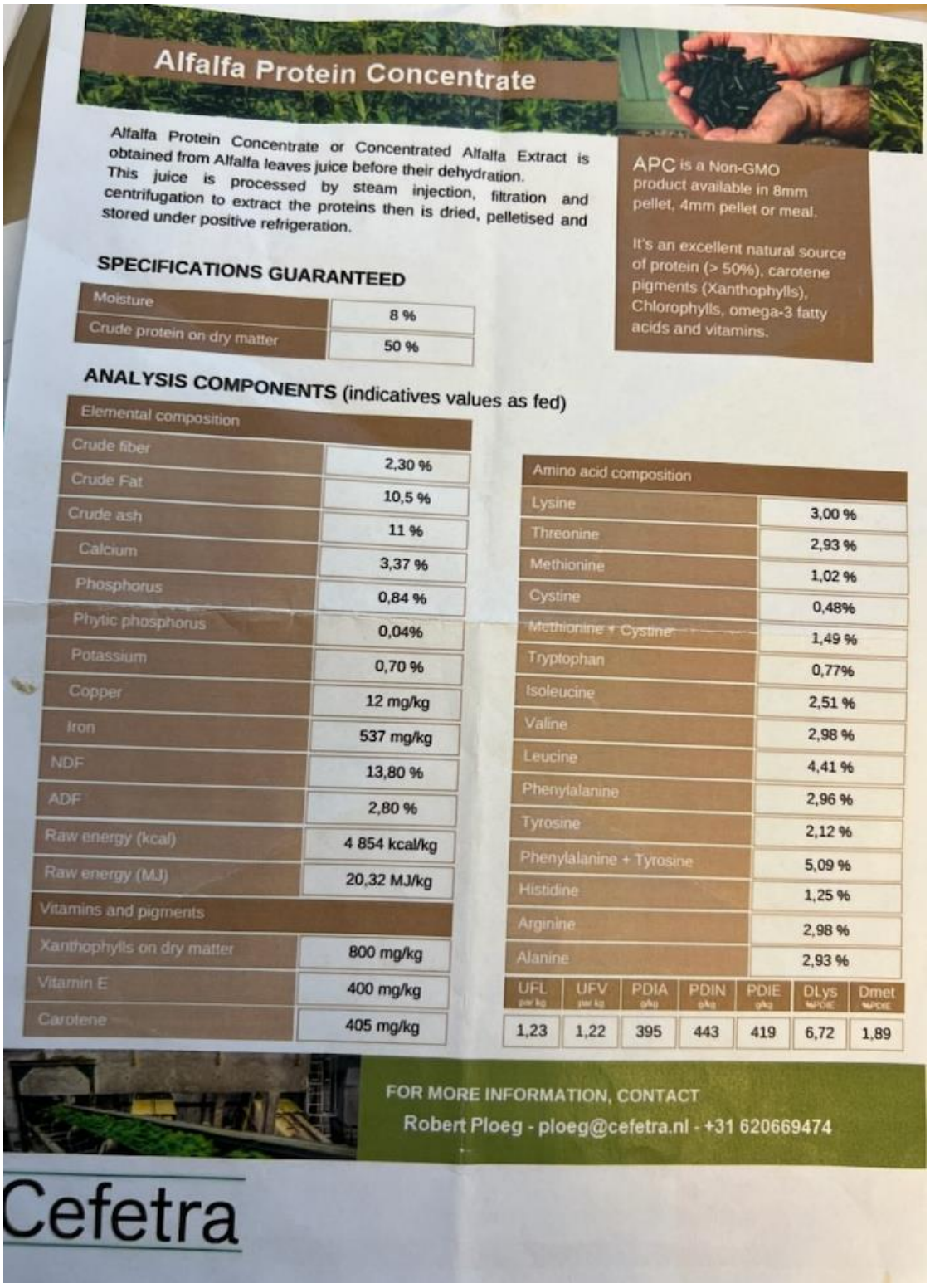
In measurements of juvenile salmon colorimetric characteristics there was relatively inconclusive results as regards luminosity of skin. Luminosity of fillet was however higher in diets fed increased GP. Fillet a*value was also significantly lower in higher GP treatments which correlated with increased b*value (i.e., yellowness). This leads us to reject H_3 as color was significantly affected by introducing GP in the feed.

Time was an important factor in the present study, occurring in a span of 45 days (6.5 weeks). This is a relatively short time for the juvenile salmon to adapt to the feed. It is of interest to conduct experiments on grass protein with a longer duration to verify the presently observed trends in the study.

7. Appendix

7.1 FISHWELL Welfare indicators

7. 2 Cefetra APC content sheet



Alfalfa Protein Concentrate

Alfalfa Protein Concentrate or Concentrated Alfalfa Extract is obtained from Alfalfa leaves juice before their dehydration. This juice is processed by steam injection, filtration and centrifugation to extract the proteins then is dried, pelletised and stored under positive refrigeration.

APC is a Non-GMO product available in 8mm pellet, 4mm pellet or meal.

It's an excellent natural source of protein (> 50%), carotene pigments (Xanthophylls), Chlorophylls, omega-3 fatty acids and vitamins.

SPECIFICATIONS GUARANTEED

Moisture	8 %
Crude protein on dry matter	50 %

ANALYSIS COMPONENTS (indicatives values as fed)

Elemental composition	
Crude fiber	2,30 %
Crude Fat	10,5 %
Crude ash	11 %
Calcium	3,37 %
Phosphorus	0,84 %
Phytic phosphorus	0,04%
Potassium	0,70 %
Copper	12 mg/kg
Iron	537 mg/kg
NDF	13,80 %
ADF	2,80 %
Raw energy (kcal)	4 854 kcal/kg
Raw energy (MJ)	20,32 MJ/kg
Vitamins and pigments	
Xanthophylls on dry matter	800 mg/kg
Vitamin E	400 mg/kg
Carotene	405 mg/kg

Amino acid composition	
Lysine	3,00 %
Threonine	2,93 %
Methionine	1,02 %
Cystine	0,48%
Methionine + Cystine	1,49 %
Tryptophan	0,77%
Isoleucine	2,51 %
Valine	2,98 %
Leucine	4,41 %
Phenylalanine	2,96 %
Tyrosine	2,12 %
Phenylalanine + Tyrosine	5,09 %
Histidine	1,25 %
Arginine	2,98 %
Alanine	2,93 %

UFL	UFV	PDIA	PDIN	PDIE	DLys	Dmet
1,23	1,22	395	443	419	6,72	1,89

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Cefetra

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