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Assessing the potential of *Aurantiochytrium limacinum* as a novel source of DHA for pre-smolt Atlantic salmon (Salmo salar): evaluating its impact on fish performance, nutrient digestibility, nutrient retention, and utilization.

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Master of Science in Aquaculture

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List of abbreviations

A. limacinum *Aurantiochytrium limacinum*

ADCs Apparent digestibility coefficients

ALA Alpha linolenic acid

ANOVA Analysis of variance

ANFs Anti-Nutritional Factors

BSFL Black soldier fly larvae

DHA Docosahexaenoic acid

EPA Eicosapentaenoic acid

FAO Food and Agricultural Organization

FCR Feed conversion Ratio

FI Feed intake

FO Fish oil

GHG Green House gases

IPN Infectious Pancreatic Necrosis

LCA Life Cycle Assessment

PUFA Polyunsaturated fatty acid

R&Ds Research and Developments

RO Rapeseed oil

SGR Specific growth rate

WSI Water stability index

Abstract

Docosahexaenoic acid (DHA) is essential for the normal growth of Atlantic salmon, with fish oil (FO) being the primary source. The expanding aquaculture industry increases pressure on wild fish stocks, necessitating novel and sustainable DHA sources. *Aurantiochytrium limacinum*, a microbial organism, holds potential as an alternative DHA source for aquaculture. This study aims to assess *A. limacinum* potential as a novel DHA source for Atlantic salmon, considering factors such as fish performance, nutrient digestibility, nutrient retention, and utilization. Pre-smolt Atlantic salmon were fed one of four experimental diets for nine weeks. Diet 1(control) was a commercial-like diet based on fish meal and soy protein concentrate as main protein source, and FO and rapeseed oil as fat sources. Diets 2-4 *included A. limacinum* at 5%, 10%, and 15%, respectively, with equal FO to meet DHA requirements. No differences in feed intake were observed across dietary groups. However, fish fed the highest inclusion level of *A. limacinum* (15%) showed reduced growth performance. Apparent digestibility of total fat and DHA also decreased in fish fed higher levels of *A. limacinum*. Despite these results, the study using cold-pelleted diets provides a foundation for future research on novel DHA sources. The findings suggest that moderate inclusion levels of *A. limacinum* can support sustainability in aquaculture. Further optimization of its production and processing could enable higher FO replacement levels as a DHA source for Atlantic salmon.

CHAPTER 1: Introduction

1.1 Aquaculture's Modern and Global Development

The global development of aquaculture has become more relevant in the context of a growing global population, as highlighted by the predicted growth in this sector from 7.4 billion in 2017 to 9.7 billion in 2050 according to the United Nations (Arora et al, 2019). The pressing question of how to meet the food demands of this expanding population has gained prominence, especially since the 2008 food price surge (FAO, 2009). Aquaculture plays a significant role in addressing this challenge. The Food and Agriculture Organization (FAO) estimates that 17% of the world's total animal protein used for human consumption was derived from aquaculture and fisheries in 2009. This underscores the substantial contribution of aquaculture to global protein production due to its contribution in providing a sustainable and diverse source of nutrition.

To meet the demand for food security, substantial efforts have been made in developing global aquacultural models in the seafood industry. Researchers, such as Bodrisky et al. (2010), have engaged in significant modeling endeavors to anticipate and project the world's food supply demand up to the middle of the century. These modeling efforts help in assessing the potential role of aquaculture in meeting the increasing demand for seafood and protein.

The development of aquaculture is not just an economic and ecological imperative but also a response to the broader global challenge of ensuring food security for a growing population. As the aquaculture sector continues to evolve, innovations in technology, sustainable practices, and international collaboration will be pivotal in shaping its modern trajectory and its capacity to contribute to the world's food needs in a responsible and environmentally conscious way (Diana et al., 2013). It is of importance to also note that aquaculture is the fastest growing sector of the global food system, increasing at a rate far higher than that of dairy and terrestrial livestock production (FAO, 2020). Also, more than half of the fish and shellfish produced directly for food worldwide are currently sourced from aquaculture, as production of aquaculture exceeds captured fisheries (Figure. 1). By 2030, it is predicted that aquaculture will provide up to two thirds of the world's fish demand for food according to the World bank.

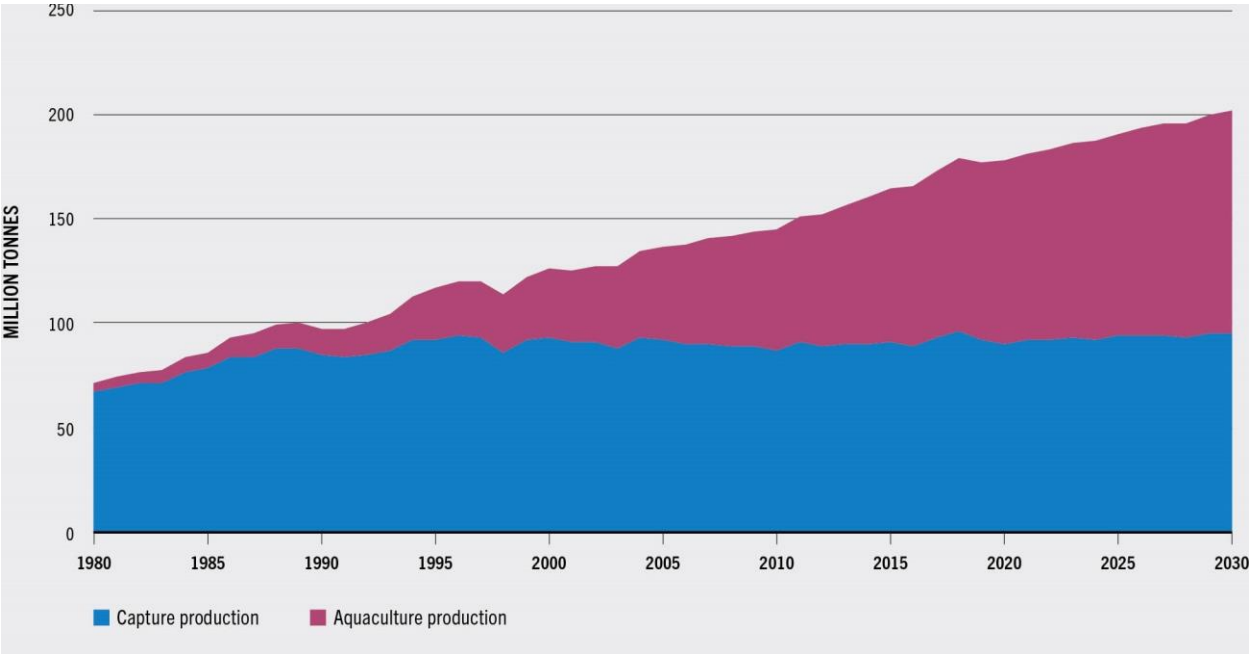

Figure 1: Global captured fisheries and aquaculture production (1980 – 2030), (FAO, 2022).

1.2 Salmon Aquaculture in Norway

Salmon aquaculture in Norway has become an important contributor towards global fish production. In 2022, the global production of salmon was approximately 2 863 700 million tons, where Norway alone produced 1 511 100 million tons (FAO, 2022). It is also noteworthy that Norway did not completely cease the practice of captured fisheries. According to FAO 2022, the global forage fish capture remains approximately 22,000,000 tons. However, Norway has strict regulations, shifting the economic focus towards sustainable aquaculture, and promoting sustainable standards and practices (Hersoug B. 2021). The Norwegian aquaculture sector made modifications from the whole value chain by effectively following these standards:

Firstly, the industry has placed a high priority on safeguarding marine ecosystems, taking proactive measures to reduce the impact of salmon farming on the environment. Intensive salmon production has a large impact on the environmental footprint, such as green-house gas (GHG) emission (Ellingsen et al., 2009), nutrient spillage from fish sludge and uneaten feed (Acosta 2016). Norway enforces strict standards for salmon aquaculture to avoid such

problems, in order to promote sustainable operations that are aligned with environmental preservation goals (Olaussen 2018).

Secondly, sustainability in Norwegian salmon aquaculture extends across the health and welfare of the fish. The industry emphasizes responsible veterinary practices and disease monitoring, particularly during outbreaks. According to Torrissen et al. (2013), health concerns such as parasitic salmon lice, viral infectious pancreatic necrosis (IPN), and Bacteria gill complex, pose challenges to the Norwegian salmon fish farming sector. Therefore, the industry focuses on providing optimal conditions to promote the overall well-being of salmon (Sommerset et al., 2022).

In addition, there is a dedicated effort in Norwegian salmon farming to develop sustainable feed formulations**.** Feed is the most substantial input factor in Norwegian farmed salmon aquaculture costs (Iversen et al., 2020). However, net cost of production in salmon aquaculture industry have seen a decline of feed cost to 45% as other costs have risen, particularly those associated with disease challenges and increased contractor usage (Iversen et al., 2020). Nevertheless, feed still represents a significant factor in fish production. This initiative involves reducing dependence on fishmeal and fish oil derived from wild stocks, exploring alternative protein and fat sources, and maintaining the nutritional quality of salmon feed, which will be explained in detailed in this thesis (Glencross et al., 2024).

In summary, all these points further underscore the industry's commitment to balancing the nutritional demands of the present, with the imperative of protecting ecosystems, and resources for the well-being of future generations, by assessing situations that may arise as a result of practicing salmon farming in Norway.

1.3 Sustainable Salmon Feed: Practices and Innovations

In 2020, Norway used approximately 1.5 tons of feed ingredients to produce salmon, were 92% of ingredients used for this production were imported (Aas et al,2020). Regarding nutritional based assessments, salmon aquaculture feed industry's historical dependence on Fish meal and Fish oil, in farmed salmon production arises from the nutritional requirements of farmed fish. Marine ingredients are valuable due to their balanced protein content and essential amino acids which are crucial for the growth and health of Atlantic salmon (Sprague et al., 2015). Wild-caught fish stock used in salmon feed production are mainly

forage fish species such as Atlantic herring (*Clupea harengus)*, Pacific herring *(Clupea* pallasii), European anchovy (*Engraulis encrasicolus*) and Northern Pacific anchovy (*Engraulis mordax)* (Huntington et al., 2009). However, these species play a major role in the marine ecosystem as they belong to lower trophic levels in the food chain (Fréon et al., 2005). Exploitation of these endangered species could lead to depletion of wild stock and the possibility of extinction in extreme situations (Deutch, 2007). Therefore, there is a need to replace marine ingredients with more sustainable sources of protein and oil.

In recent decades, there has been a significant trend within the industry towards integrating plant-based ingredient sources into fish feed (Figure. 2). However, assessments conducted through Life Cycle Assessment (LCA) and carbon footprint models (see Table 1) have raised concerns regarding the sustainability implications of relying on these plant-based sources for aquaculture, particularly regarding land usage, water consumption, and eutrophication (Aas et al., 2022; Torstensen et al., 2008). The subsequent section of this thesis will delve into the nutritional considerations associated with the utilization of plant-based ingredients.

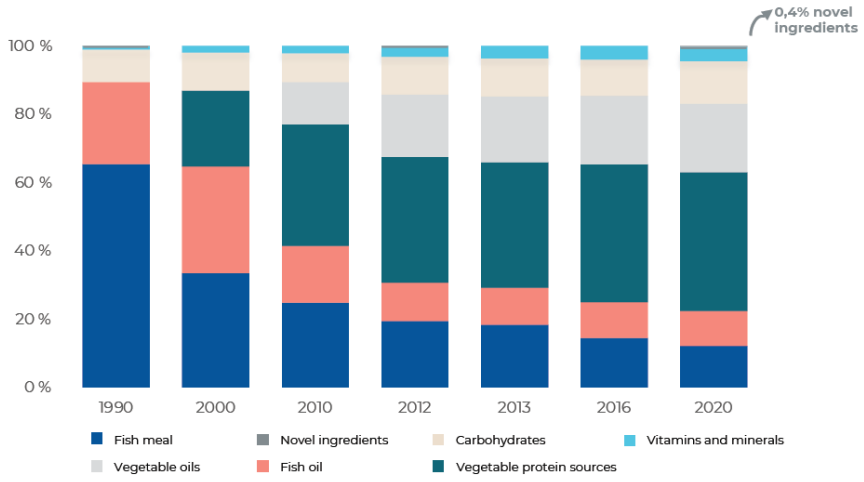

Figure. 2: Ingredients used in Norwegian salmon feed (% of total ingredients). (NCE, 2022, adapted from Aas, 2022)

Table 1: GHG emissions of agricultural products imported into Norway, land use and energy demand of the plant protein ingredients (adapted from Hognes et al., 2011).

Ingredient	Carbon footprint [kg CO_2e/kg]	Occupied agricultural area [m^2/kg]	Cumulative energy demand [MJ/kg]
Wheat grain, dried	0.35	1.65	2.20
Soy Protein Concentrate (SPC)	3.09	4.06	4.01
Wheat gluten	Confidential data		
Pea Protein Concentrate (PPC)	0.69	9.54	10.8
Sunflower meal	1.01	12.1	8.78
Rape seed oil	0.87	3.60	6.54

Currently, Norwegian salmon feed only includes 0.4% of novel ingredients (Figure 2). There is a need to increase the production of local Norwegian novel feed ingredients to reduce the dependency on imported ingredients, environmental impact and still fulfill the nutritional requirements of Atlantic salmon. NCE Seafood Innovation recently categorized these novel ingredients as harvested resources, farmed resources, and underutilized resources (Figure 3). Moreover, these novel ingredients may present opportunities to support the growth of aquaculture without imposing limitations on its projected future expansion (Almås et al., 2020).

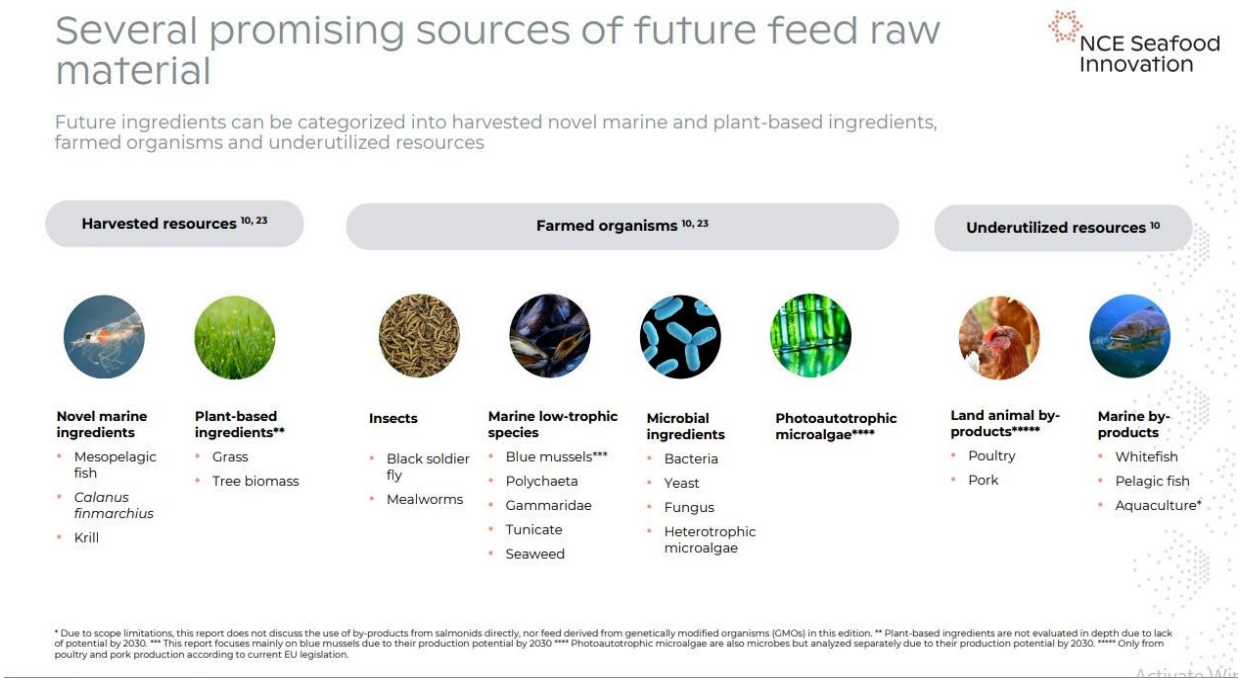

Figure 3: Suggested characterization of future ingredients by NCE Seafood Innovation (2022).

1.3.1 Plant-based ingredients in aquafeeds

Increased inclusion of plant-based ingredients in fish feed raises concerns regarding the unbalanced amino-acid content and absence of essential long chain omega-3 fatty-acids. As a possible solution, studies were conducted with blends of plant ingredients, with or without amino acid supplementation, as well as the replacement of FO with supplemented essential fatty acids (Gomes et al., 1995; Kaushik et al., 1995; de Francesco et al., 2004; Espe et al., 2006).

Aquafeed with full-fat soybean meal (SBM), containing 35 to 50% crude protein (CP), was considered the best alternative to FM in the last decades. However, soybean oil was still unable to replace FO without inducing health problems in Atlantic salmon (Moldal et al., 2014). The major limitation of using soya as a fat source is its incapacity to supply sufficient essential omega-3 fatty acids. Plants have a restricted metabolic pathway for producing omega-3 fatty acids, specifically EPA (Eicosapentaenoic acid) and DHA (Docosahexaenoic acid) (Abbadi et al, 2004; Adarme-Vega et al, 2014;).

In addition, SBM is known for containing anti-nutritional factors (ANFs) affecting digestibility and negatively impacting the distal intestine health of Atlantic salmon (Jacobsen et al., 2018; Krogdahl et al., 2003). Common examples of ANFs are lectins, phytic acid, and trypsin inhibitors, amongst many others (Huang, 2018).

As an alternative to the problems raised above, soy protein concentrate (SPC) has been successfully incorporated into Atlantic salmon feed. Soybean meal undergoes a refinery process that removes sugars, oligosaccharides, and most importantly, removes most of the anti-nutritional factors (ANFs) (Hart et al, 2010). As a result, SPC offers a concentrated protein product with improved protein apparent digestibility coefficient (ADC) in Atlantic salmon (Metochis et al., 2016; Hart et al., 2007). However, there is still the need to supply essential amino acids and fatty acids.

Due to plant-based ingredients having low level omega 3 fatty acid, genetically modified ingredients have been explored to produce omega-3s. For example, soybeans when genetically modified can accumulate more (ALA) Alpha-linolenic acid (Zhou et al 2024; Brink et al., 2014); *Brassica juncea* (Indian mustard) can have up to 15% EPA and 1.5% DHA after being genetically modified (Wu et al., 2005); canola plant oils (rapeseed) when genetically modified have an improved high level of omega 3s and low levels of erucic acid,

which is considered an ANF (Sissener et al., 2018; Bell et al., 2003); flax oil from flaxseed is naturally rich in ALA, but when genetically modified, its content of omega-3 is enhanced and stable (Hatlen et al., 2012; Nuez-Ortin et al., 2016).

However, if we want to increase the percentage of Norwegian ingredients in Atlantic salmon feed, perhaps plant-based ingredients are not the most promising alternative. Only approximately 3% of Norway's surface is arable. This small percentage of the land is mostly used for food production and land animal feed. Furthermore, importing plant-based ingredients for aquafeeds is also associated with carbon emissions due to transportation and deforestation (OECD 2021). Therefore, other alternatives should be explored.

1.3.2. Black soldier fly larvae (BSFL)

Black soldier fly larvae BSFL *(Hermetia illucens*) meal, which is a viable protein source for Atlantic salmon, can contain about 40% protein and 30% fat (Lin et al., 2022), depending on the larvae stage of development, composition of the rearing substrate and processing after harvest (Newton et al., 2005). However, the presence of omega-3 fatty acids in BSFL is contingent upon their dietary intake, as it cannot be synthesized by the organism itself. Conversely, if their diet lacks sufficient sources of omega-3 fatty acids, their omega-3 content may be lower.

In addition, BSFL represents a promising avenue for enhancing the sustainability of salmon aquaculture practices. Their desirability lies in their capacity for resource efficiency, minimal water, and land usage, with reduced environmental footprint (da Silva et al.,2020). However, despite their potential benefits, challenges related to resource consuming de-chitination arise. Chitin is a component of the exoskeleton present in BSFL and can enhance the hardness and durability of feed pellets or the physical properties of Atlantic salmon. However, its abundance can present hurdles in formulating salmon feed (Li, 2021)

Atlantic Salmon, like many fish, has limited capacity to digest chitin. Excessive chitin levels in feed can affect gut health and nutrient digestion and uptake, thus impacting the overall growth and vitality of the fish (Albrektsen et al., 2022). Therefore, managing chitin levels in feed formulations is crucial to ensure optimal digestion and nutrient absorption in salmon.

BSFL demonstrates notable capability to convert low-value substrates into high-value nutrients/biomass, thereby contributing to environmental conservation efforts (Zulkifli et al., 2022; Spranghers et al., 2017). Therefore, fruits and vegetable waste as substrate for

rearing BSFL are promising due to their abundance and potential nutrient content (Linn et al., 2024; Spranghers et al., 2017). Additionally, BSFL meal can be considered as a potential substitute for fish meal (FM) and soy protein concentrate (SPC) in aquafeeds, provided it is supplemented with essential amino acids like methionine and lysine (English, 2021).

Ongoing research endeavors are focused on optimizing both the production and utilization of BSFL, with the overarching objective of fostering a more novel ecologically sound and efficient use of BSFL in salmon aquaculture industry.

1.3.3 Microbial ingredients

Microbial ingredients, including non-human food marine ingredients such as fungi (yeasts), microalgae, protists, and bacteria, have garnered growing interest as viable alternatives. These alternative ingredients present opportunities to support the growth of aquaculture. Microbial ingredients have a rapid growth rate and boast minimal carbon footprint due to independence from agricultural land, minimal freshwater usage, and ability to be cultivated from non-food biomass,CO2 (in the case of microalgae and protists), or natural gas (methanotroph bacteria) (Wan-Mohtar et al, 2022; Nagappan et al., 2021).

Furthermore, it has been shown that processing methods influence nutritional composition of yeast. Agboola et al, (2022) studied the distinct processing methods of three non-Saccharomyces yeast species (*Cyberlindnera jadinii*, *Blastobotrys adeninivorans* and *Wickerhamomyces anomalu*), either inactivated or autolyzed. Depending on the species and the processing method used, differences in the nutritional value were observed. Regarding digestibility, inactivated *C. jadinii, W. anomalus and B. adeninivorans* had ADCs of sum amino acid of 57%, 86% and 73%, respectively. Generally, diets containing these microorganisms exhibit a favorable amino acid profile when supplemented with methionine, arginine, lysine, and phenylalanine, which are frequently the most limiting essential amino acids for juvenile Atlantic salmon. In addition to this, yeast species demonstrate efficiency in converting non-food lignocellulosic biomass. They also serve as a source of bioactive components found in their cell walls, such as β-glucans and mannans, which have been associated with various health benefits for fish, including immune modulation and improved gut health (Agboola et al., 2021).

Finally, Agboola et al, (2022) suggests that genetic modification or enhanced nutrient digestibility via exogenous enzyme supplementation, along with cost-effective downstream processing, could serve as a viable strategy to enhance overall protein quality and essential fatty acid content in yeast. However, to implement this approach on a larger scale, additional investment is required to ensure affordability and sustainability concerns for feed manufacturers in the aquaculture sector. Hence, there is a need to further investigate alternative microbial organisms that answer the mentioned problems, such as *A. limacinum* as potential substitutes for Atlantic salmon feed.

CHAPTER 2:Background and Objectives

2.1 *Aurantiochytrium limacinum*

A. limacinum, a single-cell and heterotrophic marine protist, is a *Thraustochytrid*, previously known as *Schizochytrium limacinum*. It is suggested that their ancestor may have been photosynthetic and subsequently lost their plastids in multiple lineages (Cavalier-Smith, 1999) but recent findings have more reliable data on them (Leyland et al., 2017). *A. limacinum* belongs to the class *Labyrinthulomycetes*, which are known for their ability to produce high levels of long-chain polyunsaturated fatty acids (LC-PUFAs), particularly DHA through PUFA-synthase pathway (Yuki et al., 2022; Clement et al., 2009).

2.2 A novel source of DHA in aquaculture.

EPA and DHA are essential fatty acids that salmon have a limited capacity of synthesize from their precursor ALA, thus, they must be acquired through diet. They play a pivotal role in the growth and health of salmon (Storebakken et al., 2000). The National Research Council (NRC, 2011) recommendation for Atlantic salmon of n-3 PUFA ranges between 0.50% to 1.0% EPA and DHA (Storebakken., et al 2000). Furthermore, good EPA and DHA standard in the fish diet is an important indicator of good nutrition, physiology, health, and welfare (Santigosa et al., 2023). Also, PUFAs play crucial roles in various biological processes in Atlantic salmon. Besides their contribution to energy storage, EPA and DHA play relevant functions in, for example, cell membrane structure, cellular synthesis, pigmentation, immune homeostasis, and neural function. (Tocher, 2015; Holen et al., 2018; Santigosa et al., 2023). For example, EPA and DHA are abundant in the retina of fisheyes. Their presence ensures optimal photoreceptor function, allowing fish to perceive their environment (Rosenlund et al., 1997; Noffs et al., 2009; Stoknes et al, 2004).

As mentioned above, fish oil is currently the most commonly used source of essential PUFAs. To promote sustainability in feed production, there is a growing need to explore alternative sources of DHA. Microorganisms, such as *A. limacinum*, emerge as promising sustainable candidates for replacing fish oil in aquafeed formulations.

These microbial species offer significant advantages as a source of DHA in salmon feed. *A. limacinum* does not rely on light for growth and production of omega-3, making it a valuable candidate for sustainable omega-3 and astaxanthin production (Yuki, 2022). Microbes used as fish feed are resource-efficient, thriving in controlled environments. Their production

minimizes water consumption, reduces land utilization, and has a lower carbon footprint compared to other industries (Albrektsen et al., 2022).

In the current thesis, we assessed the potential of *A. limacinum* as a DHA source in diets for Atlantic salmon. *A. limacinum* utilizes lignocellulosic sugar hydrolysates derived from Norwegian spruce tree by-products (Olsen et al., 2023). This innovative approach significantly enhances the sustainability of *A. limacinum* as a DHA source. Unlike conventional sources such as corn or wheat, which demand substantial amounts of freshwater and arable land for their production, *A. limacinum* thrives on lignocellulosic substrates, thereby reducing the strain on these valuable resources. By utilizing by-products from the forestry industry, *A. limacinum* offers a more environmentally friendly alternative, contributing to the conservation of freshwater and land resources while providing a potentially valuable source of DHA for Atlantic salmon diets.

2.3 Hypothesis:

***A. limacinum* is a microbial ingredient which may serve as a sustainable alternative source of DHA to FO in diets for pre-smolt Atlantic salmon.** However, it is unknown if Atlantic salmon can digest and utilize DHA from *A. limacinum*. To test this hypothesis, DHA requirements (NRC, 2011) will be fulfilled by FO and fish will receive increasing inclusion levels of *A. limacinum* as an additional source of DHA. **It is expected that normal growth, digestibility, nutrient utilization, and pellet quality will be supported by the increasing inclusion of *A. limacinum* in pre-smolt Atlantic salmon diets.**

2.4 Main objectives:

- To characterize the effects of diets containing increasing levels of *A. limacinum* on the growth performance, nutrient digestibility, nutrient utilization and retention of Atlantic salmon.
- To assess the physical properties of pellets containing increasing levels of *A. limacinum*, including density, hardness, sinking velocity and water stability.
- To integrate all of the information from the pellet quality analysis, growth performance, digestibility, nutrient utilization, and retention, in order to evaluate the potential of *A. limacinum* as an alternative source of DHA in salmon feeds.

CHAPTER 3: Materials and Methods

3.*1 A. limacinum* production

For this study, *A. limacinum* was produced at the Faculty of Chemistry, Biotechnology and Food Science (Ås, NMBU), as described by Olsen et al. (2021). Briefly, the microorganism *A. limacinum* ATCC-MYA 1381 SR21 was purchased from the American Type Culture Collection and stored in 20% glycerol at -80 ◦C. Its sugar source was lignocellulosic sugar hydrolysates from Norwegian spruce trees by-products, supplied from BALI™ Borregaard AS (Sarpsborg, Norway). *A. limacinum* was obtained from a continuous fermentation under sufficient oxygen supply. In this study the freeze-dried sample of *A limacinum* had a total fat content of 188.5 g/kg and a total fatty acid content of 13.2 ± 1.9% DCW (Dry cell weight) (Olsen et al., 2023). Notably, 35.8% of the total fatty acids content in the *A. limacinum* used for this experiment consisted of DHA.

Using spruce hydrolysates for cultivation resulted in lipid-to-protein ratios that were considerably lower when compared to the glucose-based media. Although spruce lignocellulosic sugars may not be as effective as commercial glucose sources, such as corn or wheat, this sugar source is more sustainable and it can still be used as a carbon source in fed-bath fermentation mode for production of *A. limacinum* SR21 biomass rich in DHA (Olsen et al., 2023).

The *A. limacinum* provided for this study was a paste with a dry matter (DM) content of 233.9 g/kg. Efforts to analyze the total fat content of the paste resulted in erroneous values (i.e., close to 0 g/kg). Therefore, a sample of the paste was freeze-dried and re-analyzed for total fat. However, analysis of the nutritional composition of the final diets indicated decreasing total fat content in the diets with an increasing level of *A. limacinum*, suggesting that analysis of the freeze-dried sample resulted in an underestimation of the fat content of the *A. limacinum* paste despite correcting for the difference in moisture content of the freeze-dried sample. Conversely, paste samples were used for analyzing other nutrients (with the exception of amino acids).

Table 2: Nutritional composition of *A. limacinum* (g/kg DM)

Analyzed chemical composition	*A. limacinum*
Dry matter	233.9
Crude Protein	220.9
Total Fat	188.5
Ash	132.3
Calcium	18.4
Potassium	9.5
Magnesium	0.4
Gross energy (MJ/kg DM)	19.7

Table 3: Analyzed content of percentage (TFA) Total Fatty acids in *A. limacinum* ingredient (freeze dried) (paste).

Fatty Acids g/Kg		*A. limacinum*	**Fatty Acids g/Kg**		*A. limacinum*
Lauric acid	C12:0	0.01	**Palmitoleic acid**	C16:1	0.00
Myristic acid	C14:0	3.83	**Heptadecenoic acid**	C17:1	0.00
Pentadecanoic acid	C15:0	0.19	**Elaidic acid**	C18:1n9t	0.00
Palmitic acid	C16:0	40.71	**Oleic acid**	C18:1n9c	9.08
Margaric acid (heptadecenoic)	C17:0	0.00	**Cetoleic acid**	C20:1n9	0.00
Stearic acid	C18:0	1.97	**Nervonic acid**	C24:1n9	0.00
Arachidic acid	C20:0	0.00	**Eicosadienoic acid**	C20:2	0.69
Heneicosanoic acid	C21:0	0.00	**Behenic acid**	C22:0	0.00
Linolelaidic acid	C18:2n6t	0.00	**α-Linolenic acid**	C18:3n3	0.00
Linoleic acid	C18:2n6c	0.00	**Eicosapentaenoic acid (EPA)**	C20:5n3	2.62
γ-Linolenic acid	C18:3n6	0.00	**Docosahexaenoic acid (DHA)**	C22:6n3	36.03

3.2 Feed formulation.

Four experimental diets (Diet 1 – Diet 4) were formulated to contain increasing levels of *A. limacinum* on a dry matter basis: 0%, 5%, 10% and 15%, respectively (Table 4). Since the effectiveness of *A. limacinum* as a source of DHA is not yet known, FO was included in the experimental diets to meet the DHA and EPA requirements of pre-smolt Atlantic salmon. In order to accommodate the inclusion of *A. limacinum* in the diets, RO, and potato starch were gradually decreased. Additionally, major protein sources such as FM and SPC did not differ between diets (Tables 4 and 5). All diets were formulated to meet or exceed the nutritional requirements of Atlantic salmon (NRC, 2011).

3.3 Feed production

Firstly, all the dry ingredients were weighed in accordance with Table 2 and mixed in an industrial paddle mixer (FôrTek, NMBU). Later on, wet ingredients such as FO, RO, *A. limacinum*, gelatine and water were mixed (in that same order). Gelatin was dissolved in water and activated at 55˚C.

Pellets were produced through cold pelleting. The mixed ingredients were placed in a pasta machine (ITALGI P35A, Italy) with a 2.5mm dye (Figure 5). The pellets were dried with hot air (below 60℃) until achieving a moisture level below 10%. In this way, water activity and microbial growth were minimized. The diets were stored at 4℃ until utilization.

Figure 5: Pelleting of mixed ingredients without cutting (left); pelleted feed after drying (right).

Table 4: Ingredient composition of the experimental diets containing increasing, graded levels of *A. limacinum* (g/kg DM)

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal [a]	226.4	226.4	226.4	226.4
Soy protein concentrate [b]	264.0	264.0	264.4	264.0
Wheat gluten [c]	60.0	46.0	33.0	20.0
Gelatinized potato starch [d]	160.0	140.0	119.0	98.0
Fish oil [e]	50.0	50.0	50.0	50.0
Rapeseed oil [f]	150.0	134.0	118.0	102.0
A.limacium [g]	0.0	50.0	100.0	150.0
MCP [h]	15.0	15.0	15.0	15.0
PREMIX [i]	5.0	5.0	5.0	5.0
GELATIN [j]	60.0	60.0	60.0	60.0
L-Lysine [k]	5.0	5.0	5.0	5.0
DL-Methionine [l]	3.0	3.0	3.0	3.0
Choline Chloride [m]	1.5	1.5	1.5	1.5
Yttrium oxide [n]	0.1	0.1	0.1	0.1
Sum Total	1000.0	1000.0	1000.0	1000.0

[a]Fishmeal: Pelagia AS, Bergen, Norway; [b]Soy protein concentrate: TRADKON SPC-500F, Sojaprotein, Bečaj, Serbia; [c]Wheat gluten meal: Lantmännen Reppe AB, Lidköping, Sweden; [d]Gelatinized potato starch: LYGEL F 60, Lyckeby, Kristianstad, Sweden; [e]Fish oil: ED&F Man, Mexico; [f]Rapeseed oil: Scanola A/S, Denmark; [g]*A. limacinum*, Olsen et al., 2023; [h]Monocalcium phosphate, Bolifor MCP-F, Oslo, Norway Yara; [i]Vitamin and mineral premix: Provides per kg of diet: vitamin A, 6800 IU; vitamin D3, 3000 IU; vitamin E, 260 mg; vitamin K3, 20 mg; vitamin B1, 18 mg; vitamin B2, 26mg; Pantothenic acid, 80 mg; vitamin B6, 17 mg; vitamin B12, 60 mcg; Nicotinic acid, 150 mg; Folic acid, 10 mg; Biotin, 791 mcg; Vitamin C, 270 mg; Inositol, 495 mg; Zinc oxide, 75 mg; Iodine, 3.2 mg; Copper, 3.9 mg; Manganese, 4.92 mg; Zinc chelate, 37.5 mg; Citric acid, 0.54 mg; BHT (Butylated hydroxytoluene), 1.6 mg; Propyl gallate, 0.84 mg; BHA (Butylated hydroxyanisole), 1 mg; Silicic acid, 12.45 mg; Calcium carbonate, 0.053 %; [j]Gelatin: GELITA Sweden AB; [k]L-lysine: L-lysine, feed grade (Biolys®), Evonik, Essen, Germany; [l]DL-methionine: DL-methionine, Evonik, Essen, Germany; [m]Choline chloride: Choline chloride, MIAVIT GmbH, Essen, Germany; [n]Yttrium oxide: Yttrium oxide (Y_2O_3), Sigma-Aldrich, Vienna, Austria.

Table 5: Nutritional composition of Diets 1-4 (g/kg DM)

Analyzed chemical composition	**Diet 1**	**Diet 2**	**Diet 3**	**Diet 4**
Dry matter	925.9	925.9	922.4	908.5
Crude Protein	473.3	471.4	473.0	474.8
Total Fat	221.1	215.2	203.9	197.5
Ash	66.4	72.4	78.5	85.2
Starch	173.9	157.4	137.2	125.6
Calcium	10.0	12.0	12.4	12.8
Potassium	8.6	9.3	10.0	10.2
Magnesium	1.8	1.7	1.7	1.8
Gross energy (MJ/kg DM)	23.8	23.6	23.4	23.1

Table 6: Analyzed amino acid content of *A. limacinum* (g kg^{-1} ingredient).

Amino Acids g/Kg (DM)	*A. limacinum*	**Amino Acids g/Kg (DM**	*A. limacinum*
Cysteine	1.9	**Valine**	5.0
Methionine	4.5	**Isoleucine**	4.5
Aspartic acid	11.6	**Leucine**	7.2
Threonine	7.9	**Tyrosine**	2.9
Serine	7.3	**Phenylalanine**	4.3
Glutamic acid	17.7	**Histidine**	2.6
Proline	5.7	**Lysine**	7.6
Glycine	5.5	**Arginine**	6.8
Alanine	6.6	**Tryptophan**	2.4

Table 7: Analyzed amino acid content in experimental diets.

Amino Acids g/Kg	Diet 1	Diet 2	Diet 3	Diet 4
Cysteine	3.7	3.5	3.6	3.5
Methionine	7.9	7.4	7.4	7.9
Aspartic acid	29.7	29.8	30.7	31.3
Threonine	13.4	13.7	14.0	14.4
Serine	14.6	15.0	14.5	15.5
Glutamic acid	67.3	64.8	61.8	59.6
Proline	25.8	23.4	22.4	20.7
Glycine	23.3	23.8	23.8	23.7
Alanine	18.0	18.7	18.8	18.9
Valine	15.2	15.0	15.3	15.1
Isoleucine	14.8	14.8	14.7	14.5
Leucine	25.16	25.0	24.7	24.3
Tyrosine	6.9	7.1	6.4	6.8
Phenylalanine	15.0	14.8	14.3	14.1
Histidine	9.6	9.8	9.9	9.8
Lysine	26.5	26.0	26.5	25.8
Arginine	25.5	24.8	25.0	25.9
Tryptophan	4.0	3.9	3.5	-

Table 8: Fatty acid composition of the experimental diets containing increasing, graded levels of *A. limacinum* (g/kg DM)

Name	**Fatty acid**	**Diet 1**	**Diet 2**	**Diet3**	**Diet 4**
Lauric acid	C12:0	0.0	0.0	0.0	0.0
Myristic acid	C14:0	2.4	2.6	2.8	3.1
Pentadecanoic acid	C15:0	0.0	0.0	0.0	0.0
Palmitic acid	C16:0	10.6	12.0	13.4	15.3
Margaric acid (heptadecenoic)	C17:0	0.0	0.0	0.0	0.0
Stearic acid	C18:0	2.8	2.9	2.8	2.9
Arachidic acid	C20:0	1.9	1.9	1.9	2.0
Heneicosanoic acid	C21:0	0.3	0.4	0.4	0.4
Behenic acid	C22:0	1.0	1.3	1.4	1.5
∑ **SFA**		19.1	21.0	22.8	25.2
Palmitoleic acid	C16:1	2.9	3.0	3.1	3.3
Heptadecenoic acid	C17:1	0.0	0.0	0.0	0.0
Elaidic acid	C18:1n9t	43.5	41.3	39.0	36.1
Oleic acid	C18:1n9c	3.1	3.0	2.9	2.8
Cetoleic acid	C20:1n9	0.0	0.0	0.0	0.0
Nervonic acid	C24:1n9	0.0	0.0	0.0	0.0
∑ **MUFA**		49.5	47.3	45.1	42.2
Eicosadienoic acid	C20:2	0.0	0.0	0.0	0.0
Linolelaidic acid	C18:2n6t	0.0	0.0	0.0	0.0
Linoleic acid	C18:2n6c	16.3	15.4	14.5	13.2
γ-Linolenic acid	C18:3n6	0.0	0.0	0.0	0.0
∑ n-6		16.3	15.4	14.5	13.2
α-Linolenic acid	**C18:3n3**	5.2	4.8	4.5	4.2
Eicosapentaenoic acid (EPA)	**C20:5n3**	4.9	5.2	5.5	5.8
Docosahexaenoic acid (DHA)	**C22:6n3**	3.3	4.4	5.8	7.5
∑ **n-3**		13.4	14.5	15.9	17.4
∑ **PUFA**		29.7	29.9	30.4	30.7

3.4 Experimental design.

The experiment took place at the Center for Fish trials (Ås, NMBU). The experimental procedures were performed following the Norwegian regulations on the use of animals in experiments (FOR-2015-06-18-761) and European Union Directive 2010/637EU.

For nine weeks, a total of 420 pre-smolts with an initial average weight of 29.8 g ± 0.08 g, were randomly distributed in 12 fiberglass tanks (300L). Each tank was stocked with 35 fish. Fish received one of the four experimental diets, with three replicate tanks per diet (Table 5). Fish were fed with automatic belt feeders for a period of 8h/day (5:00-13:00) and monitored daily. Uneaten feed was collected using the wedge wire screen method described by Helland et al. (1996). The daily feed supply was dependent on the uneaten feed from the previous day, projected biomass, and additional 10% excess to guarantee that fish were fed *ad libitum* (Shomorin et al, 2019). Water quality (dissolved oxygen level and temperature) was also assessed daily and recorded to ensure proper and adequate fish welfare during the whole experiment.

Table 9: Randomization of experimental diets to the tanks.

Tank No.	13	14	15	16	17	18	19	20	21	22	23	24
Diet No.	3	4	2	1	4	3	1	4	2	3	1	2

Figure. 6: Tanks at Center for Sustainable Aquaculture (Ås, NMBU)

3.5 Final sampling

After nine weeks of the dietary trial, six fish per tank were randomly selected (totaling 18 fish per dietary group) for further analysis. These fish were euthanized using a lethal dose of M-222, following which their weight and length were measured.

After the initial nine-week feeding trial, fecal samples were collected from the posterior intestine of the remaining fish in each tank, following the method described by Austreng (1978). The stripped feces from all fish in each tank were weighed and stored at -20°C for later freeze-drying to assess digestibility. The remaining fish in the tanks were fed the experimental diets for an additional two-week period and stripped twice, with one week apart of each sampling. Yttrium oxide was used as a marker for digestibility analysis.

Following fecal collection, the fish were fasted for 72 hours. After fasting, a pooled sample of five fish per tank was collected to determine whole-body chemical composition, focusing on DHA and other fatty acid retention. The carcasses were stored at -20°C until processing and freeze-drying.

3.6 Pellet quality assessment

3.6.1 Pellet length, width, hardness

Thirty pellets were randomly selected from each diet and were placed in descending order by length. Amongst these thirty pellets, only the middle fifteen were selected to measure length, width, and hardness. Length and width were measured using electronic calipers.

The hardness of the same pellets selected for the determination of length and width was measured using a hand-held Kahl device. The force needed to break the pellet was recorded in kilograms (kg).

3.6.2 Sinking Velocity

This was measured by filling a 1.2 m long plastic tube (12 cm in diameter) with drinkable tap water to the 1 m mark at 23 °C. One pellet was dropped into the tube at a time. Time was recorded in seconds for each pellet to travel 1m. Fifteen pellets per diet were recorded to get an average sinking velocity of the particular diet.

3.6.3 Water Stability index

Water stability of the pellets was measured using a Julabo SW22 Shaking water bath according to Baeverfjord et al. (2006), but with some modifications. Baskets were weighed before and after the addition of 20 g of samples. The baskets along with beakers containing Milli-Q water (300 mL) were then placed in the water bath at 23°C and shaking was set at 120 rpm. Measurements were recorded in triplicates for each diet sample at three incubation times (i.e., 15 mins, 30 mins, 60 mins). Samples were dried for 20 hours at 100-104℃. After

oven drying, the weight of the baskets along with the plates were recorded. Dry matter retained gave an estimation of water stability of experimental diets.

3.6.4 Bulk density

The bulk density of the pellets was determined through three replicate measurements using a bulk-density cup. Firstly, the bulk-density cup was tarred on the analytical balance. Subsequently, the cup was filled with pellets, and the weight of the pellets was documented. Bulk density was reported in grams per liter (g/L).

3.7 Chemical Analysis

Chemical analysis was conducted as follows: Feed samples and freeze-dried fecal samples were ground until they reached a fine and homogeneous consistency. Whole fish samples were partially thawed, ground, freeze-dried, and re-ground before analysis. Dry matter (DM) content was determined by oven drying at 104°C until a constant weight was achieved. Samples were then sent to Labtek (NMBU) for further chemical analysis.

Ash content was determined by combustion at 550°C. Nitrogen (N) content of the feed, feces, and fish was measured using the Kjeldahl method according to Commission Regulation (EC) No. 152/2009, and crude protein (CP) content was calculated as N × 6.25.

Determination of total fat using Soxtec™ 8000 Extraction system in combination with Foss

Hydrotec™ 8000 Hydrolysis system. Gross energy was assessed using a Parr™ 6400 Automatic Isoperibol Bomb Calorimeter (Parr Instrument Company, Moline, IL, USA) based on ISO 9831.

Amino acid analysis, excluding tryptophan, was conducted on a Biochrom 30+ Amino Acid Analyzer (Biochrom Ltd., Cambridge, UK) in accordance with Commission Regulation (EC) No. 152/2009. Tryptophan content was evaluated using an UltiMate™ 3000 UHPLC system equipped with an auto-injector (Thermo Scientific, Massachusetts, USA) and a Shimadzu RF-535 fluorescence detector (Shimadzu Corporation, Kyoto, Japan) according to Commission Regulation (EC) No. 152/2009.

Levels of yttrium (Y), calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), and total phosphorus (P) were measured using a Microwave Plasma Atomic Emission Spectrometer (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA) after acid decomposition in a microwave digestion system (START D), Milestone Srl, Sorisole, Italy.

3.8 Fatty acid analysis

Lipid extraction was conducted at the Biospectroscopy Laboratory (NMBU) following a modified Lewis trans-esterification method, adapted from Olsen et al. (2023). Initially, 2 mL screw-cap polypropylene (PP) tubes were prepared by filling them with 15 ± 5 mg of biomass and approximately 250 ± 30 mg of acid-washed glass beads (710–1180 µm diameter). Subsequently, 500 µL of chloroform and 50–100 µL of internal standard, comprising a 1:1 (w/w) mixture of glyceryl tritridecanoate (C13:0) and trichloroacetate acid (C:23) in chloroform, were added to the PP tubes.

Homogenization of the biomass was carried out using a Precellys Evolution tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France) at 5,500 rpm for 6 cycles of 20 seconds each. The homogenized mixture was then transferred to glass reaction tubes via washing the PP tubes with 2,400 µL of a methanol-chloroform-hydrochloric acid solvent mixture (7.6:1:1 v/v; 3x800 µL), followed by the addition of 500 µL of methanol.

The reaction mixture was subsequently incubated at 90°C for 90 minutes and then cooled to room temperature, with the addition of 1 mL of Milli-Q water. Residual water was removed by washing the glass tubes twice with 1,500 µL of 0.01% butylated hydroxytoluene containing hexane.

Extraction of fatty acid methyl esters (FAMEs) was conducted by adding 2 mL of hexane to the reaction mixture, followed by 10 seconds of vortex mixing and centrifugation at 1,040 g for 5 minutes at 4°C. The upper organic phase containing the FAMEs was transferred to new glass tubes, while the lower aqueous phase was subjected to two additional extractions using a 2 mL hexane-chloroform mixture (4:1 v/v).

The collected solvents were evaporated under nitrogen at 30°C, and approximately 5 mg of anhydrous sodium sulfate was added to the glass tubes to ensure complete drying. The extracted FAMEs were then transferred into GC vials by washing the glass tubes with 1,500 µL of 0.01% butylated hydroxytoluene containing hexane.

Gas chromatography (GC) analysis of the extracted FAMEs was performed using a 7820A System (Agilent Technologies, Santa Clara, CA, USA) equipped with an Agilent J&W 121–2323 DB-23 column (20m × 180µm × 0.20µm) and a flame ionization detector (FID). Helium was used as the carrier gas, and the injection volume was 1 µL with a split ratio of 30:1 (flow rate was 30 mL/min). The total runtime per sample was 36 min, with a temperature

program starting at 70°C for 2 min, followed by an increase to 150°C over 8 min, then to 230ºc over 16 min, and finally to 245°C over 1 min.

Identification and quantification of different fatty acids were performed using the external standard Supelco 37 Component FAME Mix (C4-C24 FAME mixture, Sigma-Aldrich, USA), along with the C:13 and C:23 internal standards. The determination of total fatty acids (TFAs) was expressed as weight percentage (wt%) of total FAMEs of sample dry weight, and fatty acid composition was expressed as wt% of individual FAME of total FAMEs.

3.9 Growth Performance

- Specific growth rate:

$$\boldsymbol{SGR}(\%) = \frac{\boldsymbol{ln\,(FBW) - ln(IBM)}}{\boldsymbol{days}} \times \mathbf{100}$$

Where, FBW = final body weight in g fish; IBW = initial body weight in g fish^{-1} (Brett and Groves, 1979)

- Thermal-unit growth coefficient (TGC) was calculated as

$$\boldsymbol{TGC} = \mathbf{100}[(\boldsymbol{FBW})^{\frac{1}{3}} - (\boldsymbol{IBW})^{\frac{1}{3}}] \times (\boldsymbol{sum\,T \times D})^{-\mathbf{1}}$$

where: FBW = final body weight in g fish^{-1} ; IBW = initial body weight in g fish^{-1} ; sum T × D = sum degrees Celsius × days (Iwama and Tautz, 1981; Cho, 1992).

- Feed intake (g DM fish^{-1}) was calculated as:

$$\boldsymbol{Feed\ Intake\ (FI) = Total\ feed\ intake}\frac{\boldsymbol{\frac{g}{dm}\ /\ per\ tank}}{\boldsymbol{Total\ number\ of\ fish\ per\ tank}}$$

(Helland et al., 1996).

- FCR was calculated as:

$$\boldsymbol{FCR} = \frac{[\boldsymbol{Total\ feed\ intake(g/DM\ fish - 1)}]}{(\boldsymbol{FBW - IBW})}$$

Where FBW = final body weight in g fish-1 ; IBW = initial body weight in g fish-1 (Agboola et al., 2022).

- $\boldsymbol{Weight\ gain}\ (\%) = \mathbf{100} \times \frac{\boldsymbol{final\ weight(g) - Initial\ weight\ (g)}}{\boldsymbol{Initial\ weight(g)}}$

3.10 Digestibility and Retention calculations

- Apparent digestibility coefficients (ADC, %) of the nutrients in the experimental diets were calculated as:

$$\boldsymbol{ADC = 1 - F/D \times Di/Fi}$$

Where: D = % nutrient (or kJ/g gross energy) of the diet; F = % nutrient (or kJ/g gross energy) of the feces; Di = % digestion indicator (yttrium) of the diet; Fi = % digestion indicator (yttrium) of the feces (Hooft et al., 2019).

- Retained nitrogen (RN, g fish-1) and retained energy (RE, kJ fish-1) were calculated as: $\boldsymbol{RN = (FBW \times N\,f) - (IBW \times N\,i)\ and\ RE = (FBW \times GE\,f) - (IBW \times GE\,i),\ respectively}$

where: FBW = final body weight in g fish^{-1} ; IBW = initial body weight in g fish^{-1} ; Nf content final = nitrogen content (%) of the final carcass sample; Ni content initial = nitrogen content (%) of the initial carcass sample; GE content final = gross energy (kJ g^{-1}) content of the final carcass sample; and GE content initial = gross energy (kJ g^{-1}) content of the initial carcass sample (Hooft et al., 2019).

- Nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) were calculated for each tank as a percentage of ingested nitrogen (IN, g fish^{-1}) and ingested energy (IE, kJ fish^{-1}), respectively:

NRE (% IN) = $\boldsymbol{\left[(FBW \times N\,f) - (IBW \times N\,i)\right]/IN \times 100}$

and ERE (% IE) = $\boldsymbol{\left[(FBW \times GE\,f) - (IBW \times GE\,i)\right]/IE \times 100}$,

where: FBW = final body weight in g fish^{-1} ; IBW = initial body weight in g fish^{-1} ; N content final = nitrogen content (%) of the final carcass sample; N content initial = nitrogen content (%) of the initial carcass sample; GE content final = gross energy (kJ g^{-1}) content of the final carcass sample; and GE content initial = gross energy (kJ g^{-1}) content of the initial carcass sample (Hooft et al., 2019).

- Nitrogen retention efficiency (NRE) and energy retention efficiency (ERE) were calculated for each tank as a percentage of digested nitrogen (DN, g fish^{-1}) and digested energy (DE, kJ fish^{-1}), respectively:

NRE (% DN) = $\left[[(\boldsymbol{FBW} \times \boldsymbol{N\,f}) - (\boldsymbol{IBW} \times \boldsymbol{N\,i})]/\boldsymbol{DN}\right] \times \mathbf{100}$

and ERE (% DE) = $\left[[(\boldsymbol{FBW} \times \boldsymbol{GE\,f}) - (\boldsymbol{IBW} \times \boldsymbol{GE\,i})]/\boldsymbol{DE}\right] \times \mathbf{100}$,

where: FBW = final body weight in g fish-1 ; IBW = initial body weight in g fish-1 ; N content final = nitrogen content (%) of the final carcass sample; N content initial = nitrogen content (%) of the initial carcass sample; GE content final = gross energy (kJ g-1) content of the final carcass sample; and GE content initial = gross energy (kJ g^{-1}) content of the initial carcass sample (Øverland et al., 2013).

- Apparent fatty acid retention was calculated as follows:

$$\boldsymbol{Fatty\ acid\ retention}\ (\%) = \frac{\left(\boldsymbol{FBW} \times \boldsymbol{FA_{final}}\right) - \left(\boldsymbol{IBW} \times \boldsymbol{FA_{initial}}\right)}{\boldsymbol{Feed\ intake} \times \boldsymbol{FA_{diet}}}$$

Where FBW is final body weight (g $fish^{-1}$), FA_{final} is the fatty acid content of the final carcass sample, IBW is the initial body weight (g $fish^{-1}$), $FA_{initial}$ is the fatty acid content of the initial carcass sample, feed intake is the feed intake of the fish in g DM $fish^{-1}$, and FA_{diet} is the fatty acid content of the diet.

3.11 Statistical analysis

Data analyses were performed with GraphPad Prism v. 10.2.2. Before statistical comparison between diets, normality was assessed by Shapiro-Wilk test. If data were normally distributed, a one-way analysis of variance (ANOVA) was used, followed by host-hoc Tukey's multiple comparisons test. If data were not normally distributed, even after transformation, a non-parametric test was used, such as Kruskal-Wallis test, followed by a post-hoc Dunn's multiple comparisons test. Observations were considered significantly different at $p < 0.05$.

Fatty acid digestibility Apparent Digestibility Coefficients (ADCs) and retention data were analyzed using the GLM procedure of SAS (SAS OnDemand for Academics, SAS Institute Inc., Cary, NC., USA). Tank was considered the experimental unit for these analyses. The

Shapiro-Wilk test in PROC UNIVARIATE of SAS was used to assess normality and the Brown and Forsythe test in PROC GLM of SAS was used to test for homogeneity of variances for all dependent variables prior to other statistical analysis. A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to determine differences between treatment means for normally distributed data. Non-normally distributed data (i.e., ADCs for 16:1 and 18:3n3) were analyzed using the Kruskal-Wallis test in PROC NPAR1WAY in SAS. Significance was declared at $p < 0.05$.

CHAPTER 4 : Results

4.1 Physical pellet quality

In this study, after careful evaluation of the physical qualities of experimental diet pellets, the parameters considered included pellet hardness, bulk density, pellet width, pellet length, sinking velocity, and water stability index.

4.1.1 Bulk density

A significant difference was observed in bulk density associated with the inclusion levels of *A. limacinum* (Figure 8). Specifically, the bulk density of the pellets increased along the inclusion levels of *A. limacinum* in each diet.

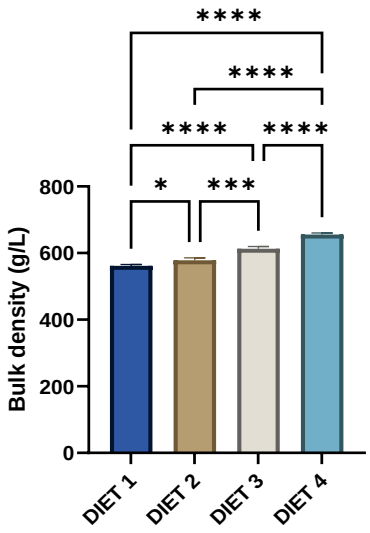

Figure 8: Bulk density in (g/L) of Diets 1-4. (n=15) *p-value <0.05; **p-value <0.01); ***p-value <0.001); ****p-value ≤0.0001.

4.1.2 Pellet width and length

It was observed that as the inclusion levels of *A. limacinum* increased, the pellet width decreased (Figure 9A). There were significant differences in pellet width among the diets with *A. limacinum* when compared to the control diet (Diet 1) with the exception of Diet 2. However, there was no clear association between pellet length and *A. limacinum* inclusion level (Figure 9B). Diet 2 and Diet 3 were significantly longer than control diet (D1) while D4 was only significantly different to D2.

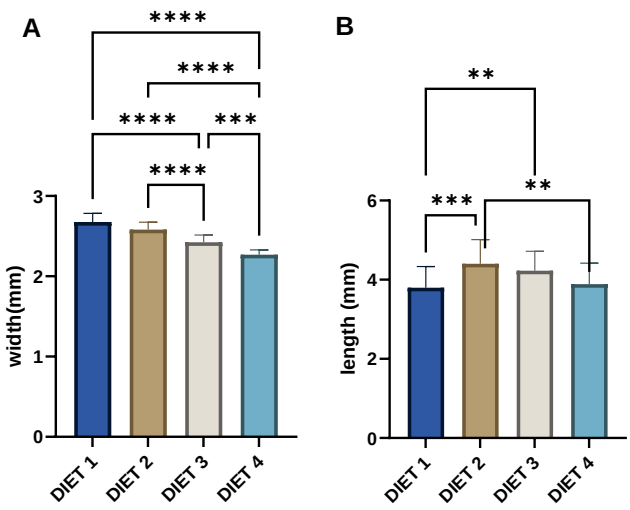

Figure 9: Pellet width and length (n=15); ** p-value <0.01); ***p-value <0.001); ****p-value<0.0001).

4.1.3 Pellet hardness

When analyzing pellet hardness between diets, there were no significant differences between the pellets from different dietary groups (Figure 10). The average hardness (± stdev) of the different diets were 2.8 (±0.78), 2.8 (±0.78), 3.2 (±1.02) and 3.67(±1.11), respectively. However, the non-parametric Kruskal-Wallis test indicated a potential trend (p = 0.0721) toward significance, where higher inclusion levels of *A. limacinum* were associated with increased hardness.

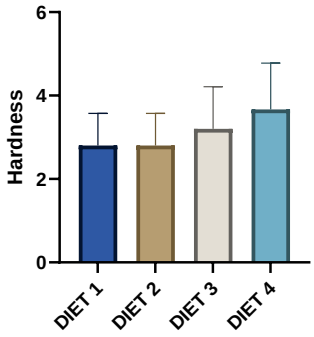

Figure. 10: Pellet hardness of the different diets. n=15.

4.1.4: Pellet sinking velocity

Regarding the sinking velocity of pellets (ms^{-1}) analysis revealed a significant difference in sinking velocity among the diets ($p = 0.0009$), where only the higher inclusion levels (D3 and D4) had significantly faster sinking velocities compared to the control (Diet 1).

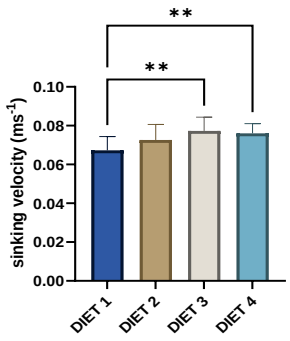

Figure 11: Pellet sinking velocity (ms^{-1}) of the different diets. (n=15) p-value ns =non significance; ** p-value=<0.001)

4.1.5 Water Stability

The Water stability index (WSI) was assessed at three different incubation times (15, 30, and 60 minutes). There were no significant differences in the WSI among the diets and time points between them.

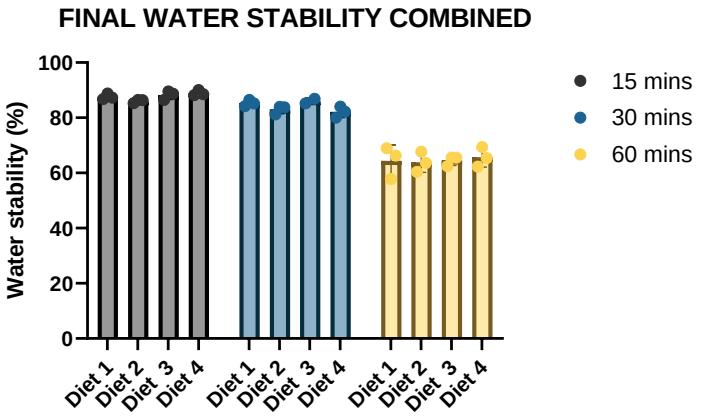

Figure 12: Water stability of dietary pellets (1-4) in 15,30 and 60 mins timepoints.

4.2: Growth performance

During the nine-week dietary trial, no mortalities were observed. The average relative feed intake was 1.10 g/fish/day and there were no significant differences between the groups (Figure 13A). Regarding the FCR across different dietary treatments, significant differences were only observed between the higher inclusion level of *A. limacinum* (Figure 13B) and the control diet (p = 0.0120). Identical differences between diets were observed in SGR analysis (p=0.0111, Figure 13C). TGC also displayed a similar pattern (Figure 13D), based on the temperature sum of 945.1 degrees Celsius over the nine-weeks period.

Table 10: Indicating Initial body weight and Final body weight in (g/fish**)**

Diet	IBW (g/fish)	FBW (g/fish)
1	29.7 ±0.07	131.9 ±4.24
2	29.8 ±0.07	127.4 ±0.43
3	29.8 ±0.09	127.3 ±1.57
4	29.8 ±0.09	117.1 ±5.44

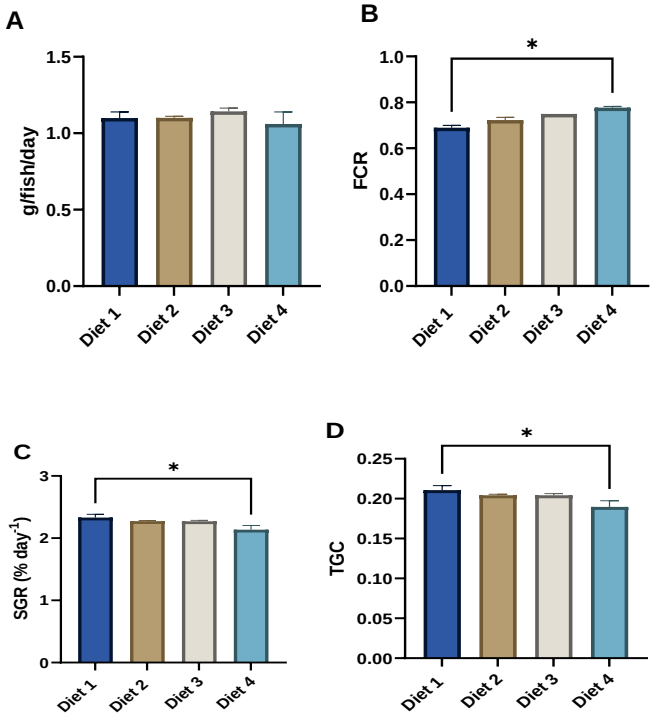

Figure 13: Growth performance. A: feed intake. B:Feed Conversion Ratio C. Specific Growth Rate. D. Thermal Growth Coefficient. (n=3) (ns= non-significant); *p-value <0.1

Differences in weight gain among the diets were observed, as depicted in figure 14. It was noted that Diet 2 and Diet 3 did not exhibit significant differences compared to the control diet. However, fish fed the highest inclusion level of *A. limacinum* (D4) showed the lowest weight gain, significantly differing from all other dietary groups ($p<0.05$).

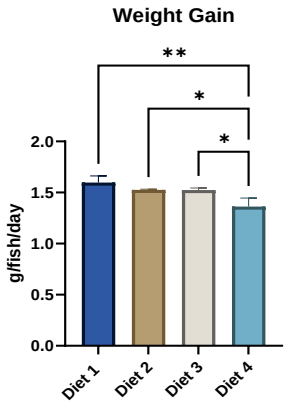

Figure 14: weight gain after 9 weeks of dietary trial. (n=12) *p-value <0.5); ** p-value <0.01)

4.3 Digestibility

Regarding the apparent digestibility coefficients (ADCs), there were no significant differences between the diets in terms of dry matter (DM), ash, and crude protein (CP) (Table 11, Figure 15A-C). However, ADCs of total fat and gross energy were negatively related with the inclusion levels of *A. limacinum*, where higher inclusion levels led to lower ADC of these parameters.

Table 11: Average Apparent digestibility of DM, ash, CP, total fat, and GE. Values expressed in percentage (mean ± SD) p-value.

Diet	Dry matter	Ash	Crude Protein	Total fat	Gross Energy
1	76.6 ±0.22	18.8 ±1.43	90.2 ±0.90	99.2 ±0.23	85.6 ±0.09
2	73.5 ±0.42	17.1 ±1.35	88.9 ±0.33	96.7 ±0.33	82.7 ±0.24
3	71.2 ±1.45	15.3 ±3.31	87.3 ±0.70	95.0 ±1.32	80.3 ±1.0
4	70.9 ±5.30	19.9 ±14.31	86.5 ±1.39	92.3 ±1.39	79.5 ±3.83
P-value	*0.115*	*0.882*	*0.672*	*0.0002*	*0.022*

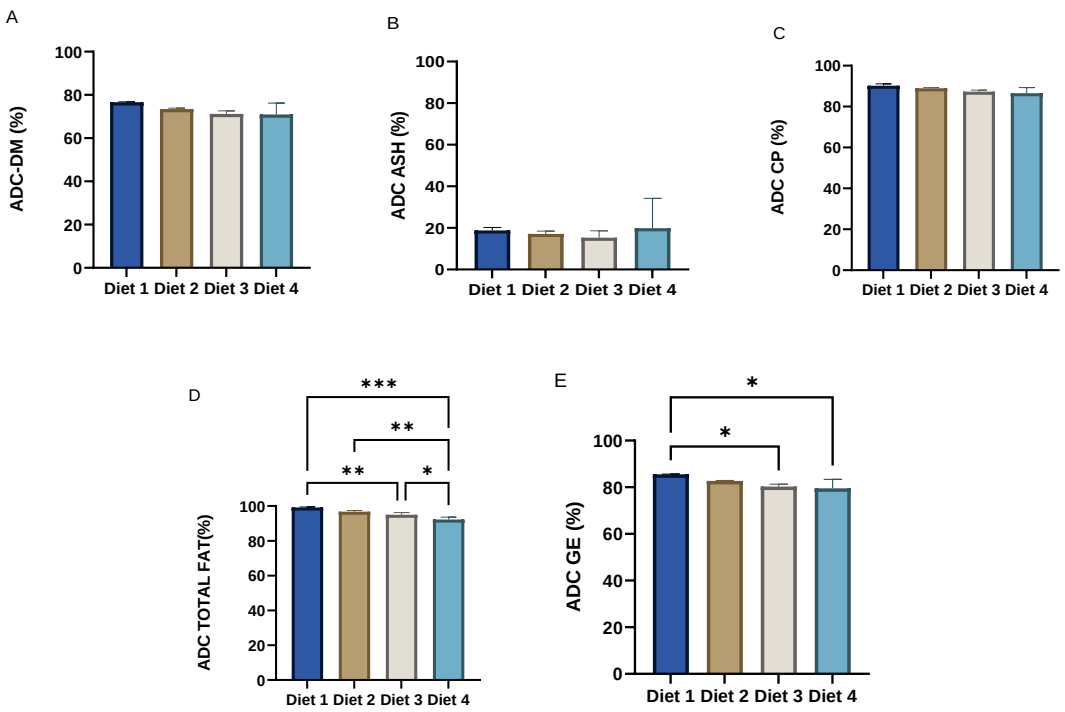

Figure 15: Apparent digestibility coefficients (ADC) fed different inclusion levels of *A. limacinum*. A: Dry matter. B:Ash C: Crude protein D: Total fat E:Gross energy with (DM,ASH,CP,TOTAL FAT and GE) in %, ANOVA quotations indicating (n=12) (ns= non-significant); *p-value =<0.5) **** p-value <0.01)***p-value <0.001); ****p-value<0.0001)

4.4 Nitrogen, total fat, and energy retention

The retention analysis (Table 12) demonstrated that there was a general tendency of lower retentions of nitrogen, energy, and total fat when fish were fed higher inclusion levels of *A. limanicum* (Figure 15). However, only D4 was significantly different to CD in the three parameters (p< 0.01). In addition, D4 was also significantly different to D2 regarding the nitrogen retention.

Table 12: Retention of nitrogen (RN, g fish^{-1}), energy (RE, Kj fish^{-1}) and total fat (g fish^{-1}) in pre-smolt Atlantic salmon fed (Diet 1-4). Data are given as mean ± S.E.M.; P value (n=4).

Diets	RN	RE	Total Fat
1	2.91 ±0.12	993.71 ±66.75	14.45 ±1.38
2	2.78 ±0.06	929.35 ±2.62	12.86 ±0.46
3	2.73 ±0.08	927.77 ±51.70	13.35 ±0.99
4	2.49±0.08	816.00 ±53.52	11.49 ±1.02
P-value	*0.0035*	*0.0152*	*0.0399*

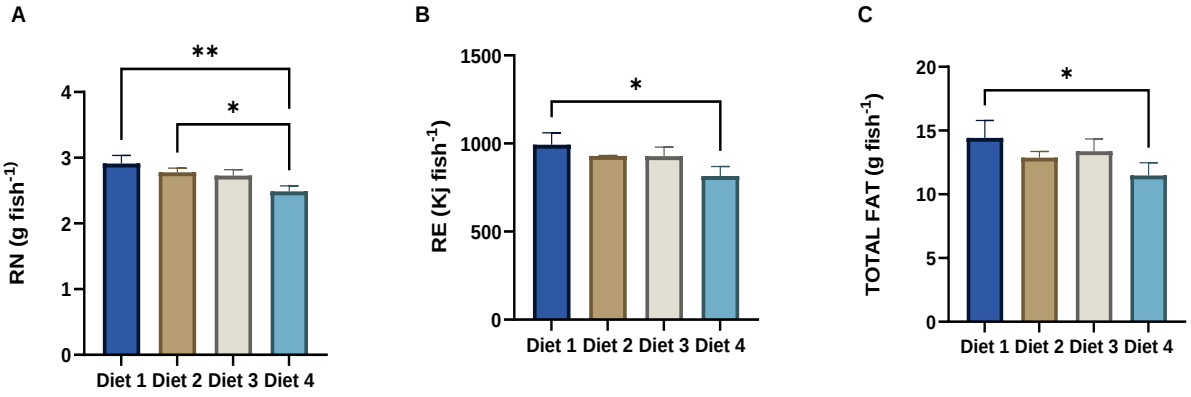

Figure 16: Retention of Nitrogen (A), energy (B) and total fat (C) RN,TOTAL FAT) in fish fed different inclusion levels of *A. limacinum* for the additional two/weeks period. (n=12) (ns= non-significant); *p-value =<0.5) **p-value <0.01).

4.5 Retention efficiency (percentage ingested)

It was observed significant differences related to the retention efficiency of nitrogen of ingested feed (Figure 17A), specifically between fish fed diets 3 and 4 when compared to the control (p-values = 0.04 and 0.01, respectively). However, the retained energy as a percentage of ingested energy (Figure 17B) showed significant differences only between Diet 1 and Diet 4. In contrast, lipid retention as a percentage of ingested total fat was not found to be significant across all groups of pre-smolt Atlantic salmon (Figure 17C).

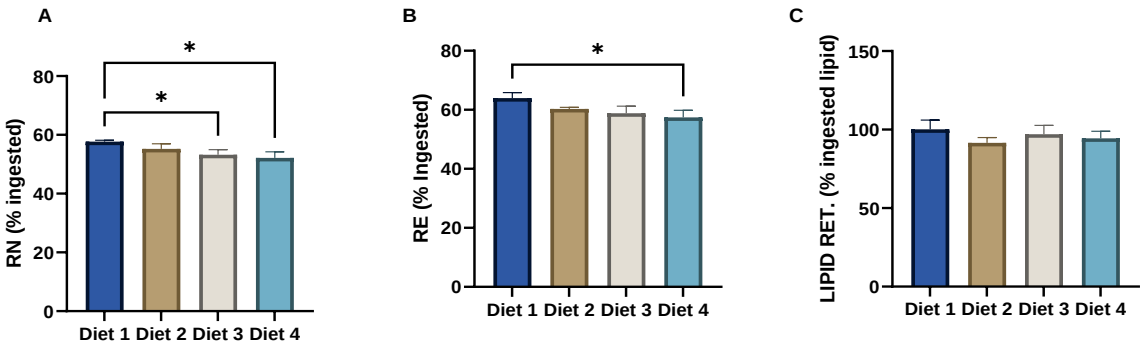

Figure 17: Retention efficiency (% ingested) of Retention of Nitrogen (A), energy (B) and Lipid retention (C) in fish groups fed different inclusion levels of *A. limacinum*. (n=12) ns= non-significant; *p-value =<0.5; **p-value <0.01.

4.6 Retention efficiency (% Digested)

The retention efficiency of nitrogen, lipid, and energy as percentages of digested nutrients or energy indicated no significant differences between fish fed diet 1-4 as shown in figure 18(A-C).

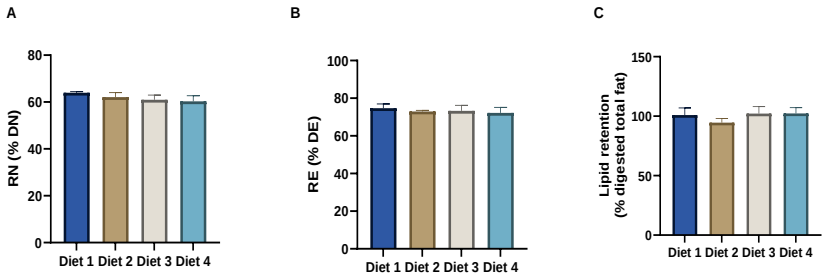

Figure 18: Retention efficiency (% digested) of Retention of Nitrogen (A), energy (B) and total fat (C) RN,TOTAL FAT in fish groups fed different inclusion levels of *A. limacinum*., (n=12) ns= non-significant; *p-value =<0.5; **p-value <0.01.

4.7 Whole body composition (WBC)

The analysis on the carcass composition of the fish did not show any significant difference between diets regarding the content of water, ash, crude protein, or total fat or gross energy (Figure 19).

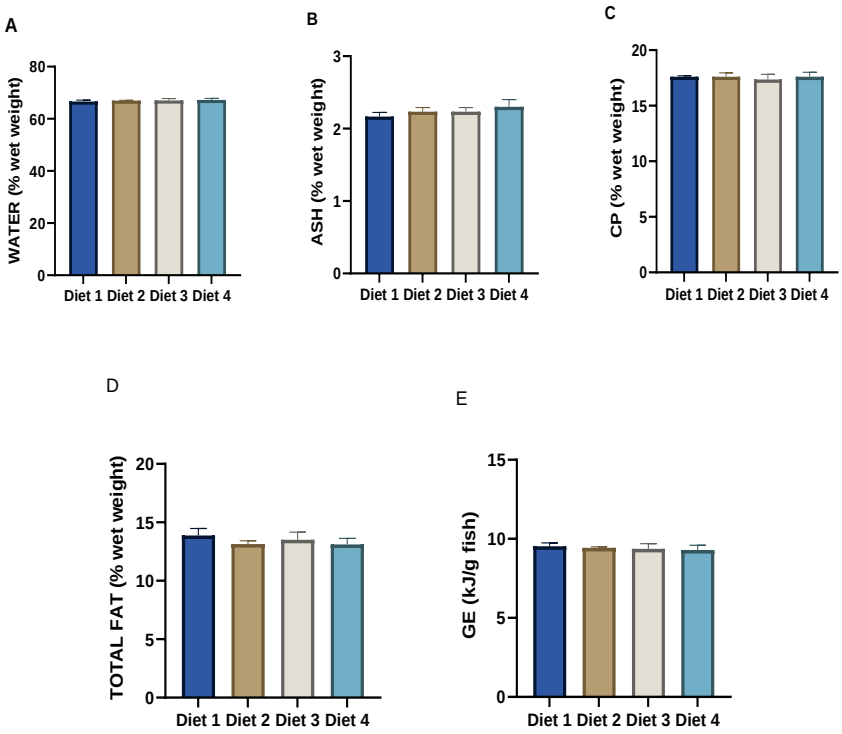

Figure 19: Whole body composition (carcass composition) of fish fed different inclusion levels of *A. limacinum*. (A) water, (B) ash, (C) crude protein, (D) total fat, expressed in (% wet weight) and (E) gross energy (kJ/g fish^{-1}).

4.8 Fatty acid analysis and retention

The ADC of fatty acids and their retention were analyzed. In the current thesis a special focus was given to EPA and DHA, due to their important roles in fish physiology and also since we are working with a novel source of DHA. We also consider ALA as it is the precursor of those fatty acids.

The ADC of EPA and DHA varied significantly across the dietary treatments. A decrease in these fatty acids with the increased inclusion of *A. limacinum* was observed (Table 13). As an example, when comparing D1 and D4, ADC of EPA was reduced by 2% while ADC of DHA was reduced more than 25%. In contrast, ALA maintained consistent high digestibility across all dietary treatments with no significant difference observed.

Table 13. Apparent digestibility coefficients (ADCs, %) of fatty acids for Atlantic salmon (*Salmo salar*) fed diets containing increasing, graded levels of *A. limacinum* (n=3 replicates per diet).

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4	SEM[1]	p-value[2]
14:0	96.9^{a}	93.3^{b}	89.6^{c}	88.3^{c}	0.7	< 0.0001
16:0	96.0^{a}	85.8^{b}	76.4^{c}	72.1^{c}	1.5	< 0.0001
18:0	93.0^{a}	90.4^{ab}	87.8^{b}	88.5^{b}	1.0	0.0227
20:0	97.9	97.5	97.6	97.2	0.3	0.5024
16:1	98.8	99.6	99.7	98.7	0.4	0.3073
18:1n9c	81.4	78.8	75.6	77.7	1.9	0.2480
18:2n6c	99.1^{a}	98.9^{ab}	98.6^{b}	98.5^{b}	0.1	0.0126
18:3n3	**99.7**	**100.0**	**99.8**	**100.0**	**0.1**	**0.3535**
20:5n3	**99.3^{a}**	**98.5^{b}**	**97.7^{bc}**	**97.2^{c}**	**0.2**	**0.0002**
22:6n3	**96.3^{a}**	**81.5^{b}**	**73.4^{bc}**	**71.5^{c}**	**1.8**	**< 0.0001**
∑ SFA[3]	95.8^{a}	88.6^{b}	81.7^{c}	78.4^{c}	1.2	< 0.0001
∑ MUFA[4]	88.7	88.0	87.0	88.0	1.1	0.7730
∑ PUFA[5]	98.9	96.5	93.8	91.9	0.5	< 0.0001
∑ n-3	98.8^{a}	93.8^{b}	89.4^{c}	86.9^{c}	0.8	< 0.0001
∑ n-6	99.1^{a}	98.9^{ab}	98.6^{ab}	98.5^{b}	0.1	0.0185

[1]Standard error mean.
[2]Significance of the one-way ANOVA or the Kruskal-Wallis test. Values in the same row with different superscripts are significantly different ($p < 0.05$).
[3]Sum saturated fatty acids.
[4]Sum monounsaturated fatty acids.
[5]Sum polyunsaturated fatty acids.

The analysis of WBC revealed that the percentage of EPA and DHA, when compared to all body total fatty acids, was significantly higher when fish were fed diets with increasing levels of *A. limacinum* (Table 13). Post-hoc analysis on EPA have shown that diets with higher inclusion levels of *A. limacinum* (D3 and D4) were significantly different when compared to control (D1), while for DHA, all dietary groups where significantly different from each other. ALA content remained consistent across all diets.

Table 14. Whole body fatty acid composition (% total fatty acids) of Atlantic salmon (*Salmo salar*) fed diets containing increasing, graded levels of *A. limacinum* (n=3 replicates per diet).

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4	SEM[1]	p-value[2]
14:0	2.53[a]	2.66[b]	2.80[c]	2.97[d]	0.01	< 0.0001
15:0	0.16	0.20	0.21	0.22	0.01	0.1869
16:0	12.93[a]	13.35[b]	13.54[b]	13.95[c]	0.05	< 0.0001
18:0	10.61	9.61	9.00	8.16	0.93	0.3607
21:0	0.56[a]	0.53[ab]	0.47[bc]	0.45[c]	0.01	0.0033
16:1	2.94[a]	3.07[b]	3.19[c]	3.39[d]	0.01	< 0.0001
18:1n9t	2.71	2.76	2.80	2.86	0.08	0.5634
18:1n9c	37.62	37.16	36.88	36.28	0.81	0.7080
20:1n9	3.51[a]	3.63[ab]	3.65[ab]	3.72[b]	0.04	0.0296
22:1n9	1.75[a]	1.90[ab]	2.02[bc]	2.22[c]	0.05	0.0015
18:2n6c	12.81[a]	12.47[b]	12.24[c]	11.79[d]	0.05	< 0.0001
20:2	0.87[ab]	0.89[a]	0.84[b]	0.79[c]	0.01	< 0.0001
20:3n6	0.36	0.36	0.36	0.36	0.01	0.9536
18:3n3	**3.23**	**3.22**	**3.21**	**3.15**	**0.02**	**0.0908**
20:5n3	**1.70[a]**	**1.85[ab]**	**1.97[b]**	**2.14[c]**	**0.04**	**0.0002**
22:6n3	**5.72[a]**	**6.39[b]**	**6.83[c]**	**7.55[d]**	**0.05**	**< 0.0001**
∑ SFA[3]	26.79	26.35	26.02	25.74	0.93	0.8682
∑ MUFA[4]	48.52	48.52	48.53	48.48	0.86	1.0000
∑ PUFA[5]	24.68[a]	25.16[ab]	25.46[bc]	25.78[c]	0.11	0.0006
∑ n-3	10.65[a]	11.45[b]	12.01[c]	12.84[d]	0.06	< 0.0001
∑ n-6	13.16[a]	12.82[b]	12.60[b]	12.15[c]	0.05	< 0.0001

[1]Standard error mean.
[2]Significance of the one-way ANOVA or the Kruskal-Wallis test. Values in the same row with different superscripts are significantly different ($p < 0.05$).
[3]Sum saturated fatty acids.
[4]Sum monounsaturated fatty acids.
[5]Sum polyunsaturated fatty acids.

When assessing the apparent retention (%) of essential fatty acids, different trends from different fatty acid analysis was observed as presented (Table 14). The apparent retention of EPA was consistent among the dietary groups. However, the apparent retention of DHA decreased with the increasing inclusion levels of *A. limacinum*. This decrease was significantly different between all dietary groups (from 151.3% to 90.3%). Regarding ALA,

diets with higher inclusion levels of *A. limacinum* (D3 and D4) were significantly different when compared to control (D1).

Table 15. Apparent retention (%) of fatty acids in the carcass of Atlantic salmon (*Salmo salar*) fed diets containing increasing, graded levels of *A. limacinum* (n=3 replicate tanks per diet).

Fatty acid	Diet 1	Diet 2	Diet 3	Diet 4	SEM[1]	p-value[2]
14:0	88.9	82.9	85.0	78.6	2.3	0.0683
16:0	110.0^{a}	96.9^{b}	91.4^{b}	79.5^{c}	2.4	0.0001
18:0	377.9	321.1	309.8	257.7	41.4	0.3070
21:0	166.8^{a}	140.0^{b}	117.9^{bc}	101.0^{c}	5.1	< 0.0001
16:1	91.9	89.2	93.1	92.6	2.6	0.7215
18:1n9t	5.9^{a}	6.1^{ab}	6.8^{bc}	7.2^{c}	0.2	0.0043
18:1n9c	1207.1	1167.3	1220.5	1191.4	33.7	0.7183
18:2n6c	77.6	76.2	82.1	82.6	1.9	0.0984
18:3n3	**59.9^{a}**	**60.7^{ab}**	**66.9^{bc}**	**68.3^{c}**	**1.5**	**0.0090**
20:5n3	**32.1**	**32.0**	**34.1**	**34.5**	**1.0**	**0.2498**
22:6n3	**151.3^{a}**	**124.7^{b}**	**107.0^{c}**	**90.3^{d}**	**2.3**	**< 0.0001**
∑ SFA[3]	155.9^{a}	132.6^{ab}	123.7^{ab}	104.6^{b}	7.7	0.0097
∑ MUFA[4]	90.6	90.4	97.8	99.5	2.5	0.0629
∑ PUFA[5]	77.9	75.8	78.5	75.7	1.7	0.5687
∑ n-3	72.1	69.7	70.1	66.4	1.4	0.1143
∑ n-6	77.6	76.2	82.1	82.6	1.9	0.0984

[1]Standard error mean.
[2]Significance of the one-way ANOVA. Values in the same row with different superscripts are significantly different ($p < 0.05$).
[3]Sum saturated fatty acids.
[4]Sum monounsaturated fatty acids.
[5]Sum polyunsaturated fatty acids.

Finally, when comparing the DHA retention with its content in the diet, supplied from FO alone in D1 or FO and *A. limacinum* in diets D2-D4, we observed a negative correlation between these two factors.

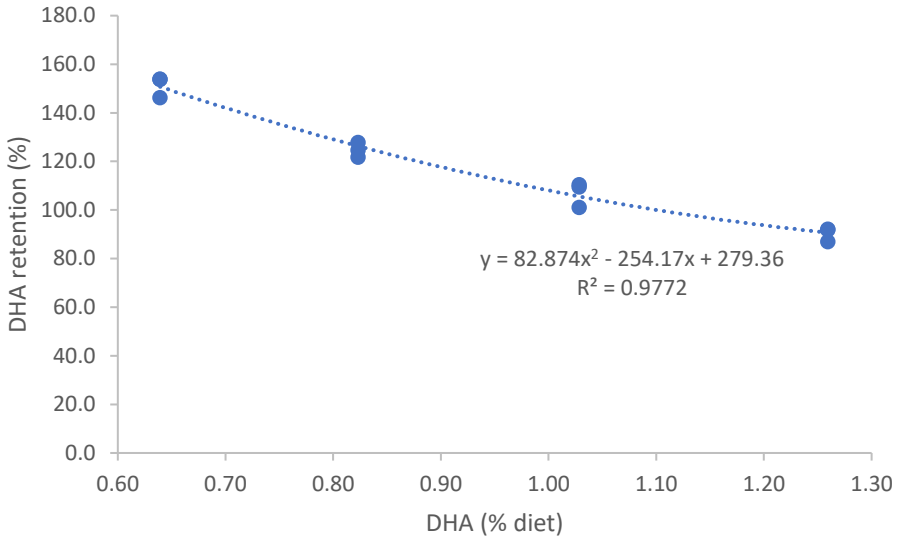

Figure 20. Polynomial trendline of the correlation between apparent retention of DHA and DHA in the feed (n=3, replicate tanks per diet).

Chapter 5 : Discussion

5.1 Pellet quality

In this study, a comprehensive screening of parameters affecting the physical quality of salmon feed pellets was conducted. The results revealed a positive relationship between the bulk pellet density of each diet (g/L) and the inclusion level of *A. limacinum* diet 1-4 having average bulk densities ranging from 561.6 to 655.8g/L. This observation could be explained by differences in pellet dimensions, specifically width and length, which would directly influence volume and density (Wolfgang et al., 2012). In this current study, diet 4 had the smallest pellet, both in length and width, which can explain the observed differences in density. However, diets were not significantly different regarding hardness. A possible cause for the differences in size could have occurred with the drying process. During pelleting, we used the same die size and similar water content. During drying, we aimed to achieve a final moisture level between 8 to 10% to reduce water activity and microbial growth. However, diets with the higher inclusion level of *A. limacinum* might have shrunk more. Consequently, these pellets were denser. The increase of density in diets with higher inclusion of *A. limacinum,* reflected in diets with higher sinking velocity. The relationship between density and sinking velocity was also observed by Milanovic (2015). Also, recent findings (Pandey, 2018) suggest that pellets with an optimal sinking velocity may ensure efficient feeding, waste reduction and maintaining a healthy environment in salmon aquaculture. These relationships highlight the importance of considering pellet density as a key determinant of sinking behavior in aquafeed formulations. The relationship between sinking velocity and feed intake will be discussed later on.

Water stability is a relevant indicator of pellet quality, especially in this current study where we worked with cold pelleting. Adequate water stability ensures that the pellets maintain their physical structure when in contact with water, and that fish is not deprived of essential nutrients. In our study, we did not observe any differences between diets in the three different time points of 15, 30 and 60 mins (Figure 12). This lack of differences indicates that inclusion levels of *A. limacinum* had no significant effect on water stability of the different diets.

5.2 Growth performance

Differences in growth performance parameters were observed among the dietary groups, including FCR, SGR, TGC, and weight gain, even though there were no significant differences in feed intake. No mortality was observed.

At the beginning of the experiment, fish had a narrow average difference in weight (29.37 ± 0.08 g/fish). After a 9-weeks period, it was observed that fish more than quadruplicate their average weight (125.9 ± 6.4 g/fish), but with differences among the dietary groups. Fish fed diets 2 and 3 showed similar SGR and TGC values as the control diet. This lack of significant differences indicates that modest inclusion levels of *A. limacinum* supported normal growth of pre-smolt Atlantic salmon. However, fish fed higher inclusion levels of *A. limacinum* had a lower weight gain, SGR and TGC compared to the control. Notably, there were no differences in feed intake between the dietary groups, still a higher FCR in fish fed D4 was observed. Similar observations were also reported in previous studies where juvenile Atlantic salmon were fed novel ingredients as a source of DHA (Ruiz et al., 2024; Zhang et al., 2020).

A higher FCR means that more feed is needed to achieve similar final weight, which in turn could increase the environmental output and cost for the aquaculture industry. The increased FCR in fish fed D4 may be attributed to various factors, such as pellet quality, sinking velocity and water stability. As Atlantic salmon prefers to feed in the water column (Milanovic, 2015) the higher sinking velocity of pellets from D4 (as discussed above) could be a factor to consider in the observed higher FCR. However, since there were not observed differences in feed intake, the differences in FCR might be due to differences in the chemical composition, nutrient digestibility, and retention among diets.

Considering the nutritional composition of the experimental diets, our observation revolved around the potential underestimation of total fat content within the *A. limacinum* paste used during formulation. This difficulty in estimation may have led to a lower inclusion of total fat in the formulated feeds than intended (Table 4 and 5). Given the essential role that fat plays as a primary energy source for fish, especially during this critical developmental stage where the fish are growing rapidly and actively accumulating energy reserves. Differences in dietary energy levels can have negative implications for the growth of pre-smolt Atlantic salmon (Tocher, 2003). Thus, we suggest that the observed differences in growth performance (FCR, TGC and SGR) among dietary groups may be partly attributed to

variations in the nutritional composition of the diets, particularly concerning fat content. Further investigation into the interplay between dietary composition, energy availability, nutrient digestibility assessment and retention is necessary to support this hypothesis and explain the observed results.

5.3 Apparent digestibility and retention

The digestibility results revealed no significant differences in the ADCs of dry matter, ash, and crude protein among dietary groups, as illustrated in Figure 15. However, clear tendencies were observed where the ADCs of total fat and gross energy decreased in fish fed higher inclusion levels of *A. limacinum* (10% and 15%). Higher ADC values for gross energy and total fat are desirable in the Aquaculture industry. These lower ADCs indicate poor digestibility and feed efficiency when Atlantic salmon are fed higher inclusion levels of *A. limacinum*. Similar results, lower ADCs of lipid and energy, were also observed when rainbow trout were fed different strains of *Nannochloropsis oceanica*, *Phaeodactylum tricornutum* and thraustochytrid as alternative sources of DHA and EPA (Sevgili et al., 2019). However, there are some studies showing the opposite. Tibbetts et al., (2020) observed increasing ADC levels for lipids and energy when juvenile Atlantic salmon were fed diets with complete or partial replacement of fish oil. Therefore, this discrepancy suggests that ADC differences could be species-related, regarding various fish species and different microorganisms species used as source of DHA, as well as their production process/treatment.

Retention of nutrients (nitrogen, energy, and total fat) follows a similar trend as digestibility, with higher inclusion levels of Microbial ingredients resulting in lower retention, also as observed in previous studies (Tibbetts et al., 2020). However, when considering retention efficiency, relative to the percentage digested, in this study, no differences among the dietary groups were observed (Figure 18). The same was also observed in whole-body carcass composition for ash, water, crude protein (CP), total fat, and gross energy (Figure 19). These results suggest that regardless of the inclusion levels of *A. limacinum*, fish utilized the digested nutrients equally.

Observations of nutrient retention efficiency as a percentage ingested diets indicated similar results, where ingested nitrogen and energy at higher inclusion levels were significantly lower than the control group (except for total fat, which shows no significant differences). These differences suggest that during fish production, nitrogen and energy retention may be

negatively influenced when pre-smolt Atlantic salmon are fed high inclusion levels of *A. limacinum*, even though total fat retention remains consistent across the dietary treatments.

5.4 EPA, DHA, and ALA retention

The apparent retention of ALA, precursor of EPA and DHA, was significantly increased following the increased inclusion of *A. limacinum*. Its apparent digestibility and its proportion in whole-body composition were not significantly different. These results could indicate that fish did not utilize ALA for energy production and/or synthesis of EPA and DHA. In fact, it may support our hypothesis where DHA requirements were fulfilled by FO, with the same inclusion levels in all diets. Regarding the apparent retention of EPA, it was similar among all dietary groups. However, EPA's apparent digestibility was significantly reduced in fish fed D4 (2% less) and present in higher percentage of whole-body composition of fish fed D4 when compared to fish fed the control diet (D1).

Interestingly, the apparent retention (%) of DHA was significantly reduced in fish fed *A. limacinum* from 151.3% to 90.3% (Table 15). Furthermore, there was a negative correlation between DHA retention and DHA content in the diets (figure 20). Even though there was a significant dose-dependency of the amount of DHA in WBC and fish fed *A. limacinum* (Table 14), there was a relevant reduction (25%) of the apparent digestibility of this fatty acid (Table 13), which might have had a larger influence. Similar results of ADC were observed by Zhang et al (2023), where they found a decreasing trend in EPA and DHA digestibility in diets containing *A. limacinum* on both Atlantic salmon and rainbow trout.

Chapter 6 : Conclusion

Based on the findings, the initial hypothesis are partially supported. The inclusion of *A. limacinum* did not affect the water stability of the pellets. Although pellet size varied, feed intake remained uniform across all diets. Fish fed higher levels of *A. limacinum* exhibited lower growth performance, indicated by higher FCR and lower SGR, potentially due to underestimated fat content in the raw materials. However, the study provided insights into the digestibility of *A. limacinum*. Fish fed higher levels showed a lower ADC of total fat but no change in retention efficiency. For DHA, there was a significant decrease in both ADC and apparent retention in diets with *A. limacinum.*

These findings from cold-pelleted diets serve as a starting point for future research on DHA sources, especially with extrusion technology, which may improve ingredient digestibility. The study underscores the importance of optimizing *A. limacinum* inclusion levels in Atlantic salmon diets and improving its production processes to enhance its nutritional value and digestibility. This could make *A. limacinum* a viable, sustainable source of DHA for pre-smolt diets.

Declaration

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

In the course of preparing this master's thesis, the author utilized Grammarly, Quillbot and ChatGPT to identify and correct spelling errors, improve grammar, enhance clarity, and refine other aspects of the English text. Following the use of these tools, the author thoroughly reviewed and edited the content as necessary and assumes full responsibility for the final publication.

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