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Estimation of endogenous loss from soy variants using the ^{15}N isotope dilution method in Atlantic salmon (*Salmo salar*)

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Master of Science in Aquaculture

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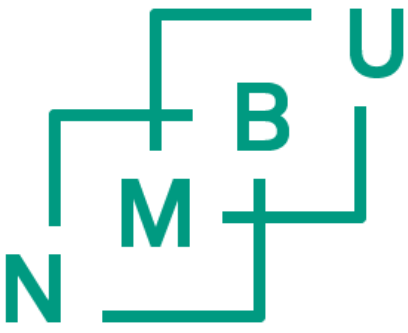

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Erica Curles

Abstract

True digestibility of feeds and feed ingredients is difficult to measure in fish, partially due to methodological challenges in quantifying endogenous losses that are mixed with undigested materials originating from the feed. This difficulty is why apparent digestibility, not corrected for the endogenous losses, is predominantly used to evaluate the quality of animal feeds. Endogenous loss has historically been estimated with several different methods to attempt to determine true digestibility. This study used the ^{15}N-isotope dilution method, which involves enriching the feed or the animal's tissues with an isotope, in this case nitrogen-15, and then measuring the nitrogen-15 in the excreted material. This isotope dilution method has been used in pigs and rats to calculate true digestibility. However, it has never been used to measure endogenous losses in fish. Soy is a common feed ingredient that may result in high endogenous loss, partially due to the Kunitz and Bowman-Birk protease inhibitors that inhibit hydrolysis by binding to the active site of digestive enzymes and prevent both enzyme and inhibitor from being absorbed. *Candida utilis* was chosen to be the vehicle for ^{15}N due to its known palatability to salmon, favorable amino acid profile, and suitability for fast, high density culture. Over one kilogram of yeast was grown in a 42 l fermenter using a 24-hour fed-batch fermentation protocol. The yeast was labeled by replacing 35.3% of the required ammonium sulfate with $(^{15}NH_4)_2SO_4$. The ^{15}N-enriched *C. utilis* was incorporated into a feed where it was the sole protein source, and this feed was given to Atlantic salmon fingerlings to label their tissues. The labeled fingerlings were then fed 3 diets with varying expected levels of protease inhibition: a diet with *C. utilis* as the sole protein source, a 30% commercial soy diet, and a diet containing 30% triple null soy, a strain of soy that lacks the Kunitz protease inhibitor. No high temperature treatment was employed during feed processing, in order to maintain activity of the protease inhibitors in soy. The percentages of ^{15}N in the feces and in the muscle and pyloric caeca were analyzed. Endogenous loss was calculated using the isotope dilution equation with both muscle and pyloric caeca as the ^{15}N pool. The muscle was enriched in ^{15}N by a factor of approximately 5 times, while the pyloric caeca was enriched by a factor of approximately 25 times. The percentage of ^{15}N in the muscle did not significantly change while the fish were fed unlabeled feeds, while the percentage of ^{15}N in the pyloric caeca decreased quickly. Both the corrected feces ^{15}N percentages and the endogenous nitrogen losses partially matched the expected pattern with the commercial soy-fed fish having higher values than both the triple null soy- and yeast-fed. The diet comprised of 30% commercial soy caused approximately double the endogenous nitrogen loss of the diet with yeast as the sole protein source. The diet made with 30% triple null soy did not cause significantly more endogenous nitrogen loss than the yeast diet. This was unexpected

because the triple null soy still contained the Bowman-Birk protease inhibitor while the yeast diet was not intended to contain protease inhibitors. The main point of contention when using the isotope dilution method is whether the chosen source for ^{15}N (i.e. plasma, tissue type, organ) is representative of the amount of ^{15}N secreted into the digestive system. Using whole pyloric caeca has not been attempted before. The results indicate that whole pyloric caeca may be a relatively accurate source pool for endogenous loss of enzymes, such as trypsin and chymotrypsin, that are secreted by the pancreatic tissue embedded in the pyloric caeca. The endogenous loss calculated with the isotope dilution equation can be used to calculate true digestibility by subtracting the endogenous nitrogen from the total fecal nitrogen. The data obtained on pyloric caeca uptake and loss from this study were sufficient to rank the endogenous losses of the diets but not sufficient to accurately use it as a ^{15}N source pool. This means that any estimate of true digestibility from this data alone would likely be inaccurate. To increase the accuracy of true digestibility estimates using pyloric caeca as the source of ^{15}N, further studies should be done using feeds with known true digestibilities and compare the digestibility estimated by using isotope dilution. An accurate regression model of the rate of loss of ^{15}N from pyloric caeca should also be calculated using frequent sampling of labeled tissues while consuming an unlabeled simple (i.e. fishmeal) diet until the tissue returns to normal enrichment levels.

Keywords: Endogenous loss, isotope dilution, nitrogen-15, apparent digestibility, true digestibility, Atlantic salmon (*Salmo salar*), soy, triple null soy, protease inhibitors, Kunitz, Bowman-Birk, *Candida utilis*, fermentation

Table of Contents

List of Figures

List of Tables

1. Introduction

1.1. Endogenous loss

1.1.1. Digestibility

When discussing the energy an animal obtains from food, the energy is broken down into four categories: gross, digestible, metabolizable, and net energy (NRC 1998). Gross energy is the heat produced by combustion of the feed, digestible energy is the gross energy minus the energy lost in feces, and metabolizable energy is the digestible energy minus the energy lost in urine and gases (and ammonia excreted via the gills in fish). The net energy is the energy that the animal retains after subtracting heat increment caused by the feed intake from metabolizable energy. This experiment focuses on digestible energy, as it is a common first estimate for availability of the nutrients and energy provided by a feed. Apparent digestibility of nutrients and energy is a commonly used estimate for digestible energy that does not account for endogenous losses (FAO 1985). The equation to estimate apparent digestibility is therefore relatively simple and is shown below:

$$Apparent\ digestibility\ (\%) = \frac{I - F \times 100}{I}$$

where I = intake of the nutrient, F = fecal output of the nutrient (FAO 1985). The formula for calculating true digestibility of a feed is:

$$True\ digestibility\ (\%) = \frac{I - (F - F_k) \times 100}{I}$$

where F_k = the amount of the nutrient in the feces that comes from endogenous sources (FAO 1985).

When measuring protein digestibility, there is a wide variety of endogenous components that apparent digestibility does not include, such as endogenous enzymes and amino acids, bacterial protein, mucin protein, serum albumin, and epithelial cells that have been sloughed off the intestinal wall (Nyachoti et al. 1997). It is debatable whether bacterial protein truly represents a type of endogenous loss, however it is always present in feces (Souffrant 1991). Several dietary factors will increase endogenous protein loss including fiber (Larsen et al. 1993) and the level of protein in the feed (Nyachoti et al. 1997). Another important cause of endogenous loss, and the cause focused on in this study, are antinutrients present in certain plants. Many studies have shown that an increase in protease inhibitors in the feed, usually from soy or peas, causes an increase in endogenous nitrogen loss in pigs (Leiner & Kakade 1980, Huisman et al. 1992, Grala et al. 1998) and in Atlantic salmon (Krogdahl et al. 2003). Not accounting for endogenous loss can lead to underestimating the digestibility of nutrients (Adeola et al. 2016), which can lead to a

higher than necessary inclusion level of the nutrient in question in the diet. It is important to be able to assess these endogenous losses and use them to find the true digestibility of feed components in order to optimize feed design, reducing both cost and waste.

1.1.2. Atlantic salmon digestive anatomy

Atlantic salmon (*Salmo salar* Linnaeus), the species used in this study, is carnivorous and therefore proteases, such as pepsin, trypsin, and chymotrypsin, are of high importance in the digestion process (Einarsson & Davies 1996, Rungruangsak-Torrissen et al. 2006). In Atlantic salmon, pepsinogen is secreted from the cardiac region of the stomach, while trypsinogen and chymotrypsinogen are secreted by pancreatic cells diffusely located in the fat between the pyloric caeca (Einarsson & Davies 1996). Like most carnivorous animals, salmon have a relatively short, simple digestive tract with the important sections to this study being the pyloric caeca, mid-intestine, and distal intestine (Figure 1). Atlantic salmon do not demonstrate a high amount of microbial activity in their distal intestine (Ringø et al. 2015) partially because their body temperature is too low to support high microbial activity (Cahill 1990). Additionally, natural feed organisms for salmon diets are low in indigestible sugars and non-starch polysaccharides and the fish therefore do not need microbes to break down these compounds (Cahill 1990). Atlantic salmon has the capacity to reabsorb some amino acids in the distal intestine (Ingham & Arme 1977, Bakke-McKellep et al. 2000), however they do not absorb lipids there (Denstadli et al. 2004). This means that salmon cannot reabsorb endogenous compounds, including proteins and their catabolites from fermentation, to the same extent as herbi- and omnivores and could not absorb the medium chain fatty acids that would be produced by microbial fermentation even if activity was high (Boisen & Eggum 1991, Collinder et al. 2003). The low microbial activity means that the amount of endogenous nitrogen in the feces should not be significantly different to the endogenous nitrogen at the end of the small intestine, unlike in animals with hindgut fermentation, like pigs (Darcy-Vrillon et al. 1991). This means that, when estimating endogenous loss in salmonids or other fish without significant microbial fermentation, external fecal collection methods can be used instead of surgically inserting fistulae to collect digested material from the small intestine, as is being done in pigs (Nyachoti et al. 1996). This makes collection of digested material needed to estimate digestibility and endogenous loss from fish much simpler.

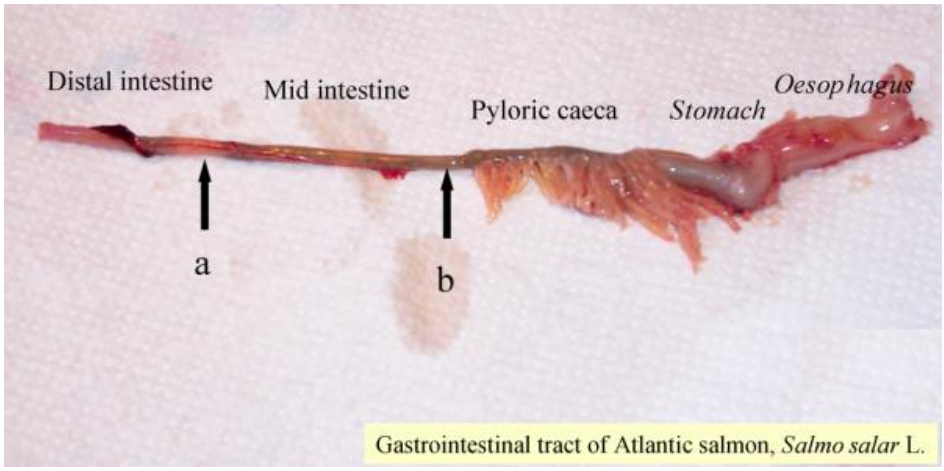

Figure 1. The digestive tract of an Atlantic salmon (~100g). The (a) and (b) labels indicate the starting point of the mid and distal intestine respectively (Sanden & Olsvik 2009).

1.1.3. Protease inhibitors

There are several prominent compounds in plants that cause an increase in endogenous loss by binding to internal molecules or minerals, such as protease inhibitors which bind to digestive enzymes, including trypsin and chymotrypsin (Norton 1991). Protease inhibitors in soy, namely the Kunitz and Bowman-Birk-type inhibitors, and the endogenous loss they cause is the focus of this study.

There are two main strategies when it comes to removal of antinutrients from feeds made from plant material: genetic modification of the source crop or additional processing of the feed ingredient, either physical, chemical or both. The Kunitz protease inhibitor is heat-labile, meaning that heat renders it inactive by altering its structure so it can no longer bind to trypsin (Chen et al. 2014). The Bowman-Birk inhibitor is heat-stable and thus is much more difficult to inactivate or remove from soy (Norton 1991). However, the Kunitz inhibitor is responsible for most of the trypsin-inhibiting activity (Kassell 1970, Kumar et al. 2018) making Bowman-Birk less damaging than Kunitz in unprocessed soy. The United States Department of Agriculture USDA has developed a strain of soy known as "triple null", referring to the fact that it has been bred to lack three negative compounds: soy agglutinin, Kunitz protease inhibitors, and P34 (an allergen) (Schmidt et al. 2015). However, Teimouri (2018) showed that there was no difference in intestinal histology or growth rate between Atlantic salmon fed triple null soy versus commercial full-fat soybeans. Genetic modification has the advantage of reducing processing costs and only having to be accomplished once to create an entire crop but involves expensive research. Inactivation of antinutrients by heat requires precision because it must balance the destruction of useful components, such as essential amino acids, with the desired inactivation of harmful compounds. Therefore, heat stable antinutrients like Bowman-Birk cannot be completely

inactivated with processing without also reducing the quality of the feed (Gatlin et al. 2007). Heating all of the feed required for such a large industry also requires a lot of energy and is costly for feed manufacturers (Clarke & Wiseman 2000, Gillman et al. 2015).

This study aims to quantify endogenous nitrogen losses (representing the lost trypsin and chymotrypsin) in Atlantic salmon fed soy diets containing different levels of protease inhibitors.

1.2. Methods for measuring endogenous loss

Basal endogenous loss, which is the loss of a given nutrient due to basic metabolic activity such as oxygen exchange or movement, can be estimated relatively easily. For example, endogenous loss of nitrogen has been estimated in many species by feeding a nitrogen-free diet to the animal in question and calculating the fecal loss of the nutrient while on this diet (Wünsche et al. 1987, Sanz et al. 1994, Stein et al. 2007). This is not representative of real-world conditions and therefore may not be accurate (Spindler et al. 2016). Estimates of endogenous loss from feeding an N-free diet are lower than those calculated when feeding diets that do contain protein (Boisen & Moughan 1996), suggesting that this method underestimates basal endogenous nitrogen loss under normal feeding conditions. Feeding a nitrogen-free diet also only detects basal endogenous loss and not endogenous loss caused by compounds in feed that bind to internal molecules and then are excreted together. Measurement of endogenous loss caused by feed ingredients is more difficult and many methods have been developed to try and quantify this loss. When measuring endogenous loss in pigs or any animal with significant intestinal microbial activity, the contents at the end of the small intestine, called the ileal digesta, should be used because the fermentation in the hind gut can alter the amount of endogenous material in the feces (Boisen & Eggum 1991, Darcy-Vrillon et al. 1991). The N-free diet method cannot be used to determine endogenous loss in salmon because salmon would not accept a nitrogen-free diet (Storebakken, Pers. Comm.).

The second most simple method, after the N-free diet method, is the regression method. In studies using this method, animals are fed diets with varying levels of an ingredient to the animal to generate a regression line based on the endogenous losses at those levels. The basal endogenous loss is therefore the y-intercept of this line, where the ingredient would theoretically be missing from the feed (Taverner et al. 1981). This method produces estimates similar to the N-free diet method, indicating that the regression also underestimates endogenous loss (Leibholz & Mollah 1988, Donkoh et al. 1995).

The third commonly employed method is called enzyme-hydrolyzed casein (EHC) with ultra filtration. This method involves adding peptides and free amino acids that are meant to

mimic the products of the normal hydrolysis of casein as the sole protein source for the animals. The products of digestion are then centrifuged and filtered and the precipitate of what passes through the filter and the high molecular weight fraction in the filter are measured as the endogenous loss (Moughan et al. 1992). This produces slightly higher values for endogenous loss but some endogenous products are lost in filtering, though this is thought to be negligible (Butts et al. 1991). It is thought that the presence of dietary peptides in the diet somehow leads to more realistic estimations compared to the N-free experiments (Boisen & Moughan 1996).

Another method is the homoarginine/guanidination method. In this method, lysine in the feed is converted to homoarginine (Hagemeister & Erbersdobler 1985), which is an amino acid that is not metabolized or usable by animals for protein synthesis (Schmitz et al. 1991). This means than any homoarginine in the digestive tract is exogenous and homoarginine content is therefore a proxy for the percentage of dietary material not digested at the point of sampling. Various points in the digestive tract can be measured by this method and the amount of dietary protein present can be calculated. Endogenous loss can then be determined as well by subtracting the exogenous nitrogen from the total nitrogen present. The disadvantages of this method are that it relies on the assumption that all amino acids are absorbed at the same rate, which is true for most but not all, and that the experiments cannot last very long because homoarginine may be toxic (Boisen & Moughan 1996). Modifying the lysine may also affect digestibility, but this is not definitive (Drescher et al. 1994).

Boisen and Eggum described another method for estimating endogenous loss, which is to compare *in vivo* values of nitrogen loss in digesta to digestibilities determined *in vitro* (1991). Boisen and Fernandez (1995) developed a method to assess digestibility of feeds *in vitro* using pancreatin that generated estimates similar to known true values. If the *in vitro* estimates are close to true values, then the endogenous loss can be calculated by simply subtracting the *in vitro* estimate from the total nitrogen in a sample from an experimental animal fed the same material. However, the *in vitro* estimates are not reliable for several amino acids (Boisen and Fernandez 1995), making it a less than ideal method.

The final method, and the method this experiment aims to use to identify endogenous loss from soy in fish, is known as isotope dilution and will be discussed at length in the next section.

1.2.1. The uses of stable isotopes

Stable isotopes have been used to track isotopic signatures because even small differences can be detected due to the precision of the analysis (Verschoor et al. 2005). Stable

isotopes of nitrogen can be used to deduce the position of an animal in a food web if the isotope ratio of the base of the web is known. This is because the ratio between ^{15}N and ^{14}N (the most abundant form of nitrogen) increases in a predictable fashion in animal tissues as trophic level increases (Deniro & Epstein 1981, Perkins et al. 2014). Consumer tissues show an enrichment in ^{15}N compared to their diet, though the mechanism for this retention of ^{15}N is unclear (Kelly 2000, O'Connell et al. 2012). More efficient retention of ^{15}N may be due to the faster reaction speed of $^{14}NH_2$ during transamination (Macko et al. 1986). Excreted nitrogen is also lower in ^{15}N than the tissues of the animal (Minagawa and Wada 1984), and detritivores and animals that produce ammonia as the waste product have less ^{15}N in their tissues (Vanderklift & Ponsard 2003). Animal tissues also become enriched in ^{15}N during periods of fasting (Hobson et al. 1993) or when their diet is low in protein due to the recycling of endogenous amino acids, which are already enriched compared to the diet (Vanderklift & Ponsard 2003). Muscle is most commonly accepted to be representative of the whole-body composition and is the least variable tissue in terms of assimilation rate (Pinnegar & Polunin, 1999). There is evidence in Epp et al. (2002) to suggest that animals absorb ^{15}N more effectively when it is incorporated into a biological feed ingredient, like algae, versus when ^{15}N is supplied in a synthetic form. In their study, shrimp in tanks where the water, and thereby algae, was enriched with ^{15}N had a higher level of ^{15}N in their muscle by the end of the experiment than those fed ^{15}N-amino acids. The researchers attributed this difference to the high solubility of crystalline amino acids – the amino acids were lost to the water before the shrimp could assimilate the ^{15}N. Algae cells would not dissolve and therefore ^{15}N was transferred more effectively to the shrimp by the enriched algae (Epp et al. 2002).

The well-established precise methodology associated with stable isotope analysis makes "labeling" animals with stable isotopes an appealing method to observe internal processes, along with the safety and simplicity of handling stable isotopes compared to radioactive isotopes (Verschoor et al. 2005). There have been several studies involving enriching the ^{15}N content of algae, diatoms or *Artemia* to investigate the nutritional contributions of different feed ingredients to the diet of herbivores, mostly shrimp. Differentially enriching feed ingredients or using feed ingredients with different isotopic signatures allows for the study of relative contributions of different protein or lipid sources, as in Gamboa-Delgado (2009). He determined the difference in nutritional contribution of live feeds versus inert feeds to Senegalese sole (*Solea senegalensis*) larvae. D'Avanzo et al. (1991) used ^{15}N to enrich dissolved organic material (DOM) and used that labeled DOM to quantify how important such material is in the nutrition of the sheepshead minnow and the grass shrimp. Stable isotope analysis has many benefits but does still require significant sample preparation and is relatively expensive to analyze (Verschoor et al. 2005).

Enriching diets or animals with ^{15}N is also the set up for the isotope dilution method. This method in general uses the concentration of an isotope in a source material and measurements of the concentration of the same isotope after digestion to estimate how much of the element is from the source. For measuring endogenous protein loss, the animal is labeled, which in pigs involves continuously intravenously injecting synthetic ^{15}N-enriched amino acids, and the percentage of ^{15}N in the ileal digesta is measured. This ^{15}N is then compared to the ^{15}N in the source material to produce an estimate of the amount of endogenous N in the feces. The most debated part of this method is what to use as the source for ^{15}N (Moughen 1992). Many studies have used plasma, specifically the TCA-soluble part (De Lange et al. 1990, Schulze et al. 1995, Lien et al. 1997, Grala et al. 1998). However, this may not be an accurate estimate for the source of digestive enzymes, as Souffrant et al. (1993) found that pancreatic juices were much higher in ^{15}N than TCA-soluble plasma (Lien et al. 1997). The isotope dilution method has been shown to produce results similar to the EHC method (Schulze et al. 1995). However, Hodgkinson et al. (2003) demonstrated that when pigs were fed ^{15}N, calculated endogenous loss was lower than EHC values and when pigs were given ^{15}N intravenously.

The main barrier to using the isotope dilution method in fish has been how to label the fish, since it would be difficult to attach IVs to fish and would cause stress to inject them multiple times, which would impact feeding. Additionally, oral labeling of animal tissues is said to be superior due to the fact that it also labels the lumen and thus endogenous proteins originating there can also be tracked (Leterme et al. 1998). The compounds used when feeding pigs or rats ^{15}N, namely amino acids (Herrmann et al. 1986), or ammonium acetate or ammonium chloride (Berger et al. 1994), are soluble in water and may not be stable in fish feed. Leterme et al. also suggested providing the animal with a wide variety of amino acids so that all of the amino acids will be equally labeled and not affected by transamination bias (1998).

This study investigates a potential solution for this problem. All of the methods for measuring endogenous loss are difficult to apply accurately to fish because feces collection methods in small fish involve dissection (time-consuming and can only happen once per fish), potential contamination with gut contents (stripping) or some residence time in water, which means some fecal contents are lost to the water. Stripping and dissection are effective in large fish but those would require more ^{15}N to sufficiently label their tissues, which is expensive and takes a while to absorb.

1.2.2. Yeast as a source of protein

Candida utilis, also known as Torula yeast, has been used as a protein source for humans and animals for decades and has many positive characteristics that are desirable for an alternative to fishmeal. *C. utilis* is a fast-growing yeast that is Crabtree-negative, meaning that it does not convert excess glucose into ethanol. It is also Kluyver-positive, meaning it needs high levels of oxygen to make complex sugars (Nie et al. 2010). Crabtree-negative yeasts are ideal for generating large amounts of biomass using simple fermentation styles because they can be provided with large amounts of glucose at once and be left to grow instead of requiring the glucose to be fed at a precise rate. Due to its Crabtree-negative status, *C. utilis* is ideal for high cell density culture, which requires little processing post-fermentation and does not require much equipment to produce large yields (Nie et al. 2010). *C. utilis* also can have a high protein content and favorable amino acid profile as a protein source for fish depending on the media used and style of fermentation, and it is palatable to salmon (Øverland et al. 2013). *C. utilis* can also be grown on sustainable material that would not otherwise be consumed by humans, such as seaweed and wood hydrolysates, making it a sustainable alternative protein source (Øverland & Skrede, 2017).

Candida utilis was chosen in this study due to the success of replacing 40% of the crude protein in a typical Atlantic salmon feed from fishmeal with *C. utilis* in Øverland et al. (2013). The growth rate and feed conversion ratio (FCR) of the salmon, and the nutrient digestibility of the feed with *C. utilis* were not significantly different from salmon fed the conventional feed with only fishmeal. The diets including *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* were not as comparable to the conventional fishmeal diet and had negative effects on the salmon, such as reduced growth and higher FCR in the case of *S. cerevisiae* and higher distal intestine and gut weight with both yeast feeds (Øverland et al. 2013).

In this study, yeast enriched in ^{15}N was used to label the tissues of Atlantic salmon in order to calculate endogenous nitrogen losses from soy using the isotope dilution method. It was expected that feed made with commercial soy would cause greater level of endogenous loss due to the presence of both Kunitz and Bowman-Birk protease inhibitors. The USDA triple null soy was expected to cause a moderate amount of endogenous loss due to the effect of the Bowman-Birk inhibitor. A feed made with unlabeled yeast was expected to inflict the least amount of endogenous loss due to the lack of known antinutrients in that feed. This pattern was anticipated to be reflected in the tissues and the feces. Using the isotope dilution method to determine the ^{15}N loss, the amount of endogenous loss caused by each of the protease inhibitors can be separated and quantified.

2. Methods

2.1. Inoculum/Yeast culture

Candida utilis LYCC 7549 was obtained from Lallemand (Salutaguse, Estonia). Single yeast colony grown on yeast extract peptone dextrose (YPD; Sigma Aldrich, St. Louis, MO, USA) agar plates was used to inoculate 5 ml YPD medium and grown overnight at 30°C. One ml of this culture was used to inoculate 50 ml YPD in 250 ml baffled shake flasks and grown overnight at 30°C and with shaking set to 200 rpm. Starting OD_{600} of the fermentation was set to 0.5.

2.2. Fed-batch fermentation

The media composition and fermentation protocol were based on the media and protocol developed by Nie et al. (2010) that resulted in the highest final biomass of *C. utilis*. The fermentation media was comprised of (g l^{-1}): glucose 70, $(NH_4)_2SO_4$ 10, KH_2PO_4 4, $MgSO_4$ 0.25, $FeSO_4$, $MnSO_4$ 0.01, $ZnSO_4$ 0.003, $CuSO_4$ 0.001. All ingredients came from Sigma Aldrich (St. Louis, MO, USA) except for the glucose, which came from the Center for Feed Technology (Fôrtek) at the Norwegian University of Life Sciences (NMBU). The glucose was dissolved into dH_2O at a concentration of 600 g l^{-1} and sterile filtered using a 0.22 µm Millipore Express Plus filter. All other solutions were autoclaved, with each of the metals stored separately in 1000x stock solutions, due to precipitation occurring when they were combined. The $FeSO_4$ solution was kept at pH 2.1 to prevent precipitation when autoclaving. The starting volume in the fermenter was 1 l.

The yeast culture was grown at 30°C in 2.5 l Minifors bioreactors (Infors HT, Bottmingen, Switzerland). pH was set to 5.5 and controlled using 1M NaOH and 1M H_2SO_4. Dissolved oxygen was maintained at 20% by stirrer cascade (200-700 rpm) and manual airflow control. Glanapon DB 870 (Busetti, Austria) was diluted 2 times and added automatically to reduce foaming.

Additional media was pumped into the fermenters starting at the 16 hour time point. This feeding medium consisted of the same ingredients but different concentrations due to the shorter fermentation time (8 hours). Glucose was increased to 80 g l^{-1} to reach a final concentration of 150 g l^{-1}, $(NH_4)_2SO_4$ was decreased to 8 g l^{-1} and KH_2PO_4 was decreased to 3 g l^{-1}. Minerals were added individually by sterile needle and syringe, the other ingredients were mixed and put into a feed bottle. Approximately 150 ml of feeding medium was added. The feeding rate was 5 from 16 to 19 h and then 4 until it ended at the 21.5 h mark.

The 30 l fermentation was run in a 42 l Techfors S bioreactor (Infors HT, Bottmingen, Switzerland) with the same protocols as the 2.5 l batch except that the ammonium sulfate in the initial medium was at a concentration of 10 g l^{-1} with 48% of that being $(^{15}NH_4)_2SO_4$, and in the feeding medium the ammonium sulfate was 100% unlabeled and at a concentration of 3 g l^{-1}. The feeding rate started at 25, then was increased to 30 at the 20 hour mark. The final culture was centrifuged down in 1 l containers for 10 minutes at 15,900 G using a Beckman Coulter (Brea, California, United States) Avanti J-26 S XP centrifuge equipped with a JLA-8.1 rotor. The pellet was then washed once, resuspended with dH_2O and poured into containers for freeze drying.

2.3. Yeast sampling

From the 2.5 l fermenters, 15 ml samples were withdrawn every 4 hours until the 16 h timepoint after which the frequency was increased to 2 h. Samples were immediately stored on ice and 3 x 1ml aliquoted into tared Eppendorf tubes. Samples were centrifuged at 10,000 G for 5 min with a Thermo Scientific Heraeus Pico 21 Microcentrifuge (Waltham, Massachusetts, United States). The pellet was washed twice with dH_2O and dried completely at 105°C for dry weight estimation. The remaining sample was centrifuged at 4,150 G for 10 min and the supernatant stored at -20°C for glucose and ammonium analysis.

For estimation of nitrogen and crude protein content by the Kjeldahl method, 50 ml of culture was withdrawn at time points 16 h, 20 h, and 24 h from the fermenter and spun down at 4,150 G for 10 minutes with a VWR Mega Star 1.6R centrifuge (Lutterworth, Leicestershire, UK). The pellet was washed twice with dH_2O and dried completely at 105°C.

When sampling the 30 l fermenter, approximately 35 ml of culture was extracted every 4 h and 3 x 1 ml aliquots were taken from that 35 ml and put in tared Eppendorf tubes. Each sample was centrifuged at 4,150 G for 10 minutes. The supernatant was taken for ammonium and glucose analysis and the pellet was washed twice with dH_2O and frozen at -20°C. The Eppendorf tube samples were treated the same as in the small scale run. The Kjeldahl method was used on the collected yeast after freeze drying to determine crude protein content of the end product. The Kjeldahl analysis was performed using a Kjeltec 8400 fully automated Kjeldahl analyser (Foss, Denmark). The methodology used is from AOAC International (2002) with slight modifications: 15ml H_2SO_4 was used, the boiling time was changed to 45 min, and 65 ml water was used.

2.4. Fermentation analysis

The dry weight of *C. utilis* over time was calculated by subtracting the original weight of the Eppendorf tubes from the weight of the tube with the pellet and then corrected for drying loss. Ammonium concentration in the supernatant was determined with a colorimetric NH_4^+ freshwater test (Merck Spectroquant Ammonium test 0.010-3.00 mg l^{-1} NH_4-N, Darmstadt, Germany) and using a Merck Spectroquant NOVA 60 spectrophotometer (Darmstadt, Germany). Glucose concentration in the supernatant was determined using a Megazyme GOPOD colorimetric assay and a Thermo Scientific (Waltham, Massachusetts, United States) Genesys 10S UV-Vis spectrophotometer. The Kjeldahl results were multiplied by 6.25 to obtain crude protein content.

2.5. Making the labeled yeast feed

800 g of the ^{15}N-labeled yeast from the 30 l fermentation was lysed by passing it though a Microfluidics LM20 Microfluidizer High Shear Fluid Processor (Westwood, Massachusetts, United States) once at 20,000 psi. 50 ml samples were taken before and after lysing to be analyzed using the Zen 2 software with a Zeiss Axio Vert.A1 microscope (Jena, Germany) to determine the percentage of broken versus intact cells and the Bradford protein assay on the supernatant. The yeast was then freeze dried and milled using a 1mm screen to obtain particles less than 1mm in size. The yeast was then incorporated into feed using the formulation in Table 1.

Table 1. Composition of labeled feed given to *S. salar*, made from *C. utilis* grown on media highly enriched in ^{15}N. Amino acid supplementation was based on the amino acid composition of *C. utilis* as determined by Øverland et al. (2013).

Ingredient	**Percentage of feed (%)**
C. utilis	69.0
Fish oil	18.0
Pregelatinized potato starch	5.0
Sodium alginate	2.0
Vitamin premix	0.5
D,L methionine	3.5
L lysine	0.7
L threonine	0.68
L valine	0.3
L histidine	0.3
L tryptophan	0.06

All dry ingredients were first mixed in a kitchen mixer with fish oil, and then water was added until the feed became sufficiently sticky to hold a solid pellet shape. The feed was pelleted

using an Italgi (Carasco, Genoa, Italy) P35A pasta extruder with a 1.5 mm die and then kept frozen at -20°C. This resulted in 1,235 g of feed.

2.6. ^{15}N uptake rate from feed

300 Atlantic salmon (*Salmo salar*, Aquagen, batch 14, 11, Trondheim, Norway) approximately 10.5 g in size were maintained in 2 70-l tanks. The fish were given 20-25 g per day of feed made from unlabeled yeast Lallemand (Salutaguse, Estonia) to test the feeding response and acclimate them to eating yeast for 2 weeks. After this, fish below 8 g and above 17 g were graded out and the remaining 224 fish with an average weight of 12.1 ± 0.31 g (mean ± s.e.m) were given 25 g per day of feed made from the ^{15}N labeled yeast grown as described above. The fish were fed continuously using automatic belt feeders to which feed was added once a day. During the time period when the fish were fed the labeled feed, the water was switched to flow-through with municipal water as the source to prevent altering the nitrogen isotope ratio in the biofilter bacteria. Water flow was manually adjusted to maintain oxygen saturation levels in both tanks consistently above 80% in the inlet (and usually above 90%) and the water temperature ranged from approximately 10.8 °C to 12 °C.

The fish were sampled at 6 time points to determine the rate of uptake of ^{15}N in different tissues. Approximately 24 hours before each sampling (except day 1), 10 fish were moved to a separate tank to starve them for a day to empty the gut. The fish were sampled the day before the labeled feed was started, then day 1 (12 hours after the labeled feed started), day 4, day 7, day 11, and right before the soy feed was started (day 25). The tissues collected were pyloric caeca, liver, mid-intestine, muscle, skin, and gill arches. For weighing the fish or moving them between tanks, the fish were sedated using Finquel MS-222 (Tricaine Methanesulfonate) buffered with sodium bicarbonate ($NaHCO_3$). For fish who were dissected, they were euthanized using an overdose of the same buffered Finquel. At the end of the experiment, all the remaining fish were euthanized with Finquel and stored at -20 °C.

2.7. Making the unlabeled feeds

The unlabeled feeds were made with the same methodology, pasta extruder, and die as the labeled feed. Components with larger particles, such as the soy and the fishmeal, were either milled or sieved using a 0.5 mm mesh. Three feeds were made: a *C. utilis* diet (CU) using the same formulation as the labeled feed but with unlabeled yeast, a soy diet using commercial soybeans (CS), and a soy diet using the triple null soy from the USDA (ES). The two soy diets

were made using the same ingredients (except for the different soybeans) and in the same percentages as listed in Table 2.

Table 2. Composition of soy feeds given to *S. salar*.

Ingredient	**Percentage of feed (%)**
Soy (full fat or triple null)	30.0
Fish meal	27.6
Fish oil	15.0
Pregelatinized potato starch	15.0
Vital wheat gluten	7.0
Mono calcium phosphate (MCP)	1.82
Vitamin premix	0.5
Limestone	0.4
Stay C 35%	0.1
Choline chloride	0.03
Y_2O_3	0.01
D,L methionine	1.3
L lysine	0.7
L valine	0.3
L threonine	0.18
L tryptophan	0.06

2.8. Feces sampling

The fish were moved on day 25 after the final ^{15}N uptake sampling from tanks 8 and 9 to tanks 1-10 (except 5) and each tank was assigned to one of the three diets (see Figure 1). There were 19 fish in each tank and they were fed the unlabeled diets for 18 days. The water from the tank outlet was run over a wedge wire screen (Oluwaseun et al. to be published) before filtering and reuse in order to separate out the feces and uneaten feed. Feed consumption was monitored by observing uneaten feed on the screens and when the fish began to eat the soy feed at the same level as the yeast feed (at day 5 of the unlabeled diet portion of the experiment), sampling of the feces began. The fish were fed twice daily for 1 hour and the feeders were checked at the end of the hour to ensure they were empty. Fifteen minutes after the feeder belts were checked, the screens were wiped clean and the material discarded. Then after 45 minutes, the material on the screen was scooped off into a labeled aluminum tray (two for each tank, one for the first 9 days of unlabeled feed and one for the last 5 days) and kept at -20°C.

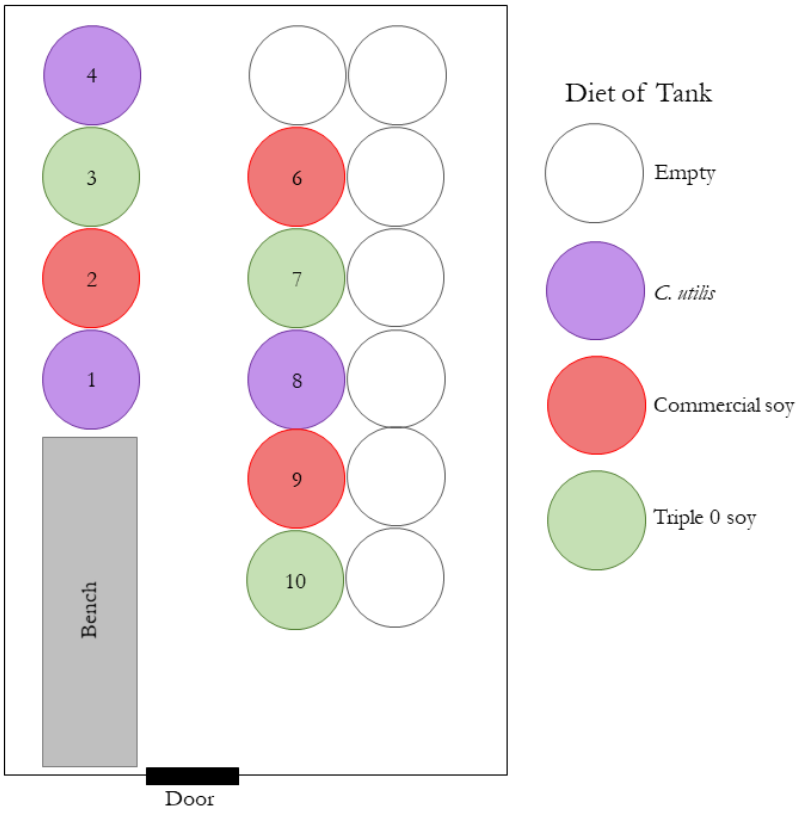

Figure 2. Layout of tanks in the experimental room, color coded by which tanks were used and which type of feed given to the tank.

2.9. Digestibility analysis

Yttrium content of the feces was analyzed using a Microwave Plasma Atomic Emission Spectrometer (MP-AES). Nitrogen content of feed and feces was calculated by the Stable Isotope Laboratory at IFE (Institute for Energy Technology, Kjeller, Norway). Apparent nitrogen digestibility was calculated using the following equation:

$$100\% - 100 \times \left(\frac{Y_2O_3 \; in \; feed}{Y_2O_3 \; in \; feces} \times \frac{N \; in \; feces}{N \; in \; feed} \right)$$

2.10. ^{15}N analysis

Samples of muscle and pyloric caeca tissue from days 0, 4, 7, 11, 25, and 42 were prepared using either the Qiagen Tissuelyser II (Hilden, Germany) (muscle, feces, and yeast) or grinding using a porcelain mortar and pestle while submerged in liquid nitrogen (pyloric caeca). Samples of feed were prepared by drying 10 g of feed for 4 h at approximately 50°C in an oven, then grinding the dried feed with a porcelain mortar and pestle. The prepared samples were sent to IFE for analysis of total nitrogen percentage and atom ^{15}N percentage. Briefly, they analyzed

the samples using the following method. Approximately 1.0 mg of each sample was transferred to a Sn capsule. The capsules with samples were combusted in the presence of O_2 and WO_3 at 1,700 ºC in a FlashEA elemental analyzer (Thermo Scientific), followed by reduction of NO_x to N_2 in a Cu oven at approximately 650 ºC. H_2O was removed in a chemical trap of $Mg(ClO_4)_2$ before separation of N_2 and CO_2 on a 0.8 m GC column. The remaining N_2 was injected on-line to a Delta V isotope ratio mass spectrometer (IRMS) from Thermo Scientific for determination of $\%^{15}N$. The absolute N amount was determined using the peak at mass 28 ($^{14}N^{14}N$) and corrected for the amount of ^{15}N present in the sample. The accuracy and precision of $\%^{15}N$ analyses was measured by replicate analysis of the international reference material IAEA 311, using IAEA 305B and DP61720 (Isolife bv) as scale anchors. The three feed samples (natural abundance) were analyzed using IAEA N-1 and N-2 as scale anchors. Further information on the standards can be found in Supplementary Table 7.

2.11. Statistical analysis

Differences between fish weights, final ^{15}N percentages in muscle, feces, and pyloric caeca, and endogenous nitrogen losses were analyzed with one-way ANOVA tests and Tukey honestly significant difference tests. Fish growth was determined with linear regression (during labeled feed) and a combination of one-way ANOVA and a one-sided t-test for the unlabeled feed period. When means over time were different, Welch's two sample t-tests were used to determine which diets had significantly different means over time. Significant differences were determined using a 95% level of confidence. The statistical tests, regression models, and figures were generated using R (version 3.5.1). The 95% confidence interval around the linear regression model was automatically generated using ggplot2. Means are expressed as [mean] ± [standard error].

The ^{15}N percentage in the feces was corrected using a combination of digestibility and a correction for total nitrogen content to express the ^{15}N as a percentage of the entire feces composition, instead the percentage of nitrogen that is ^{15}N versus ^{14}N. The following equation was used for this correction:

(Digestibility and Total Nitrogen Correction) $$\frac{\%^{15}N \times \frac{yttrium\ in\ feces}{yttrium\ in\ feed}}{100} \times \frac{\%\ nitrogen\ in\ feces}{100}$$

The endogenous nitrogen percentage was calculated using the following isotope dilution equation from Leterme et al. (2000):

$$Endogenous\ N\ (\%N) = \frac{^{15}N_{digesta} \times 100}{^{15}N_{source}}$$

The ENL (endogenous nitrogen losses) were calculated by multiplying the above endogenous N by the percentage of nitrogen in the feces, as in Leterme et al (2000).

3. Results

3.1. Yeast growth

The fermentation protocol adapted from Nie et al. (2010) did produce a high amount of biomass and the yeast exhibited a rapid growth rate (Figures 3 and 4). A linear regression model provided the best fit for the dry weight over time in the small fermenters (Figure 3) while a logarithmic regression model was the best fit for growth in the large fermenter (Figure 4). The final yeast biomass in the 2.5 l fermenters was approximately 39 g l^{-1} and the final biomass in the 42 l fermenter was approximately 43.5 g l^{-1}.

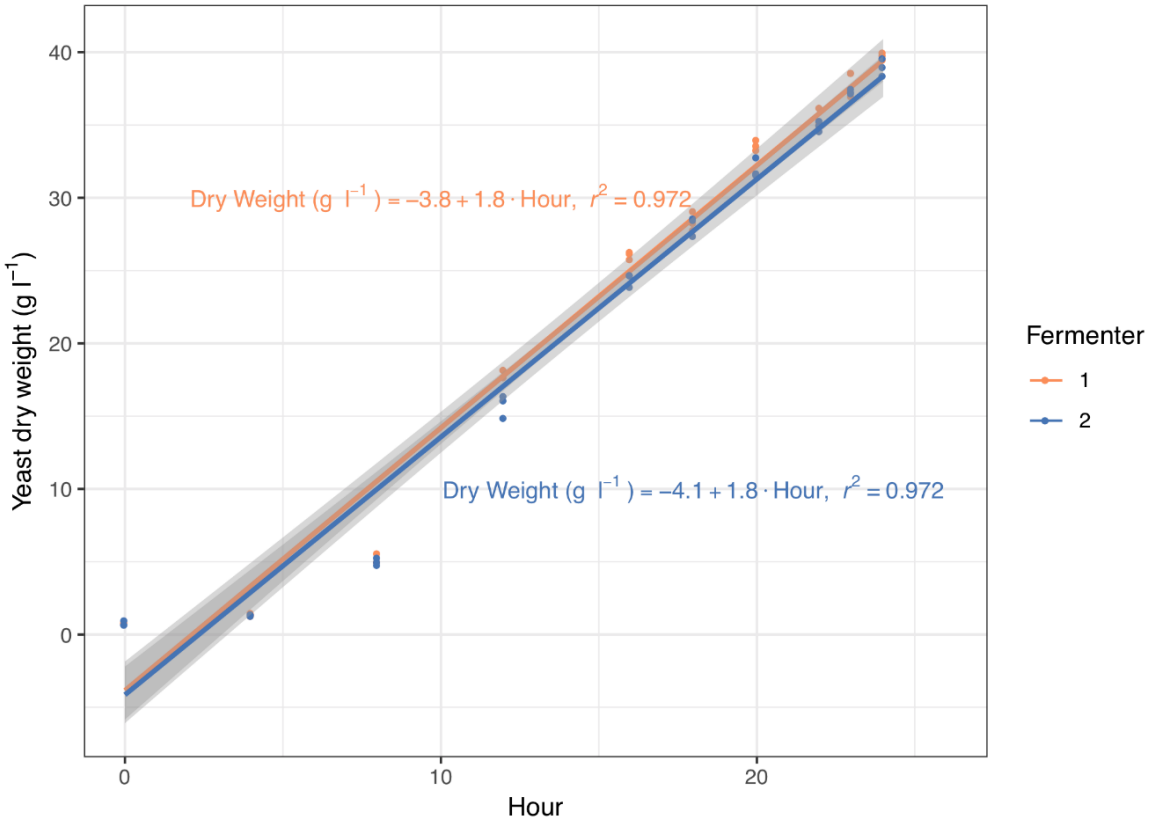

Figure 3. *C. utilis* growth over the course of 24 hours in two 2.5 l fermenters. The grey area represents the 95% confidence interval around the linear regression model.

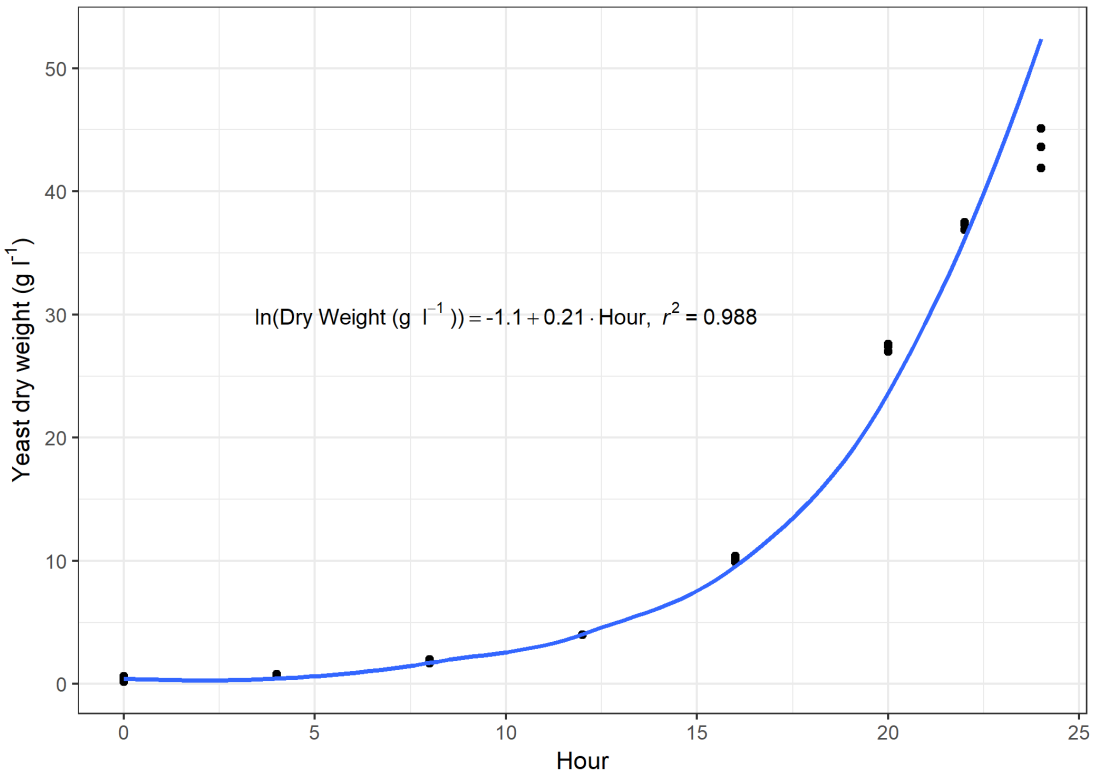

Figure 4. *C. utilis* growth over the course of 24 hours in a 42 l fermenter.

3.2. Yeast glucose

The amount of initial glucose was sufficient for the yeast, but glucose was very low during the feeding period, indicating that the glucose feeding rate and/or fed amount should have been higher to maintain a high yeast growth rate (Figure 5, 16-24 hours). The 20 hour time point was retested twice and the values remained consistently different. All other time points are almost identical, indicating that overall the two small fermenters were close replicates (Figure 5).

The glucose concentration in the 42 l fermenter never reached 0 g l^{-1}(Figure 6), meaning the feed rate and amount of glucose fed during the large scale fermentation were above the necessary amounts for maintaining a high growth rate.

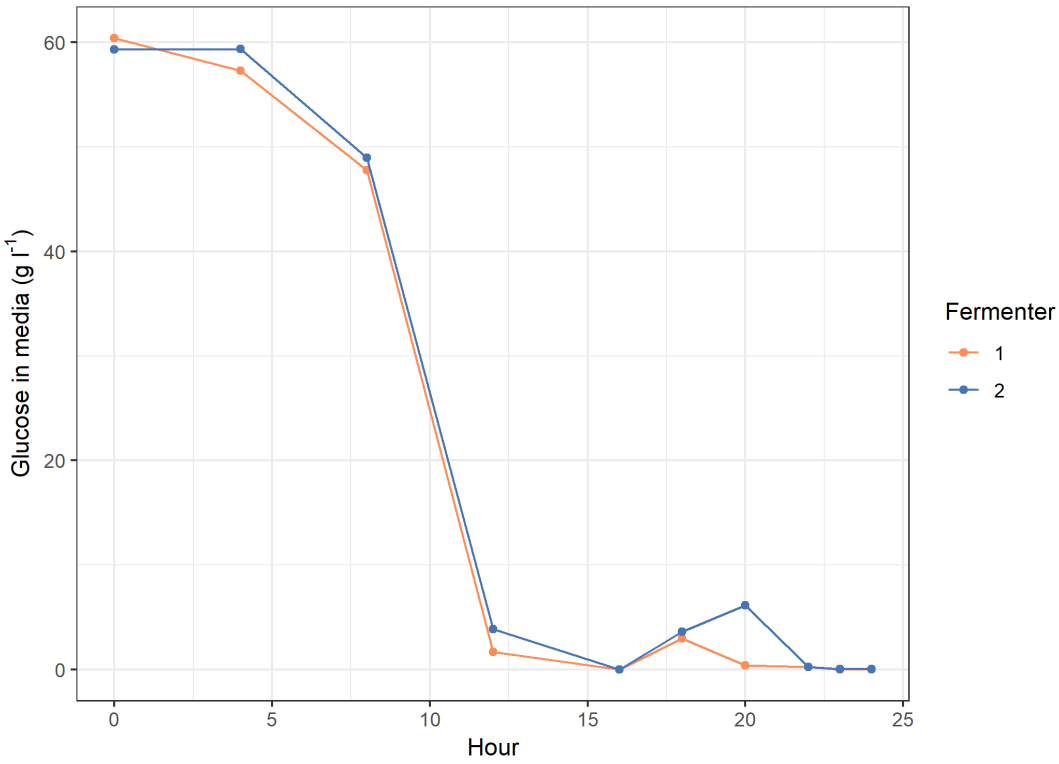

Figure 5. Glucose concentration over time in the 2.5 l fermenters.

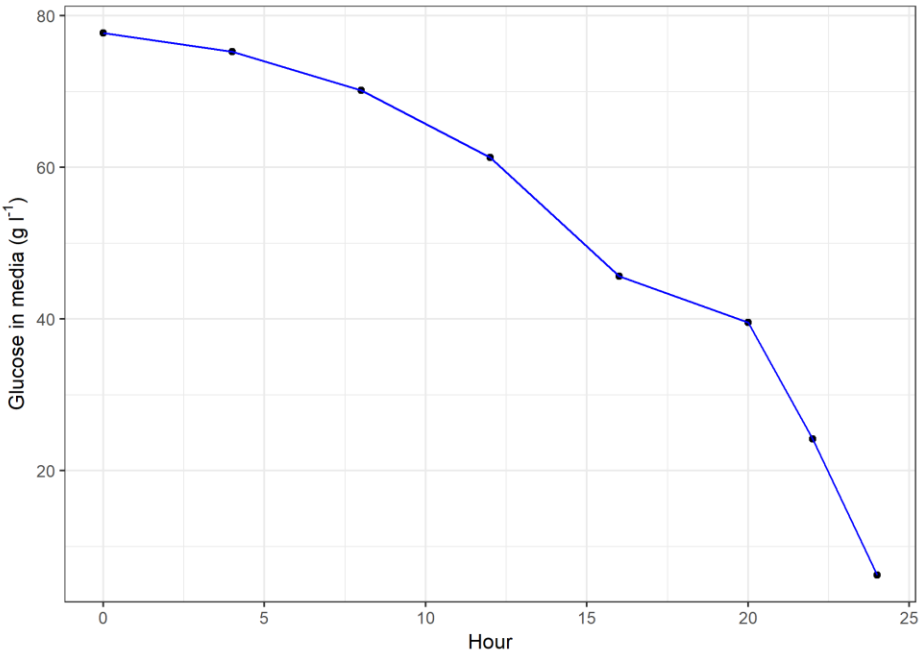

Figure 6. Glucose concentration over time in the 42l fermenter.

3.3. Yeast ammonium

In the 2.5 l fermenters, the ammonium in the media reached zero sometime between 4 and 8 hours, indicating the initial ammonium provided was not sufficient to maintain the growth

rate of the yeast (Figure 7). The ammonium was greater than zero at the end of the fermentation, indicating the amount of fed ammonium was higher than necessary. Seeing this pattern led to the decision to increase the amount of initial ammonium in the media and decrease the amount in the feeding media for the 30 l fermentation. The goal was to ensure that all of the $(^{15}NH_4)_2SO_4$ would be absorbed while also maintaining growth. The ammonium values match well at each time point, which is the same as the glucose, further indicating the fermenters were consistent duplicates.

When determining the ammonium content in the media of the 42 l fermenter, the ammonium test used failed on the samples after the 8 hour time point (Figure 8). This was potentially due to the samples being too salty for the freshwater NH_4^+ test from the high amount of NaOH that was pumped in to maintain the pH. The tested samples turned translucent and white to whiteish yellow, which is not a valid option on the colorimetric scale for this test and has been said to occur by aquarium hobbyists when a freshwater test is used on saltwater. There was not enough excess supernatant for most time points to re-test with a saltwater test. The last time point had enough excess to test at a sufficiently fresh dilution to obtain a valid result. The color demonstrated that the ammonium content was truly zero, meaning the ammonium was entirely incorporated into the yeast cells by the end of the fermentation.

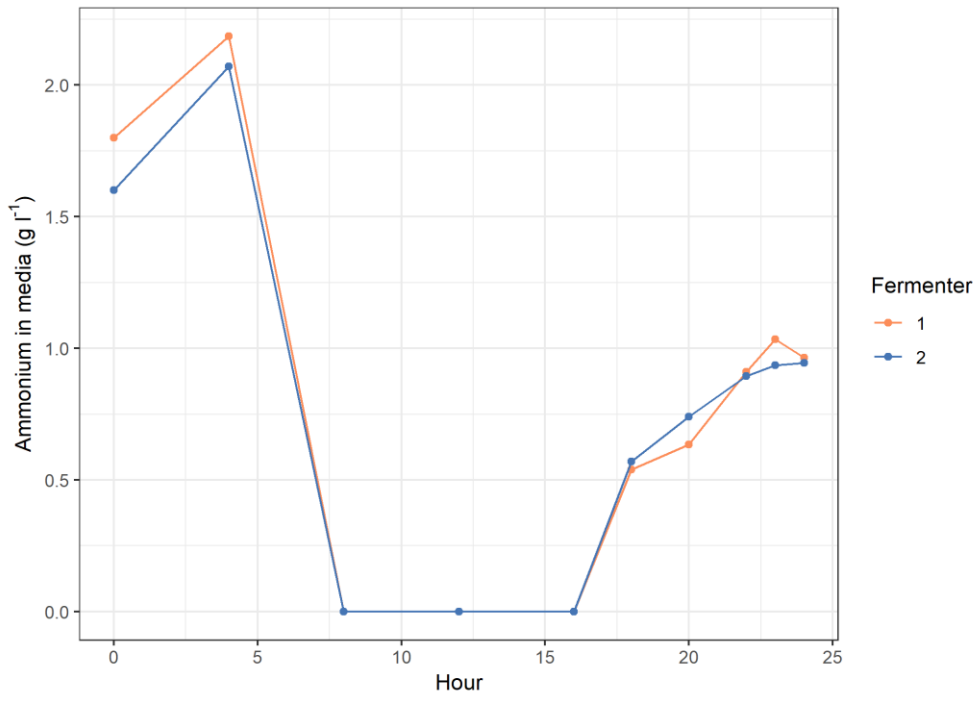

Figure 7. Ammonium concentration in the 2.5l fermenter media over time.

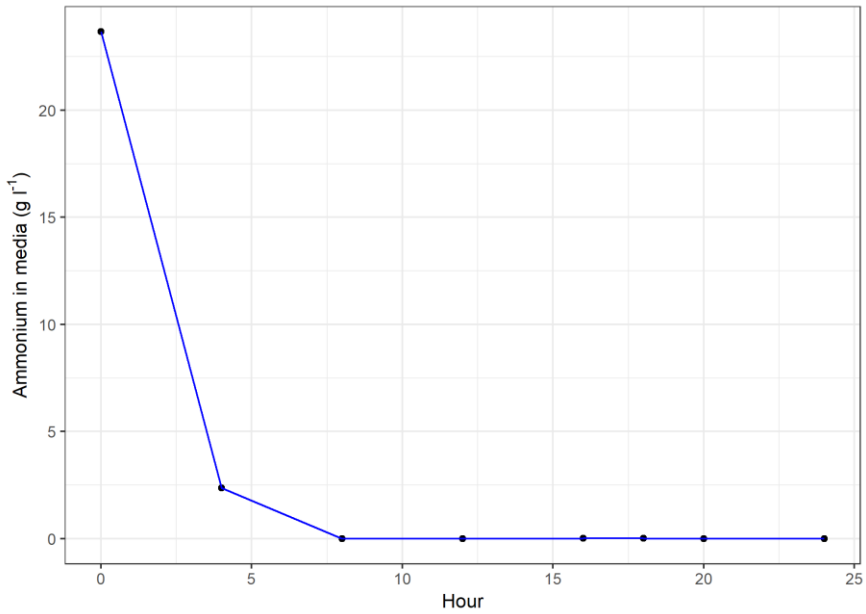

Figure 8. The amount of ammonium in the media of the 42 l fermenter over the course of 24 hours. The values between 8 and 24 hours are not truly representative of the ammonium level.

3.4. Yeast lysing

The final ^{15}N percentage in the freeze-dried *C. utilis* was 33.6%. The single pass through the Microfluidizer did increase the number of ruptured yeast cells compared to the unlysed yeast, as shown in Figure 7. At 50 times magnification, the number of broken cells (the black dots) was greater in the lysed yeast (Figure 9b) versus the unlysed yeast (Figure 9a). This was reflected in the Bradford protein test performed on the supernatant of the yeast suspension before and after lysing. Before lysing, the supernatant contained an average of 1.59 mg ml^{-1} protein and after lysing the supernatant contained an average of 5.44 mg ml^{-1} protein, indicating that lysing did increase the availability of protein.

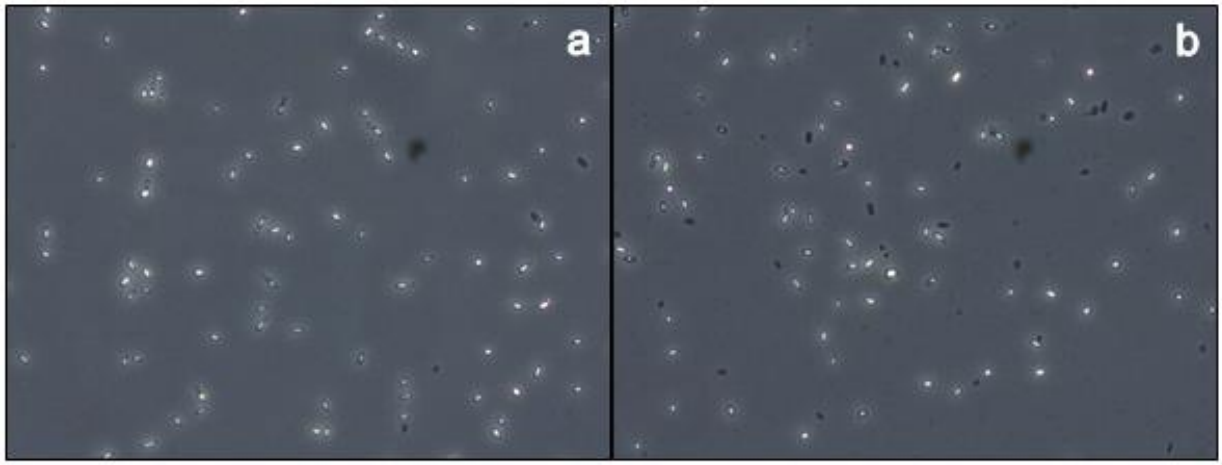

Figure 9. Light microscope images at 50 times magnification of *C. utilis* before (left) and after (right) being lysed with a Microfluidizer. The white/bright spots indicate intact cells and the dark spots indicate dead (lysed) cells. The spots with a bright center and dark edges indicate cells with perforated (but not broken) cell walls.

3.5. Fish growth

The fish did not grow significantly while being fed the yeast feed enriched in ^{15}N ($p=0.931$). The average weight of the fish on day 0 was 12.1 ± 0.15 g and the average weight on day 26 was 12.9 ± 0.19 g. The growth of the fish during the period of feeding unlabeled feeds did not differ significantly between diets (Table 4), however the fish did significantly increase in weight in this period, from a mean of 12.9 ± 0.19 g to 15.1 ± 0.50 g ($t(38)=3.98$, $p = 1.50 \times 10^{-4}$). The starting weights of the groups of fish assigned to each diet were not significantly different (one-way ANOVA, pooled standard error (SE) = 2.60, $p=0.991$). The end weights of the fish after the unlabeled feeding period (day 25 to day 42) also did not significantly differ between the diets (one-way ANOVA, pooled SE = 2.65, $p=0.133$). The diet did not have a significant effect on the weight change of the fish ($p=0.143$) (Table 3).

Table 3. The results of an ANOVA test between the fish weights at the beginning of the unlabeled feed period and the end. The test also shows the lack of a significant effect of diet on the fish weights.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Day	1	118.0	118.02	17.396	4.57×10^{-5}
Diet	2	4.1	2.05	0.303	0.739
Day x Diet	2	26.7	13.34	1.966	0.143
Residuals	195	1323.0	6.78		

3.6. Digestibility

The average apparent digestibilities of nitrogen of the unlabeled feeds are presented in Figure 10. The apparent N digestibility of the *C. utilis*-based feed was 84.6 ± 1.14% in the

beginning and 83.1 ± 0.78% for the second time period. The N digestibility of the triple null soy feed was 69.2 ± 3.88% for the first time period and 71.0 ± 2.64% for time period 2. The commercial soy had the lowest digestibility at 49.8 ± 1.71% to start and 51.7 ± 1.94% during the second sampling period. The digestibilities were significantly different between the diets (one-way ANOVA, pooled SEs 4.39, 3.37 and p values 2.15×10^{-4}, 8.16×10^{-5} for sampling periods 1 and 2 respectively). The digestibilities were not significantly different over time within the same diet (Table 8).

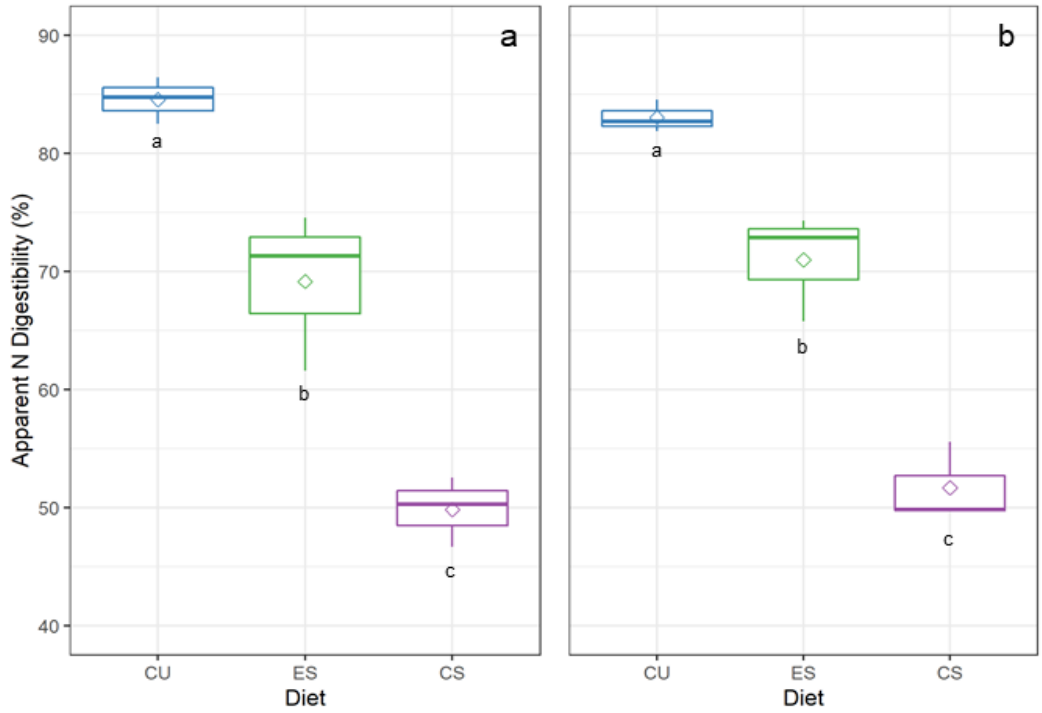

Figure 10. Boxplots depicting the digestibility of nitrogen of the 3 unlabeled feeds. (a) shows the digestibilities calculated using feces from the first 9 days of sampling and (b) shows the digestibilities over the last 5 days. Different letters indicate significantly different groups at the 95% confidence level. The open diamond points denote the arithmetic means.

Table 4. The results of the ANOVA test for digestibilities by diet and time showing that the digestibilities did not significantly change over time (p>0.05).

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Diet	2	3308	1654.0	107.948	2.13×10^{-8}
Time	1	2	2.4	0.155	0.701
Diet x Time	2	11	5.6	0.365	0.701
Residuals	12	184	15.3		

3.7. Muscle uptake and loss of ^{15}N

After 25 days of labeled feed, the muscle had increased in percent ^{15}N by approximately 5 times the initial value. The average percentage of ^{15}N in the muscle of the fish before feeding the labeled feed was 0.368 ± 2.15×10^{-4}%. The average percentage of ^{15}N in the muscle after feeding

the labeled feed was 2.07 ± 0.22% (Figure 11a). The uptake rate of ^{15}N in the muscle was well-described by a linear model ($r^2 = 0.848$), indicating that the percentage of ^{15}N in the muscle had not begun to approach the saturation point within this time period, as this would have been indicated by a logarithmic relationship.

The average percentages of ^{15}N in the muscle after the unlabeled feeding period were 2.79 ± 0.22%, 2.30 ± 0.09%, and 1.97 ± 0.41%, for the *C. utilis* feed, the triple null soy feed, and the commercial soy feed respectively (Figure 11b). According to these average percentages, the fish fed the yeast increased the amount of ^{15}N in their muscle, the fish fed the commercial full-fat soybean lost some ^{15}N from their muscle, and the fish fed the triple null soy fell in between these two. However, none of the differences between the final ^{15}N percentages (day 42) and the initial ^{15}N percent (at day 25) in muscle tissue were significantly different (Supplementary Table 2) and none of the final percentages were significantly different from each other (one-way ANOVA, pooled SE = 0.583, p = 0.160).

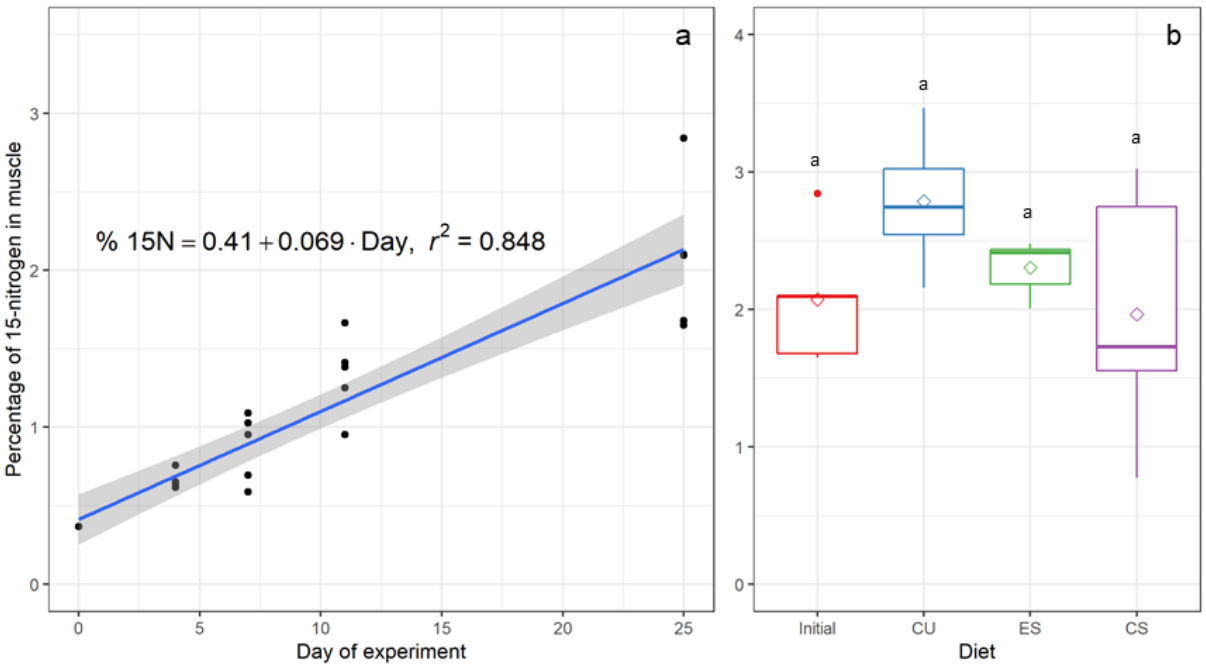

Figure 11. Plot (a) shows the uptake of ^{15}N into the muscle of the fish. Plot (b) shows the boxplot of ^{15}N percent in the fish muscle at the start of the unlabeled feeds and the boxplots at the end across the three different diets. "Initial" indicates the ^{15}N in muscle on day 25, the other boxplots represent levels at day 42. CU indicates the *C. utilis*-based feed, CS indicates the commercial soy-based feed, and ES indicates the triple null soy-based feed. Different letters indicate significantly different groups at the 95% confidence level. The open diamond points denote the arithmetic means.

3.8. Pyloric caeca uptake and loss of ^{15}N

The pyloric caeca had a much higher rate of uptake than the muscle and began to reach the saturation point by the end of the 25 days of labeled feed, as shown by the uptake rate beginning to slow in Figure 12a. The rate of loss of ^{15}N was also faster than the muscle, as shown in Figure 12b.

The average ^{15}N percentage in the pyloric caeca at the end of feeding the labeled feed was 13.9 ± 0.53%. The average ^{15}N percentages in the pyloric caeca after feeding the unlabeled feeds were 4.03 ± 0.19%, 3.29 ± 0.29%, and 3.54 ± 0.24% for the yeast-, triple null soy-, and commercial soy-fed fish respectively. The final ^{15}N percentages in the pyloric caeca of all three of the diets were significantly different than the average percentage at day 25, but the percentages were not significantly different across the diets (one-way ANOVA, pooled SE = 0.680, p-value = 6.10×10^{-13} and Supplementary Table 3).

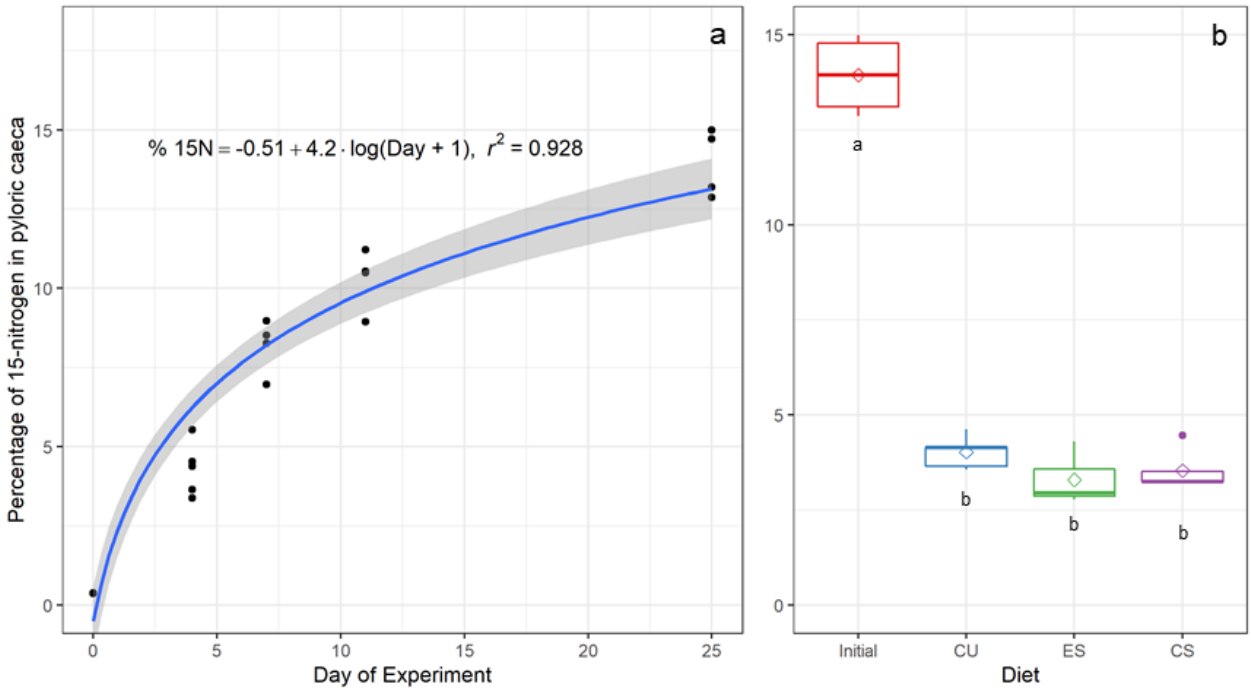

Figure 12. Plot (a) shows the uptake of ^{15}N into the pyloric caeca of the fish. Plot (b) shows the boxplot of ^{15}N percent in the pyloric caeca at the start of the unlabeled feeds and the boxplots at the end across the three different diets. "Initial" indicates the ^{15}N in pyloric caeca on day 25, the other boxplots represent levels at day 42. CU indicates the *C. utilis*-based feed, CS indicates the commercial soy-based feed, and ES indicates the triple null soy-based feed. Different letters indicate significantly different groups at the 95% confidence level. The open diamond points denote the arithmetic means.

3.9. ^{15}N percentage in feces

The average double-adjusted (see the "Digestibility and total nitrogen correction" equation in Methods) percentages of ^{15}N in the feces of the fish fed the *C. utilis*-based diet was $7.98 \times 10^{-4} \pm 4.84 \times 10^{-5}$% for the first sampling period and $5.49 \times 10^{-4} \pm 2.07 \times 10^{-5}$% for the second sampling period. The percentages of ^{15}N in the feces of the triple null soy-fed fish were $9.11 \times 10^{-4} \pm 4.74 \times 10^{-5}$% and $5.99 \times 10^{-4} \pm 2.56 \times 10^{-5}$% during the first and second sampling periods respectively. The percentages of ^{15}N in the feces of the commercial soy-fed fish were $1.26 \times 10^{-3} \pm 1.16 \times 10^{-4}$% and $0.906 \times 10^{-3} \pm 5.58 \times 10^{-5}$%.

The percentage of ^{15}N in the feces was significantly different between the three diets in each sampling period according to two one-way ANOVA tests (for sampling period 1: pooled SE = 1.90×10^{-4}, p-value= 2.14×10^{-3}, for sampling period 2: pooled SE = 9.16×10^{-5}, p-value = 1.13×10^{-5}). The percentages of ^{15}N in the feces were also significantly different between sampling periods within the same diet (Welch's two sample t-test, p-value of CU = 2.38×10^{-3}, p-value of ES = 4.77×10^{-4}, and p-value of CS = 2.87×10^{-2}).

When looking at the averages, the feces from fish fed the yeast-based diet contained the highest unadjusted percentage of ^{15}N while the commercial soy had the lowest (Figure 13a and b). When the percentage of ^{15}N in the feces is corrected for both digestibility and total nitrogen content in the feces (see Figure 13 and the "Digestibility and total nitrogen correction" equation in Methods), the yeast-fed fish had the lowest ^{15}N percent average, the commercial soy had the highest, and the experimental soy was intermediate. The differences between the commercial soy and the yeast and experimental soy are significant after this adjustment, but there was not a significant difference between the corrected ^{15}N percentage in the feces of the fish fed triple null soy versus commercial soy. This can be seen in the Tukey HSD plots where the 95% confidence interval between ES and CS overlaps zero in both time periods (Figure 13c and d).

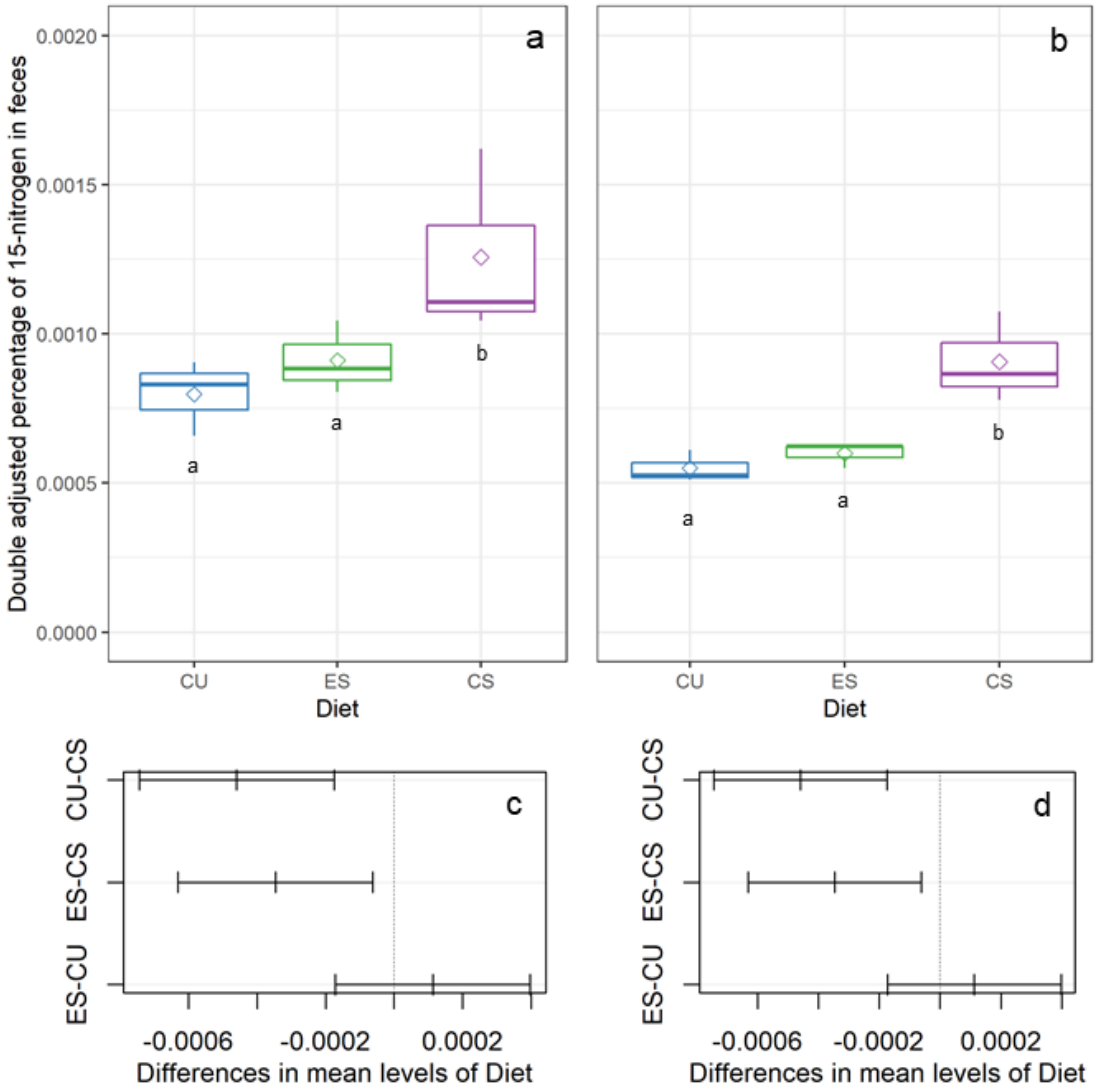

Figure 13. The plot shows the ^{15}N percentage in the feces from fish fed the yeast (CU), triple null soy (ES), and commercial soy (CS) feeds adjusted for the different digestibilities of the feeds and the total nitrogen content of the feces. (a) represents the percentages over the first 9 days and (b) represents the percentages over the last 5 days. CU indicates the *C. utilis*-based feed, CS indicates the commercial soy-based feed, and ES indicates the triple null soy-based feed. Different letters indicate significantly different groups at the 95% confidence level. (c) shows the Tukey multiple comparisons of means at a 95% confidence level for (a) and (d) shows the Tukey comparisons for (b). The open diamond points in (a) and (b) denote the arithmetic means.

In terms of evaluating the tanks as replicates, the percentages of ^{15}N in the feces from the CU tanks were significantly different from each other after the first 9 days ($p=0.00145$) but the ES and CS tanks were not significantly different. At the end of the sampling, the CS tanks were significantly different from each other ($p=0.043$), but the ES and CU tanks were not different.

3.10. Endogenous loss determined by isotope dilution

The average amounts of endogenous loss caused by the *C. utilis* diet were 12.0 ± 1.20 g kg^{-1} and 9.79 ± 0.75 g kg^{-1} during the first and second sampling periods respectively when calculated using muscle ^{15}N enrichment. The average amounts of endogenous loss from the triple null diet were 15.0 ± 0.24 g kg^{-1} and 10.2 ± 0.90 g kg^{-1} and from the commercial soy diet, the endogenous losses were 30.0 ± 4.2 g kg^{-1} and 21.4 ± 2.8 g kg^{-1}. When using the pyloric caeca ^{15}N enrichment, the endogenous losses from the *C. utilis* diet were 8.31 ± 0.83 g kg^{-1} and 6.78 ± 0.52 g kg^{-1} for the first and second sampling periods respectively. The average amounts of endogenous loss from the triple null soy were 10.5 ± 0.17 g kg^{-1} and 7.18 ± 0.63 g kg^{-1}, and the endogenous losses from the commercial soy diet were 16.6 ± 2.30 g kg^{-1} and 11.9 ± 1.58 g kg^{-1}. The diets caused significantly different amounts of endogenous loss in both time periods and when calculated using either tissue. Using a one-way ANOVA, for sampling period 1: muscle pooled SE = 4.32 and p-value = 4.88×10^{-3}, pyloric caeca pooled SE = 3.08 and p-value = 5.79×10^{-3} and for sampling period 2: muscle pooled SE = 2.45 and p-value = 1.50×10^{-2}, pyloric caeca pooled SE = 1.78 and p-value = 2.22×10^{-2}.

The amount of endogenous loss from each of the diets calculated using the isotope dilution equation (see Methods) reflected the same pattern as the adjusted ^{15}N percentage in the feces (Figure 14). The commercial soy diet had the highest amount of loss at both time points and using either muscle or ^{15}N in pyloric caeca at the end of sampling. The triple null soy diet caused an insignificantly higher amount of endogenous loss than the yeast diet when looking at the averages, which was also true of ^{15}N percentage in the feces. The triple null soy diet significantly changed in both tissue types between sampling periods in terms of the amount of endogenous loss (Welch's 2 sample t-test, p-value for muscle = 2.78×10^{-2}, p-value for pyloric caeca = 2.78×10^{-2}).

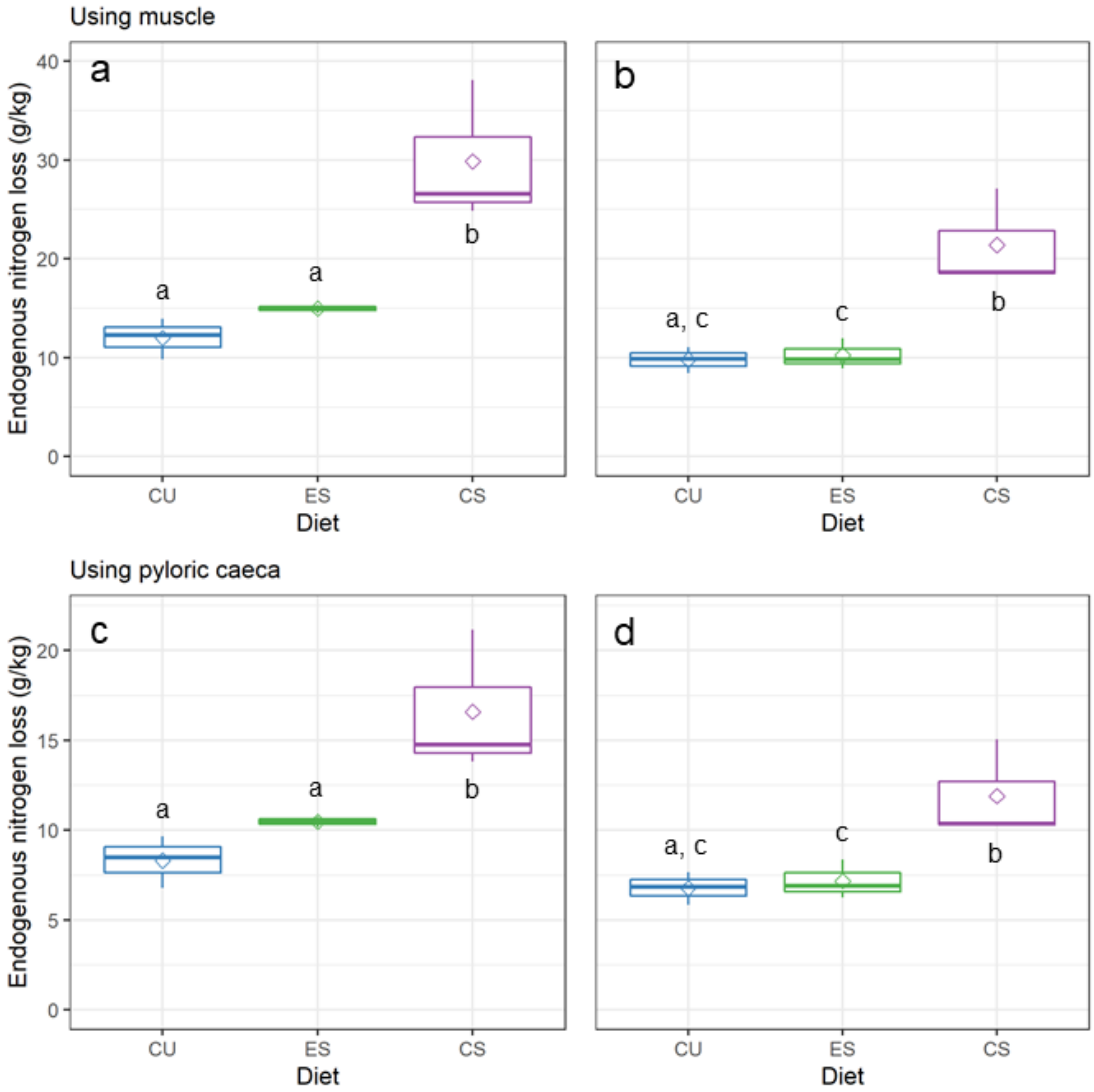

Figure 14. Boxplots showing the amount of endogenous nitrogen loss calculated using the isotope dilution equation and expressed in g kg^{-1} caused by each of the three diets. The values in (a) and (b) were calculated using the ^{15}N percentage in muscle as the source pool, and the values in (c) and (d) were calculated using the ^{15}N percentage in pyloric caeca at the end of the sampling period as the source pool. (a) and (c) present values from the first sampling period (the first 9 days) and (b) and (d) present values from the second sampling period (the last 5 days). CU indicates the *C. utilis*-based feed, CS indicates the commercial soy-based feed, and ES indicates the triple null soy-based feed. Different letters indicate significantly different groups within the same source pool at the 95% confidence level. The open diamond points denote the arithmetic means.

4. Discussion

4.1. Labeled yeast feed

The yeast fermentation was successful in terms of quick biomass production, but the final protein content was lower than in commercially produced *C. utilis*, making it a less ideal fish feed ingredient. This fermentation ended at 24 hours and the dry cell weight at that time was 43.5 g l^{-1}. Nie et al. (2010) achieved a maximum dry cell weight of approximately 65 g l^{-1} after 54 hours when using a constant feeding rate, which was also the style of feeding used in this

fermentation. The equation for the yeast growth rate in this study generated by linear regression predicts a higher biomass than 65 g l^{-1} after 54 hours. However, growth probably slowed down as the density of yeast cells increased and the necessary oxygen probably became more difficult to maintain. Therefore, the final biomass from a longer fermentation is hard to predict but would likely be at least as much as obtained by Nie et al. (2010).

The nitrogen provided to the yeast was 35.3% ^{15}N while the yeast itself contained 33.6% ^{15}N. This indicates that some ^{15}N either did not get absorbed by the yeast and remained in the media, or that some ^{15}N was lost during the processing, possibly during washing, centrifuging, freeze drying, or incorporation into the feed. The media tested at the end of the 30 l fermentation contained no ammonia, which was the only form in which nitrogen was provided to the yeast. However, the media was not tested for any other forms of nitrogen, meaning some ^{15}N may have been lost when washing the yeast if some cells had broken and leaked nitrogenous compounds into the media.

The final ^{15}N percentage in the yeast was sufficient to enrich the muscle ^{15}N of the fish by 5-fold with one 30 l fermentation worth of yeast biomass. The yeast itself, however, was quite low in protein at only 31% compared to commercially produced *C. utilis*, which can reach 56% (Øverland et al. 2013). This was likely due to the relatively low amount of nitrogen provided in the media in an attempt to maximize the ^{15}N percentage in the final product. If higher nitrogen in the media would have resulted in more protein in the yeast, this would make it a better feed ingredient for fish growth. This would have also increased unlabeled nitrogen, resulting in a lower ^{15}N percentage in the yeast, but still enough to produce fish with a detectable label. Animals are shown to absorb more ^{15}N when they are fasting or on a low protein diet, so yeast higher in protein may lead to slower tissue enrichment (Hobson et al. 1993).

4.2. Fish growth

There was no significant growth while the fish were fed the labeled *C. utilis* feed, and growth was also very low during the period of unlabeled feeds. On the other hand, the salmon did not lose weight, indicating that the feeding was close to the maintenance level. The low protein level in the labeled *C. utilis*, which was the only protein source in that feed, may have been why the fish did not grow. The lack of growth may also have been caused by an amino acid being below required levels, or the lack of palatable, marine ingredients in the feed. The yeast grown in this experiment was not analyzed for amino acid composition and not subject to assessment of essential amino acid digestibility. Therefore whether or not the feed was deficient in any amino acid was unknown. There has not been much research on *C. utilis* as a feed

ingredient at high rates of incorporation. However, some evidence suggests that the high levels of nucleic acids in yeast could impair red blood cell and immune function (Huyben 2017), which could slow or prevent growth. The yeast cells themselves may also have been poorly digestible due to incomplete lysing. Intact *C. utilis* cells have thick cell walls (Øverland et al. 2013) that make it hard for animals to break down and increasing the percentage of lysed cells through additional processing could improve the available protein and perhaps growth. The fact that the fish fed the unlabeled *C. utilis* diet (which had much higher protein) did grow indicates that the labeled diet was likely too low in digestible protein for rapid growth. Nitrogen digestibility of the unlabeled *C. utilis* diet was close to that observed in Øverland et al. (2013) – approximately 84% in this study and 88% in Øverland et al. (2013), verifying *C. utilis* as a highly digestible protein source when cell wall disruption is adequate.

4.3. Uptake and loss of ^{15}N

This study shows the rate of uptake of ^{15}N in two tissue types, muscle and pyloric caeca, in Atlantic salmon. The faster rate of uptake and loss of ^{15}N from the pyloric caeca was expected due to the high rate of cell turnover in the digestive tract versus the muscle. This is in agreeance with Souffrant et al. (1993), who found that ^{15}N was highest in the pancreatic juice versus other secretions and attributed it to high turnover. The muscle exhibited a much slower uptake rate. Though the differences between the final ^{15}N percentage in the muscle was not significant, the averages reflected the expected pattern with the ^{15}N in muscle from yeast-fed fish appearing to continue to increase. Then triple null soy fish resulted in an intermediate ^{15}N percentage at the end, and commercial soy appeared to have the lowest ^{15}N percentage at sampling time. This insignificant but expected pattern indicates a more thorough investigation should be done. If there truly was a significant increase in muscle ^{15}N among the yeast-fed fish, that would indicate that the ^{15}N was still begin deposited into the tissues from other internal sources after the enriched feed was stopped. Also, if this increase truly happens in fish fed feeds without antinutrients, the lack of this increase would indicate a loss of endogenous nitrogen above basal levels, which was reflected non-significantly in the average muscle ^{15}N of the soy-fed fish.

The lack of significant differences in muscle tissue could be due to the high amount of variation in the ^{15}N percentage. More samples should be analyzed to determine whether the error will decrease with increased sample size or if there is simply too high variation between individuals to the point that any difference between diets is too small to detect over this "noise."

The significant diet effect for ^{15}N in the feces, but not in muscle, indicates that the effect on the muscle when feeding to maintenance is either too small to detect in muscle or requires

larger sample sizes. If there is still not a significant difference between the diets after more fish are analyzed, that would indicate that measuring changes in muscle ^{15}N percent is not a precise method for observing diet effects due to individual variation eclipsing any differences caused by the diet. The fish in this study did exhibit significant variation in growth and therefore likely varied widely in metabolism and could vary in the rates at which they incorporate ^{15}N into their tissues. The final ^{15}N percentages between the three diets were not significantly different to each other in the pyloric caeca either. Earlier or more frequent sampling may have revealed a difference, or pyloric caeca could lose ^{15}N too quickly to see a difference at any point.

4.4. ^{15}N in the feces

As with any study that uses feces collected from a screen, leaching of the element in question into the water is a concern. Yue (2018) shows that ^{15}N does significantly decrease when feces are collected from a wedge wire screen versus stripped from the fish. However, there is no data yet on how quickly nitrogen would leach out from the feces of fish fed a yeast-based diet. Therefore the feces values could not be corrected for leaching rates. The feces from fish fed the two kinds of soy should leach in a similar fashion, making it reasonable to compare them even without a correction. The fish fed yeast produced feces that looked different to fishmeal or soy-fed fish feces, being almost white in color and smooth. Using a fishmeal diet in the future would allow for leaching corrections due to the speed of nitrogen leaching from fishmeal- and soy-fed fish feces being known. Stripping or dissecting for feces would give more accurate results. However with the small salmon used in this experiment, it would be hard to collect enough material if feces could only be obtained once from each fish, as in dissection. Stripping was also attempted in this study, but the fish were too small to obtain enough feces using this method, and therefore collection from a screen was used instead.

The raw ^{15}N percentage in the feces from the three different diets did not match the expected pattern, since the fish fed yeast had the highest percentage of ^{15}N, the commercial soy fish had an intermediate percentage, and the triple null soy fish had the lowest ^{15}N percentage. The difference between the two soy cultivars was not significant, which did match the results of Teimouri (2018). The feces and feeds were different from each other both in terms of nitrogen content and digestibility. These factors would influence the percentage of ^{15}N and should be corrected for to make the values more directly comparable. The lower the digestibility, the more nitrogen in the feces would have come from the feed directly, which had naturally low levels of ^{15}N, meaning that the percentage would be lower than a true representation of endogenous loss.

The feces containing a higher percentage of nitrogen would appear to be lower in ^{15}N for the same reason.

In this study, these factors were corrected for by converting the ^{15}N percentage into a percentage of the entire feces, instead of just the percentage of nitrogen that is ^{15}N. Then the values were further corrected using the yttrium content of the feed and feces, which is used to estimate the digestibility of a feed. After these corrections, the ^{15}N percentages matched the expected pattern with the feces from triple null soy and yeast fish having the lower ^{15}N values and commercial soy fish having a higher amount of ^{15}N in their feces. This would match the prediction because the yeast feed should contain no compounds that cause endogenous nitrogen loss and the triple null soy only has the Bowman-Birk protease inhibitor. The commercial soy contained both Bowman-Birk and Kunitz inhibitors, which are the main causes of endogenous nitrogen loss from consuming soy. One confounding factor in this could be that Bowman-Birk activity has been shown to increase in soy when certain Kunitz inhibitor-encoding genes are knocked out (Gillman et al. 2015).

4.5. Endogenous loss calculated by isotope dilution

Muscle and pyloric caeca tissue were used as source pools of ^{15}N in the isotope dilution equation to estimate endogenous nitrogen loss. Pyloric caeca would be the most biologically relevant tissue to use as a source pool because this study focuses on endogenous loss of trypsin and chymotrypsin, the precursors of which are stored in the exogenous pancreatic tissue diffusely located in the fat surrounding the pyloric caeca. Muscle had the advantage of being statistically consistent throughout the feces sampling period, meaning the pooled feces would all correspond to the same source value. However, the ^{15}N enrichment in muscle was lower than in pyloric caeca, therefore muscle would not be the most accurate source pool. The quick rate of loss in the pyloric caeca made it impossible to use in this study as an accurate source pool for the endogenous ^{15}N in the isotope dilution equation. If there had been more sampling points during rate of loss, a proper regression could have been done and a more accurate estimate of the ^{15}N in pyloric caeca each day would be available. The feces samples were pooled over many days. Therefore using the pyloric caeca as the value for the endogenous ^{15}N enrichment would still be inaccurate because it would likely change significantly every few days. To be able to use the pyloric caeca as a source in future experiments, daily samples of pyloric caeca and feces should be taken. In order to obtain enough feces daily for analysis, more fish or bigger fish would be needed. Even the final measurement of ^{15}N in the pyloric caeca was higher than the highest ^{15}N percentage in the muscle, so using the final ^{15}N percentage in pyloric caeca as the source to

calculate endogenous loss should give a more relevant minimum estimate than muscle. The endogenous nitrogen loss from fish fed the commercial soy was approximately double that of the *C. utilis* diet (assumed to represent basal endogenous loss) when using pyloric caeca ^{15}N and slightly more than double when using muscle ^{15}N. The amount of endogenous nitrogen loss calculated by the isotope dilution equation exhibits the same pattern as the double adjusted ^{15}N in the feces where the yeast and triple null soy are not statistically different, and the commercial soy has a statistically higher amount of endogenous loss.

5. Conclusion

The percentages of ^{15}N in the feces of Atlantic salmon fed diets with differing levels of protease inhibitors revealed significant differences. The feces and feeds were significantly different in nitrogen content and nitrogen digestibility respectively, and these two factors significantly affected the relative proportions of ^{15}N. After correcting for these differences, the feces from fish fed the commercial soy-based feed had the highest amount of ^{15}N, indicating the highest level of endogenous loss, which can be expected from their protease inhibitor composition. The feces from the fish fed yeast and the triple null soy-based feed were not significantly different in terms of corrected ^{15}N percentage. The variability in muscle tissue was too large to detect a difference between the diets with this sample size and pyloric caeca did not reflect a difference between the diets during this sampling period. This should be investigated further with more frequent or earlier pyloric caeca sampling and analyzing more samples. The fed-batch fermentation method for *C. utilis* in this study is effective at producing a large amount of biomass, however that biomass was much lower in protein than commercially produced *C. utilis*. This study also showed that Atlantic salmon can survive on a diet where fishmeal is entirely replaced by yeast, however growth was severely limited while they were fed ^{15}N-labeled diet, possibly due to a combination of the low protein and potential amino acid deficiencies. Using the isotope dilution method and both the muscle and pyloric caeca ^{15}N measurements as sources, the endogenous nitrogen loss resulting from the three different diets was determined and the fish fed the commercial soy diet showed a much higher level of loss, approximately double that of the yeast diet. Using the final sampling of pyloric caeca as the ^{15}N source offers a biologically relevant estimate of the minimum endogenous nitrogen loss due to digestive enzymes, an important component of endogenous loss, originating from the pyloric caeca.

6. References

Adeola, O., Xue, P. C., Cowieson, A. J., & Ajuwon, K. M. (2016). Basal endogenous losses of amino acids in protein nutrition research for swine and poultry. *Animal Feed Science and Technology*, *221*, 274–283. https://doi.org/10.1016/j.anifeedsci.2016.06.004

Bakke-McKellep, Anne Marie, Nordrum, S., Krogdahl, A., & Buddington, R. (2000). Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic salmon (Salmo salar L.). *Fish Physiology and Biochemistry*, *22*, 33–44. https://doi.org/10.1023/A:1007872929847

Bergner, U., Uecker, E., Rossow, N., Simon, O., & Bergner, H. (1984). Untersuchungen zu endogenen N-Umsatzprozessen an ^{15} N-markierten Schweinen. *Archiv Für Tierernaehrung*, *34*(9), 593–605. https://doi.org/10.1080/17450398409425707

Boisen, S. & Eggum, B. O. (1991). Critical Evaluation of in Vitro Methods for Estimating Digestibility in Simple-Stomach Animals. *Nutrition Research Reviews*, *4*(1), 141–162. https://doi.org/10.1079/NRR19910012

Boisen, S. & Fernandez, J. A. (1995). Prediction of the apparent ileal digestibility of protein and amino acids in feedstuffs and feed mixtures for pigs by in vitro analyses. *Animal Feed Science and Technology*, *51*(1), 29–43. https://doi.org/10.1016/0377-8401(94)00686-4

Boisen, S. & Moughan, P. J. (1996). Dietary Influences on Endogenous Ileal Protein and Amino Acid Loss in the Pig—A Review. *Acta Agriculturae Scandinavica, Section A - Animal Science*, *46*(3), 154–164. https://doi.org/10.1080/09064709609415866

Butts, C. A., Moughan, P. J., & Smith, W. C. (1991). Endogenous amino acid flow at the terminal ileum of the rat determined under conditions of peptide alimentation. *Journal of the Science of Food and Agriculture*, *55*(2), 175–187. https://doi.org/10.1002/jsfa.2740550203

Cahill, M. M. (1990). Bacterial Flora of Fishes: A Review. *Microbial Ecology*, *19*(1), 21–41.

Chen, Y., Xu, Z., Zhang, C., Kong, X., & Hua, Y. (2014). Heat-induced inactivation mechanisms of Kunitz trypsin inhibitor and Bowman-Birk inhibitor in soymilk processing. *Food Chemistry*, *154*, 108–116. https://doi.org/10.1016/j.foodchem.2013.12.092

Clarke, E. J. & Wiseman, J. (2000). Developments in plant breeding for improved nutritional quality of soya beans II. Anti-nutritional factors. *The Journal of Agricultural Science*, *134*(2), 125–136.

Collinder, E., Björnhag, G., Cardona, M., Norin, E., Rehbinder, C., & Midtvedt, T. (2003). Gastrointestinal Host–Microbial Interactions in Mammals and Fish: Comparative Studies in Man, Mice, Rats, Pigs, Horses, Cows, Elks, Reindeers, Salmon and Cod. *Microbial Ecology in Health and Disease*, *15*(2–3), 66–78. https://doi.org/10.1080/08910600310014980

Darcy-Vrillon, B., Souffrant, W., Laplace, J., Rérat, A., Corring, T., Vaugelade, P., Gebhardt, G., Köhler, R., Bernard, F., Calmes, R., Jung, J., & Morel, M. (1991). Exogenous and endogenous contributions to nitrogen fluxes in the digestive tract of pigs fed a casein diet. II. Ileal and faecal digestibilities and absorption of amino acids. *Reproduction Nutrition Development*, *31*(5), 561–573. https://doi.org/10.1051/rnd:19910509

D'Avanzo, C., Alber, M., & Valiela, I. (1991). Nitrogen assimilation from amorphous detritus by two coastal consumers. *Estuarine, Coastal and Shelf Science*, *33*, 203–209. https://doi.org/10.1016/0272-7714(91)90007-X

de Lange, C. F. M., Souffrant, W. B., & Sauer, W. C. (1990). Real ileal protein and amino acid digestibilities in feedstuffs for growing pigs as determined with the 15N-isotope dilution technique. *Journal of Animal Science*, *68*(2), 409–418. https://doi.org/10.2527/1990.682409x

Deniro, M. J. & Epstein, S. (1981). Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta*, *45*(3), 341–351. https://doi.org/10.1016/0016-7037(81)90244-1

Denstadli, V., Vegusdal, A., Krogdahl, Å., Bakke-McKellep, A. M., Berge, G. M., Holm, H., Hillestad, M., & Ruyter, B. (2004). Lipid absorption in different segments of the gastrointestinal tract of Atlantic salmon (Salmo salar L.). *Aquaculture*, *240*(1), 385–398. https://doi.org/10.1016/j.aquaculture.2004.06.030

Donkoh, A., Moughan, P. J., & Morel, P. C. H. (1995). Comparison of methods to determine the endogenous amino acid flow at the terminal ileum of the growing rat. *Journal of the Science of Food and Agriculture*, *67*(3), 359–366. https://doi.org/10.1002/jsfa.2740670313

Drescher, K., Hagemeister, H., De Vrese, M., Roos, N., Middendorf, K., & Rantamäki, P. (1994). Guanidination (homoarginine-labeling) of proteins does affect prececal 15N recovery in pigs. *FBN Schriftenreihe 3*.

Einarsson, S. & Davies, P. S. (1996). On the localisation and ultrastructure of pepsinogen, trypsinogen and chymotrypsinogen secreting cells in the Atlantic salmon, Salmo salar L. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *114*(3), 295–301. https://doi.org/10.1016/0305-0491(96)00036-3

Epp, M. A., Ziemann, D. A., & Schell, D. M. (2002). Carbon and nitrogen dynamics in zero-water exchange shrimp culture as indicated by stable isotope tracers. *Aquaculture Research*, *33*(11), 839–846. https://doi.org/10.1046/j.1365-2109.2002.00720.x

FAO/WHO/UNU, Food and Agriculture Organization of the United Nations, World Health Organization, & United Nations University. (1985). *Energy and protein requirements : report of*

a Joint FAO/WHO/UNU Expert Consultation [held in Rome from 5 to 17 October 1981]. Geneva: Geneva : World Health Organization. Retrieved from http://apps.who.int/iris/handle/10665/39527

Gamboa-Delgado, J. (2009). *Application of natural stable isotopes in aquaculture nutrition*. Bangor University, Gwynedd, Wales, UK. Retrieved from http://eprints.uanl.mx/1937/1/Gamboa_-_University_of_Wales.pdf

Gatlin, D. M., Barrows, F. T., Brown, P., Dabrowski, K., Gaylord, T. G., Hardy, R. W., Herman, E., Hu, G., Krogdahl, Å., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E. J., Stone, D., Wilson, R., & Wurtele, E. (2007). Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research*, *38*(6), 551–579. https://doi.org/10.1111/j.1365-2109.2007.01704.x

Gillman, J. D., Kim, W.-S., & Krishnan, H. B. (2015). Identification of a New Soybean Kunitz Trypsin Inhibitor Mutation and Its Effect on Bowman−Birk Protease Inhibitor Content in Soybean Seed. *Journal of Agricultural and Food Chemistry*, *63*(5), 1352–1359. https://doi.org/10.1021/jf505220p

Grala, W., Verstegen, M. W., Jansman, A. J., Huisman, J., & van Leeusen, P. (1998). Ileal apparent protein and amino acid digestibilities and endogenous nitrogen losses in pigs fed soybean and rapeseed products. *Journal of Animal Science*, *76*(2), 557–568.

Hagemeister, H. & Erbersdobler, H. (1985). Chemical labeling of dietary-protein by transformation of lysine to homoarginine – A new technique to follow intestinal digestion and absoprtion (Vol. 44, pp. A133–A133). Presented at the Proceedings of the Nutrition Society, CAB International C/O Publishing Division, Wallingford, Oxon, England OX10 8DE.

Hobson, K. A., Alisauskas, R. T., & Clark, R. G. (1993). Stable-Nitrogen Isotope Enrichment in Avian Tissues Due to Fasting and Nutritional Stress: Implications for Isotopic Analyses of Diet. *The Condor*, *95*(2), 388–394. https://doi.org/10.2307/1369361

Hodgkinson, S. M., Souffrant, W. B., & Moughan, P. J. (2003). Comparison of the enzyme-hydrolyzed casein, guanidination, and isotope dilution methods for determining ileal endogenous protein flow in the growing rat and pig. *Journal of Animal Science*, *81*(10), 2525–2534. https://doi.org/10.2527/2003.81102525x

Huisman, J., Heinz, T., Poel, A. F. B. V. D., Leeuwen, P. V., Souffrant, W. B., & Verstegen, M. W. A. (1992). True protein digestibility and amounts of endogenous protein measured with the 15N-dilution technique in piglets fed on peas (Pisum sativum) and common

beans (Phaseolus vulgaris). *British Journal of Nutrition*, *68*(1), 101–110. https://doi.org/10.1079/BJN19920070

Huyben, D. (2017, August). Effects of feeding yeasts on blood physiology and gut microbiota of rainbow trout [Doctoral thesis]. Retrieved September 7, 2018, from https://pub.epsilon.slu.se/14478/

Ingham, L. & Arme, C. (1977). Intestinal absorption of amino acids by rainbow trout, Salmo gairdneri (Richardson). *Journal of Comparative Physiology ? B*, *117*(3), 323–334. https://doi.org/10.1007/BF00691558

Kassell, B. (1970). [66c] Trypsin and chymotrypsin inhibitors from soybeans. In *Methods in Enzymology* (Vol. 19, pp. 853–862). Academic Press. https://doi.org/10.1016/0076-6879(70)19075-6

Kelly, J. F. (2000). Stable isotopes of carbon and nitrogen in the study of avian and mammalian trophic ecology, *78*, 27.

Krogdahl, Å., Bakke-McKellep, A. M., & Baeverfjord, G. (2003). Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (Salmo salar L.). *Aquaculture Nutrition*, *9*(6), 361–371. https://doi.org/10.1046/j.1365-2095.2003.00264.x

Kumar, V., Rani, A., Shuaib, M., & Mittal, P. (2018). Comparative Assessment of Trypsin Inhibitor vis-à-vis Kunitz Trypsin Inhibitor and Bowman-Birk Inhibitor Activities in Soybean. *Food Analytical Methods*, *11*(9), 2431–2437. https://doi.org/10.1007/s12161-018-1227-9

Leibholz, J. & Mollah, Y. (1988). Digestibility of threonine from protein concentrates for growing pigs. 1. The flow of endogenous amino acids to the terminal ileum of growing pigs. *Australian Journal of Agricultural Research*, *39*(4), 713–719. https://doi.org/10.1071/ar9880713

Leterme, P., Sève, B., & Thewis, A. (1998). The Current 15N-Leucine Infusion Technique Is Not Suitable for Quantitative Measurements of Ileal Endogenous Amino Acid Flows in Pigs. *The Journal of Nutrition*, *128*(11), 1961–1968. https://doi.org/10.1093/jn/128.11.1961

Leterme, P., Souffrant, W.-B., & Théwis, A. (2000). Effect of Barley Fibres and Barley Intake on the Ileal Endogenous Nitrogen Losses in Piglets. *Journal of Cereal Science*, *31*(3), 229–239. https://doi.org/10.1006/jcrs.2000.0306

Lien, K. A., Sauer, W. C., Mosenthin, R., Souffrant, W. B., & Dugan, M. E. (1997). Evaluation of the 15N-isotope dilution technique for determining the recovery of endogenous protein

in ileal digestion of pigs: effect of dilution in the precursor pool for endogenous nitrogen secretion. *Journal of Animal Science*, *75*(1), 148–158.

Macko, S., Fogel, M., H. Engel, M., & Hare, P. E. (1986). Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochimica et Cosmochimica Acta*, *50*, 2143–2146. https://doi.org/10.1016/0016-7037(86)90068-2

Minagawa, M. & Wada, E. (1984). Stepwise enrichment of 15N along food chains: Further evidence and the relation between δ15N and animal age. *Geochimica et Cosmochimica Acta*, *48*(5), 1135–1140. https://doi.org/10.1016/0016-7037(84)90204-7

Moughan, P. J., Buttery, P. J., Essex, C. P., & Soar, J. B. (1992). Evaluation of the isotope dilution technique for determining ileal endogenous nitrogen excretion in the rat. *Journal of the Science of Food and Agriculture*, *58*(2), 165–172. https://doi.org/10.1002/jsfa.2740580202

National Research Council (NRC). (1998). *Nutrient Requirements of Swine* (10th ed.). National Academies Press.

Nie, M., Wei, G., Shao, N., & Ge, X. (2010). A novel strategy on the high-cell-density cultivation of Candida utilis for the enhanced production of glutathione. *Korean Journal of Chemical Engineering*, *27*(4), 1246–1251. https://doi.org/10.1007/s11814-010-0190-y

Norton, G. (1991). CHAPTER 4 - Proteinase Inhibitors. In J. P. F. D'Mello, C. M. Duffus, & J. H. Duffus (Eds.), *Toxic Substances in Crop Plants* (pp. 68–106). Woodhead Publishing. https://doi.org/10.1533/9781845698454.68

Nyachoti, C. M., Lange, C. F. M. de, McBride, B. W., & Schulze, H. (1997). Significance of endogenous gut nitrogen losses in the nutrition of growing pigs: A review. *Canadian Journal of Animal Science*, *77*(1), 149–163. https://doi.org/10.4141/A96-044

O'Connell, T., Kneale, C., Tasevska, N., & Kuhnle, G. (2012). The diet-body offset in human nitrogen isotopic values: A controlled dietary study. *American Journal of Physical Anthropology*, *149*(3), 426–434. https://doi.org/10.1002/ajpa.22140

Oluwaseun, S. G., Storebakken, T., Kraugerud, O. F., Hansen, B. R., Øverland, & M., Hansen, J. Ø. (To be published). Evaluation of wedge wire screen as a new tool for faeces collection in digestibility assessment in fish: The impact of nutrient leaching on apparent digestibility of nitrogen, carbon and Sulphur from fishmeal, soybean meal and rapeseed meal-based diets in rainbow trout (*Oncorhynchus mykiss*).

Øverland, M., Karlsson, A., Mydland, L. T., Romarheim, O. H., & Skrede, A. (2013). Evaluation of Candida utilis, Kluyveromyces marxianus and Saccharomyces cerevisiae yeasts as

protein sources in diets for Atlantic salmon (Salmo salar). *Aquaculture*, *402–403*, 1–7. https://doi.org/10.1016/j.aquaculture.2013.03.016

Øverland, M. & Skrede, A. (2017). Yeast derived from lignocellulosic biomass as a sustainable feed resource for use in aquaculture. *Journal of the Science of Food and Agriculture*, *97*(3), 733–742. https://doi.org/10.1002/jsfa.8007

Perkins, M. J., McDonald, R. A., Veen, F. J. F. van, Kelly, S. D., Rees, G., & Bearhop, S. (2014). Application of Nitrogen and Carbon Stable Isotopes (δ15N and δ13C) to Quantify Food Chain Length and Trophic Structure. *PLOS ONE*, *9*(3), e93281. https://doi.org/10.1371/journal.pone.0093281

Pinnegar, J. K., & Polunin, N. V. C. (1999). Differential fractionation of δ13C and δ15N among fish tissues: implications for the study of trophic interactions. *Functional Ecology*, *13*(2), 225–231. https://doi.org/10.1046/j.1365-2435.1999.00301.x

Ringø, E., Zhou, Z., Vecino, J. L. G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R.E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H.L., Martinsen, L.L., De Schryver, P., Bossier, P., Sperstad, S., & Merrifield, D. L. (2016). Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? *Aquaculture Nutrition*, *22*(2), 219–282. https://doi.org/10.1111/anu.12346

Rungruangsak-Torrissen, K., Moss, R., Andresen, L. H., Berg, A., & Waagbø, R. (2006). Different expressions of trypsin and chymotrypsin in relation to growth in Atlantic salmon (Salmo salar L.). *Fish Physiology and Biochemistry*, *32*(1), 7–23. https://doi.org/10.1007/s10695-005-0630-5

Sanden, M. & Olsvik, P. (2009). Intestinal cellular localization of PCNA protein and CYP1A mRNA in Atlantic salmon Salmo salar L. exposed to a model toxicant. *BMC Physiology*, *9*, 3. https://doi.org/10.1186/1472-6793-9-3

Sanz, A., Morales, A. E., de la Higuera, M., & Gardenete, G. (1994). Sunflower meal compared with soybean meals as partial substitutes for fish meal in rainbow trout (Oncorhynchus mykiss) diets: protein and energy utilization. *Aquaculture*, *128*(3), 287–300. https://doi.org/10.1016/0044-8486(94)90318-2

Schadereit, R., Krawielitzki, K., & Herrmann, U. (1986). 15N transamination in the administration of various tracer substances. 1. Whole body studies in rats. *Archiv fur Tierernahrung*, *36*(9), 783–792. https://doi.org/10.1080/17450398609434346

Schmidt, M. A., Hymowitz, T., & Herman, E. M. (2015). Breeding and characterization of soybean Triple Null; a stack of recessive alleles of Kunitz Trypsin Inhibitor, Soybean

Agglutinin, and P34 allergen nulls. *Plant Breeding*, *134*(3), 310–315. https://doi.org/10.1111/pbr.12265

Schmitz, M., Hagemeister, H., & Erbersdobler, H. F. (1991). Homoarginine Labeling is Suitable for Determination of Protein Absorption in Miniature Pigs. *The Journal of Nutrition*, *121*(10), 1575–1580. https://doi.org/10.1093/jn/121.10.1575

Schulze, H., Butts, C. A., Moughan, P. J., & Verstegen, M. W. A. (1995). The 15N-isotope dilution method for determining ileal endogenous nitrogen excretion in the young (10 kg liveweight) pig. *Journal of the Science of Food and Agriculture*, *69*(1), 41–50. https://doi.org/10.1002/jsfa.2740690108

Souffrant, W. B. (1991). Endogenous nitrogen losses during digestion in pigs. In *Digestive physiology in pigs: proceedings of the 5. International Symposium on Digestive Physiology in Pigs* (pp. 147–166). Wageningen: Pudoc.

Souffrant, W., Rérat, A., Laplace, J., Darcy-Vrillon, B., Köhler, R., Corring, T., Gebhardt, G., Bernard, F., Jähnichen, M.L., Schneider, B., & Cointepas, F. (1993). Exogenous and endogenous contributions to nitrogen fluxes in the digestive tract of pigs fed a casein diet. III. Recycling of endogenous nitrogen. *Reproduction Nutrition Development*, *33*(4), 373–382. https://doi.org/10.1051/rnd:19930406

Spindler, H. K., Mosenthin, R., Rosenfelder, P., Jørgensen, H., Bach Knudsen, K. E., & Eklund, M. (2016). Determination of basal ileal endogenous losses and standardized ileal digestibility of amino acids in barley fed to growing pigs. *Journal of Animal Science and Biotechnology*, *7*. https://doi.org/10.1186/s40104-016-0115-7

Stein, H. H., Fuller, M. F., Moughan, P. J., Sève, B., Mosenthin, R., Jansman, A. J. M., Fernández, J.A., & de Lange, C. F. M. (2007). Definition of apparent, true, and standardized ileal digestibility of amino acids in pigs. *Livestock Science*, *109*(1), 282–285. https://doi.org/10.1016/j.livsci.2007.01.019

Taverner, M. R., Hume, I. D., & Farrell, D. J. (1981). Availability to pigs of amino acids in cereal grains. 1. Endogenous levels of amino acids in ileal digesta and faeces of pigs given cereal diets. *The British Journal of Nutrition*, *46*(1), 149–158.

Teimouri, S. (2018). Removal of three proteinaceous antinutrients from a soybean cultivar and effects of inclusion in diets processed at two heat levels on nutritional value and gut health for Atlantic salmon (Salmo salar L). (Master's). Feed Manufacturing Technology, Department of Animal and Aquacultural Sciences (IHA), Norwegian University of Life Sciences, Ås, Norway. 91 pp.

Vanderklift, M. A. & Ponsard, S. (2003). Sources of variation in consumer-diet δ15N enrichment: a meta-analysis. *Oecologia*, *136*(2), 169–182. https://doi.org/10.1007/s00442-003-1270-z

Verschoor, A. M., Boonstra, H., & Meijer, T. (2005). Application of Stable Isotope Tracers to Studies of Zooplankton Feeding, using the Rotifer Brachionus calyciflorus as an Example. *Hydrobiologia*, *546*(1), 535–549. https://doi.org/10.1007/s10750-005-4296-x

Wünsche, D. J., Herrmann, D. sc U., Meinl, D. M., Hennig, D. U., Kreienbring, D. F., & Zwierz, D. P. (1987). Einfluß exogener Faktoren auf die präzäkale Nährstoff- und Aminosäurenresorption, ermittelt an Schweinen mit Ileo-Rektal-Anastomosen. *Archiv Für Tierernaehrung*, *37*(9), 745–764. https://doi.org/10.1080/17450398709428245

Yue, S. (2018). Evaluation of the use of fractional abundance of stable carbon and nitrogen isotopes and indigestible inert marker in diets and faeces to calculate apparent digestibility of carbon and nitrogen (Master's). Department of Animal and Aquacultural Science of Faculty of Biosciences, Norwegian University of Life Sciences, Ås, Norway. 34 pp. Retrieved from https://brage.bibsys.no/xmlui/bitstream/handle/11250/2495608/master%20thesis-Yue%20Song.%202018%20.PDF.pdf?sequence=1&isAllowed=y

7. Supplementary Tables

Supplementary Table 1. The Tukey HSD table of comparisons between digestibilities of the three unlabeled diets during the first time period (Sampling Period 1) and the second time period (Sampling Period 2). They are all significantly different at the 95% level of confidence ($p<0.05$).

Compared Pairs	Sampling Period	Difference	Lower limit	Upper limit	p value
ES.CU	1	-15.41020	-26.41299	-4.407415	0.01209208734
CS.CU	1	-34.72637	-45.72916	-23.723583	0.00017123717
CS.ES	1	-19.31617	-30.31896	-8.313380	0.00405829528
ES.CU	2	-12.07014	-20.51216	-3.628124	0.01098953484
CS.CU	2	-31.37412	-39.81614	-22.932109	0.00006730857
CS.ES	2	-19.30399	-27.74600	-10.861969	0.00102042178

Supplementary Table 2. The Tukey HSD comparisons table with the 95% confidence intervals and p-values for the individual comparisons between ^{15}N percentages in muscle tissue after feeding labeled *C. utilis* feed ("Initial") and then after feeding unlabeled feeds (CU representing the yeast feed, ES representing triple null soy feed, and CS representing commercial soy feed).

Comparison Pairs	Difference	Lower limit	Upper limit	p value
CU-Initial	0.7149767	-0.3402633	1.7702168	0.2517061
CS-Initial	-0.1082343	-1.1634743	0.9470058	0.9908607
ES-Initial	0.2310353	-0.8242048	1.2862754	0.9220669
CS-CU	-0.8232110	-1.8784511	0.2320291	0.1567800
ES-CU	-0.4839414	-1.5391815	0.5712987	0.5688255
ES-CS	0.3392696	-0.7159705	1.3945097	0.7947375

Supplementary Table 3. The Tukey HSD comparisons table with the 95% confidence intervals and p-values for the individual comparisons between ^{15}N percentages in pyloric caeca after feeding labeled *C. utilis* feed ("Initial") and then after feeding unlabeled feeds (CU representing the yeast feed, ES representing triple null soy feed, and CS representing commercial soy feed).

Compared Pairs	Difference	Lower limit	Upper limit	p value
CU-Initial	-9.9147110	-11.229829	-8.5995933	0.000000000005876410
CS-Initial	-10.4053171	-11.720435	-9.0901994	0.000000000002599365
ES-Initial	-10.6472253	-11.962343	-9.3321076	0.000000000001642242
CS-CU	-0.4906061	-1.730511	0.7492988	0.671328077619819386
ES-CU	-0.7325143	-1.972419	0.5073906	0.356322173924558450
ES-CS	-0.2419082	-1.481813	0.9979967	0.941690038013294095

Supplementary Table 4. Tukey comparisons table between double adjusted ^{15}N percentages in feces (95% confidence level) over the first 9 days (Sampling Period 1) and over the last 5 days (Sampling Period 2). This is the table of numbers for Figure 15c and 15d.

Compared Pairs	Sampling Period	Difference	Lower limit	Upper limit	p value
CU.CS	1	-0.00045928466	-0.00074409403	-0.00017447529	0.00214558521
ES.CS	1	-0.00034614864	-0.00063095801	-0.00006133926	0.01687055812
ES.CU	1	0.00011313602	-0.00017167335	0.00039794540	0.56901286101
CU.CS	2	-0.00035711369	-0.00049450056	-0.00021972682	0.00001838070
ES.CS	2	-0.00030747980	-0.00044486667	-0.00017009293	0.00009567333
ES.CU	2	0.00004963389	-0.00008775298	0.00018702076	0.6252964118

Supplementary Table 5. Tukey HSD comparisons table with the 95% confidence intervals and p-values for the individual comparisons between endogenous nitrogen loss from the three diets calculated by isotope dilution using muscle ^{15}N as the source pool (95% confidence level) over the first 9 days (Sampling Period 1) and over the last 5 days (Sampling Period 2). CU represents the yeast feed, ES represents triple null soy feed, and CS represents commercial soy feed.

Compared Pairs	Sampling Period	Difference	Lower limit	Upper limit	p value
CU.CS	1	-17.8450692	-28.677371	-7.012767	0.005570997
ES.CS	1	-14.8902802	-25.722582	-4.057978	0.013176663
ES.CU	1	2.9547889	-7.877513	13.787091	0.695797933
CU.CS	2	-11.6149970	-19.320433	-3.909561	0.008567869
ES.CS	2	-11.1468543	-18.852290	-3.441419	0.010404612
ES.CU	2	0.4681427	-7.237293	8.173578	0.981085443

Supplementary Table 6. Tukey HSD comparisons table with the 95% confidence intervals and p-values for the individual comparisons between endogenous nitrogen loss from the three diets calculated by isotope dilution using pyloric caeca ^{15}N as the source pool (95% confidence level) over the first 9 days (Sampling Period 1) and over the last 5 days (Sampling Period 2). CU represents the yeast feed, ES represents triple null soy feed, and CS represents commercial soy feed.

Compared Pairs	Sampling Period	Difference	Lower limit	Upper limit	p value
CU.CS	1	-8.2738386	-14.424135	-2.12354189	0.01453548
ES.CS	1	-6.1223502	-12.272647	0.02794646	0.05086621
ES.CU	1	2.1514883	-3.998808	8.30178501	0.56284722
CU.CS.1	2	-5.1150700	-9.570914	-0.65922604	0.02893356
ES.CS.1	2	-4.7185189	-9.174363	-0.26267493	0.04008996
ES.CU.1	2	0.3965511	-4.059293	4.85239506	0.96000042

Supplementary Table 7. Information about standards used for 15N analysis.

Standard:		**%15N**
IAEA311	Average	2,116
International Atomic Energy	1sd	0,003
Agency		
n=18	From certificate:	
	True:	2,05
	95% CI:	2,03-2,06
IAEA 305B	Average	0,5029
International Atomic Energy	1sd	0,0031
Agency		
n=6	From certificate:	
	True:	0,5029
	95% CI:	0,5022-0,5039
DP61720	Average	11,92
Isolife bv, Netherlands	1sd	0,05
n=6		
	From certificate:	
	True:	11,92
	1sd	0,01
Standard:		**delta15N**
IAEA N-1	Average	0,54
International Atomic Energy	1sd	0,23
Agency		
n=3	From certificate:	
(only used for feed samples)	True:	0,54
	1sd	0,2
IAEA N-2	Average	20,34
International Atomic Energy	1sd	0,03
Agency		
n=3	From certificate:	
(only used for feed samples)	True:	20,34
	1sd	0,2

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