DISSERTATION

NANNOCHLOROPSIS OCULATA: A SAFE PROTEIN FEED FOR GROWING RATS AND RABBITS

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ABSTRACT

NANNOCHLOROPSIS OCULATA: A SAFE PROTEIN FEED FOR GROWING RATS AND RABBITS

The challenge to replace fossil fuels as the primary source of energy has been a long and complicated task. In recent years, following a historic increase in crude oil (>$100/barrel in 2008) the focus has been shifted to the use of microalgae as a source of oil for biofuel. The utilization of algae over other biofuel sources is advantageous as algae require less water and land than traditional crops. Some microalgae species can produce upwards of 10,000 gallons of oil per acre and when compared to corn which can produce ~18 gallons of ethanol per acre, algae becomes quite interesting. The National Renewable Energy Lab (NREL) in Golden, Colorado has been working diligently on utilizing algae as an energy source. In 2010, NREL explained that replacing all the gasoline in the U.S.A. with corn ethanol would require a corn field 1600 km^2, while replacing all the gasoline in the U.S.A. with algae oil would (theoretically) take an area only 176 km^2. One of the algae species that is being closely investigated as a source of oil is *Nannochloropsis oculata*, from the phylum Heterokontophyta. This algal species has oil content greater than 20% (DM basis). A secondary benefit to utilizing algae as a source of biofuel is the high protein (>30% DM basis), mineral rich co-product that is produced after the oil is extracted

In order to further investigate the full potential of algae, a project was designed to determine the usability of the oil-free meal as a protein feed for animals. Within this project, two studies were done, one with 24 young, growing male Sprague-Dawley®™ rats, and one with 24 adolescent male New Zealand White rabbits. Both studies were conducted for 36 days, with 12 animals in each group. In each study, a diet was prepared with 10% *Nannochloropsis oculata* meal, and one without algal meal. The diets were formulated to be isocaloric and isonitrogenous.

The study conducted with rats showed that the intake of DM, Crude Fat, ADF, NDF and ash was decreased in the algal fed rats ($P \leq 0.05$). The apparent digestibility of DM, Crude Fat and ADF was also decreased in the algal fed rats ($P < 0.05$), while NDF apparent digestibility was increased (58.28% v. 51.60%) ($P > 0.05$). More N was excreted in the feces ($P > 0.05$) and urine of the algal group ($P < 0.05$). The apparent digestibility of macro minerals was unaffected ($P > 0.10$). Overall the rats fed the algal diet displayed no measureable nutritional deficiencies, and no toxic effects were noted

In comparison, the study conducted with rabbits resulted in the intake of DM, Crude Fat, NDF and ash being similar between the two groups of rabbits ($P > 0.05$), while ADF intake was decreased in the algal fed rabbits ($P < 0.01$). The apparent digestibility of DM, NDF and ash was increased for the animals fed the algal diet ($P < 0.05$), while Crude Fat and ADF apparent digestibility was decreased ($P < 0.05$). No difference was seen in the fecal or urinary excretion of N between the groups ($P > 0.10$), while fecal P excretion was decreased in the algal fed rabbits ($P < 0.01$). The apparent digestibility of Ca, Mg and P was increased in the rabbits fed the algal diet ($P < 0.05$), while K and Na apparent digestibility was unaffected ($P > 0.10$). Similar to the rat study, no toxic or diagnosed metabolic distress was noted.

In both studies, the histology of the liver, spleen and kidneys ($P > 0.10$) was not negatively affected by feeding a diet with 10% algal meal.

The GE of the diets fed to the rats was similar (4.40 Mcal/kg v. 4.33 Mcal/kg) and the GE of the diets fed to the rabbits was also similar (4.33 Mcal/kg v. 4.37 Mcal/kg). The energy lost in the urine was greater in the algal fed rabbits (4.50 Mcal/kg v. 3.17 Mcal/kg) ($P < 0.05$), while the overall effect on DE was negligible ($P > 0.10$) between the groups.

Utilizing the algal meal from *Nannochloropsis oculata* as a source of protein in growing livestock rations is a possibility when the algal meal is priced the same as DDGs. At the same

market price, the cost per kilogram of protein is quite competitive ($0.66/kg v. $0.69/kg). The algal meal could also be considered a potentially competitive source of energy compared to DDGs ($0.15/Mcal NEg v. $0.14/Mcal NEg).

In summary, the utilization of oil-free algal meal from *Nannochloropsis oculata* can be considered a safe and possibly economic protein source for growing animals. In order to fully understand the potential of algal meal in livestock rations, more research needs to be conducted in metabolically different animals.

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CHAPTER 1

LITERATURE REVIEW

The living world has been classified into "Kingdoms" since 1735, when Carl Linneaus became known as the father of modern taxonomy. Linneaus originally identified two kingdoms; "plant" and "animal". This was the groundwork for the extensive biological ranking system that is utilized today to identify and define living organisms. Since Linneaus, several biologists have made contributions to the kingdom system, including Copeland's 4 Kingdom System. The 4 Kingdom System was introduced in 1938, and Copeland split life into two Domains: Eukaryotes and Prokaryotes, and the Kingdoms then fell within those two domains. Copeland also offered the additional Kingdoms: Monera (bacteria), Protista, to be included with the Kingdoms Plantae and Animalia. These taxonomic categories were used until 1969 when Whittaker proposed the 5 Kingdom System. Whittaker felt that there was too large a difference between fungi and plants to be combined under one Kingdom, thus resulting in 5 Kingdoms; Monera (bacteria), Protista, Plantae and Animalia, and Fungi. Whittaker's focus was more on the organism's nutrition as a point of differentiation in contrast to solely considering the cellular composition of the organism. The Whittaker 5 Kingdom System is still used today in most British, Latin and Australian textbooks. However, in the United States of America, the Cavalier-Smith 6 Kingdom System has been adopted Throughout the 1990s, Cavalier-Smith proposed several alternative Kingdom systems, and in 1998 presented his final 6 Kingdoms. The 6 Kingdoms are recognized as: Plant, Animal, Protozoa, Fungi, Plant (includes red and green algae), Chromista (all algae with chlorophyll a and c, includes Heterokonts), and Bacteria.

After the gross classification into Domain and then Kingdom, life is separated further into Phylums, which classify life based on developmental or internal organization. After Phyla classification, the taxonomic ranks are as follows; Class, Order, Family, Genus and Species.

It is still common to see references to both systems, as consistency has not quite been achieved in the research community. Therefore, when considering algae, it is important to note that this is a term that is used to describe a huge number of organisms, all which fall under the Kingdom Plant or Chromista (Whittaker vs. Cavalier-Smith), and the multiple phylums within. All true algae fall in the Eukaryote domain, and have a nucleus that is enclosed within a membrane, and chloroplasts that are bound to one or more membranes. All algae also use photosynthesis to produce energy and they produce oxygen as a byproduct of photosynthesis. Algae are much less complex (cellularly) than land plants and also lack many of the organs and structural components that are seen in land plants. For the purpose of this discussion which is focused on algae, those in both Kingdoms; Plant and Chromista, it is worthwhile to identify and understand a few of the better referenced algal groups; Cyanobacteria, Rhodophyta and Heterokontophyta and the research that has been conducted

Cyanobacteria

Cyanobacteria are often referred to as “blue-green” algae. The reference to “algae” is misleading, as this organism is a bacteria, although they do obtain their energy through photosynthesis. Bacteria are prokaryotic organisms that lack membrane bound organelles as well as a nucleus. A main reason that cyanobacteria has been so well researched and has become a mainstream term is largely due to the utilization of cyanobacteria in the human food nutritional supplement field Spirulina, a cyanobacteria and a very common human nutritional supplement and can be found in numerous “healthy” foods and nutritionally enhanced foods. Spirulina offers considerable nutritional benefits, for example it is 55-77% protein (DM basis), and it is a

complete protein, differentiating itself from most plant protein sources. This organism is also a source of essential fatty acids, such as alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), as well as arachadonic acid (AA) (Ciferri et al. 1983). With the popularity of Spirulina as a health food, it has become more widespread than other algae sources as potential food supplements.

Rhodophyta

The phylum Rhodophyta (commonly referred to as "Red Algae") is a group of 5000-6000 species of algae. Red Alga is somewhat unique in that it has a double cell wall, with an inner wall that is very high in cellulose, and an outer wall that is high in a pectin like substance (Cole et al. 1990). This unique cellular composition makes Red Algae a valuable material for the production of agar, which is used in many food manufacturing processes.

A natural phenomenon, referred to as the "Red Tide" is commonly attributed to Red Algae. However, the Red Tide is actually caused by the overabundant growth of algae from the phylum Dinoflaggelate, genus *Karenia* species *brevis*. This harmful algal bloom has been seen in both the Atlantic and the Pacific Oceans in recent years. These algal blooms can also produce a neurotoxin called brevetoxin (Sellner et al. 2003) that result in marine animal death, and economic devastation to the seafood industry. These blooms are thought to be a result of changing water temperatures and chemical composition, which makes the algae grow out of control.

Heterokonts: *Nannochloropsis* and *Schizochytrium*

This phylum consists of >10,000 known species, and these species range in size from a tiny unicellular organism to the giant multicellular kelp seen in many oceans. It is this phylum that encompasses a couple of algal genus' that are the focal point of nutritional research today. For example; *Nannochloropsis oculata*, and *Schizochytrium* (a golden algae). Other organisms in

this group are brown algae, yellow-green algae and parasitic *Phytophthora*, which was the cause of the infamous Irish potato famine. This brief synopsis supports the wide breadth of this phylum.

Algae in Research

Research utilizing certain microalgae as a potential therapeutic treatment and/or supplement for the human food chain has been explored in great detail. The potential health benefits attributed to specific microalgae range from anticancer properties to glucose metabolism enhancement. Some microalgae of the phyla Chlorophyta, such as *Dunaliella* sp. and *Chlorella* sp. have indicated the capacity as a favorable source of β-carotene to act as an anticancer agent (Mokady et al. 1991), a prophylactic treatment in Crone's disease (Lavy et al. 2003), an in-vivo antioxidant (Murthy et al. 2004), and a possible food supplement treatment for insulin resistance (Lee et al. 2009). The majority of the research that has been conducted has focused on the potential health benefits to humans and a more holistic, natural relief of common ailments. This research has looked principally at the consumption of the whole microalgae by animals to produce research data that can be applied to human health.

Two specific microalgae of the phyla Heterokontophyta that have been explored in a narrow context for other uses are, *Nannochloropsis* sp., and *Schizochytrium* sp.. In fact, the sole focus of the previous research utilizing these two algae species has been on the possible beneficial effects of their high levels of PUFA (EPA and DHA).

Both of these algae have high levels of long chain fatty acids, which have made them quite interesting as feed supplements. *Nannochloropsis sp*. was first mentioned in 1981 by Hibberd and is characterized by its lack of chlorophyll b as well as its high levels of EPA. For this specific reason, *Nannochloropsis sp*. is currently being studied as a potential source of oil for the biofuel industry, as subsequently a source of a high protein feed supplement after the oil has

been extracted In contrast, *Schizochytrium*, a golden alga that was developed and successfully marketed into the animal and human feed supplement industry as the product DHA-Gold® (Martek). For example, this product is now fed to poultry to produce DHA rich eggs.

The group of organisms that are referred to as "algae" is clearly a large and complex array of many different organisms and microorganisms. This discussion is a very condensed review of a few of the "algae" groups that are in the mainstream food and research industry today. Some of the groups are much better understood than others, and there are some very potentially healthy species within the many phyla in existence. However, the research surrounding the use of the residual protein meal after the oil has been extracted from any alga is minimal, and has not been explored in detail as a potential protein supplement for animal feed This literature review will primarily focus on these two microalgae; *Nannochloropsis oculata* and *Schizochytrium*. The utilization of both of these algal species has grown, and are now the focal points for biofuel synthesis (*Nannochloropsis sp.)* and as a nutritional source of DHA(*Schizochytrium)*. The current data for these two algal species provides the framework for more extensive research to begin. With the change in focus on algal use, the need for more data concerning the utilization of the co-products from the production of biofuel of some of these algal species is becoming necessary

***Nannochloropsis* sp.:**

This microalga is a rich source of eicosapentaenoic acid (EPA) which can be extracted out of the algae and used as a biofuel, leaving an algal meal that could be utilized as a protein feed for animals. Currently, feeds such as soybean meal, steam flaked corn, and corn gluten meal are added into livestock diets to provide protein. The use of an algal meal could be a valuable, economic, and environmentally sustainable substitution for these common agricultural crops. The utilization of *Nannochloropsis* o. meal as a protein meal to replace current protein meals fed to animals has not been explored on a large scale. Markovits et al. (1992) fed Sprague-Dawley®™

rats whole *Nannochloropsis* o. incorporated into diets at levels of 5% and 10%. The rats were fed for two, three, and four weeks, to determine if *Nannochloropsis* o. could be considered a safe dietary supplement. Significant differences ($P<0.05$) were noted in the total cholesterol and high density lipoprotein (HDL) levels, both of which were increased in the rats fed 5% or 10% algae. Although total cholesterol levels and HDL levels were increased by feeding 5% and 10% algae, there was no increase in triglyceride (TG) levels. This study did not note any toxic effects in the growing rats from the consumption of the algae, as well as no significant effect ($P>0.05$) on initial body weights and feed efficiency. Feed intake was also unaffected at 2 and 4 weeks between control and 5% algae addition ($P>0.05$), however at 3 weeks a significant difference was recorded between control and 5% algae addition ($P<0.05$). For all three feeding periods, there was a difference in feed intake between control and 10% algae addition ($P<0.01$). Markovits et al. (1992) reported results showed that this algae species could be a potential source of a healthy dietary supplement by providing the health benefits associated with increased HDL levels and unchanged TG levels. Additionally, *Nannochloropsis* o. could also be a viable source of cholesterol free EPA as well as other nutrients such as amino acids, and minerals.

A similar study was conducted by Sukenik et al. in 1993 at the National Institute of Oceanography in Israel to determine the bioavailability of the ω3 fatty acids found in specific algal species, and compare it to the ω3 fatty acids from capelin oil. Several dietary treatments were tested in this study. Diets containing 10% algae from *Nannochloropsis* o. or *Isochrysis galbana*, or a combination of 5% *Nannochloropsis* o. and 5% *Isochrysis galbana* were fed in comparison to a control diet with no algae material and 5% soybean oil. The weanling rats were fed for two weeks, and were consuming hypercholesterolemia diets throughout the study. The whole algae from the *Nannochloropsis* o. and *Isochrysis galbana* were dried and added to the diet at a level of 10% as separate treatments, and combined (5% of each algal species) as a

replacement for 1.5% of the soybean oil in the standard diet. The results showed a significant reduction *(P*<0.05) of arachidonic acid (AA) in plasma, red blood cells (RBC) and liver lipids, as well as a significant increase *(P*<0.05) in the total amount of ω 3 polyunsaturated fatty acids (PUFA) in the animals fed an algae enriched diet. The ω6:ω3 also decreased in the liver lipids from 5.7 in the control group, to 2.50-2.72 in the algae enriched diets. This decrease in the ω6:ω3 was also seen in the plasma fatty acid composition, 11.3 from the control diet, and 3.6-3.9 in from the algae enriched diets. The RBC ω6:ω3 was reduced from 17.5 to 3.6-4.4 in the control and algae diets respectively. Sukenik et al. (1993) also included a comparison feeding the free fatty acids (FFA) of *Nannochloropsis* oil and capelin oil. *Nannochloropsis* FFA or capelin FFA were added the diet at 2% as a partial substitution for the soybean oil or 5% as a complete replacement for the soybean oil. The control diet contained 5% soybean oil. The ω6:ω3 in both the liver and the plasma was significantly different *(P*<0.05) in the algal FFA or capelin FFA enriched diets. The AA levels in the algal (17.7%) and capelin FFA (6.1%) fed animals was significantly less *(P*<0.05) than the control diet (22.6%), and the capelin FFA resulted in a larger decrease than the algal FFA treatment. This study did show a difference in AA levels and ω6:ω3 in the animals when fed either whole algae or the FFA of the algal oil suggesting that algae could be a reliable source of dietary ω3 PUFA, and should be considered for further testing in the modification of plasma fatty acid composition in humans and other animals. Additionally, no adverse health effects or toxicity were noted in the rats that were fed either whole *Nannochloropsis* or *Isochrysis,* or when fed the FFA of *Nannochloropsis*.

Further examination of the ω3 benefits from algal products was explored in 1995 by Mokady et al.. This study offered data that whole *Nannochloropsis* sp. biomass could be used as a source of ω3 fatty acids, particularly EPA for pregnant and nursing female rats. The *Nannochloropsis* sp. biomass was added to the diet at 20 g/kg and fed to the female rats

throughout mating, pregnancy and lactation. The data offered evidence that the ω3 fatty acids from the algal source were transferred to the developing fetuses and subsequently the newborn pups. This dietary ω3 source was able to provide adequate long chain fatty acids for normal brain development in the young rats, specifically seen as increased levels of DHA in the brain tissue. There was no mention of adverse health effects on the dams or pups from the algal diet, and growth of the pups proceeded at a normal rate. The researchers also made note that no toxicity was observed

In 2003 Werman et al. developed a study to evaluate the effect of *Nannochloropsis* sp. biomass, the extracted lipids, and the remaining fraction after the lipids were extracted (residue) on the reduction of cholesterol in rats fed high cholesterol diets[3]. The male Sprague-Dawley®™ rats were separated into four groups and fed a standard diet enriched with cholesterol (10 g/kg) and cholic acid (2 g/kg diet). The control group consumed the high cholesterol diet with no added algae (whole, lipid fraction or residue). The remaining three groups were fed the high cholesterol diet, with either whole freeze dried *Nannochloropsis* (100 g/kg diet), algal lipid extract (35 g/kg diet) or the algal residue (65 g/kg diet). The study results showed that the rats fed the whole *Nannochloropsis* and the lipid extract had a marked reduction in plasma and liver cholesterol. The rats fed the algal residue had only a minor reduction in liver cholesterol compared to the control group, however a significant reduction ($P<0.05$) in plasma cholesterol compared to the control group. It was hypothesized that this reduction in plasma cholesterol when feeding the whole algal could have been due to the increased propionic acid production in the cecum, due to the fermentation of the soluble fiber content in the algal residue. The increase in this short chain fatty acid (SCFA) has been shown to inhibit hepatic cholesterol synthesis. The plasma cholesterol reduction that was observed when the rats were fed the algal meal was considered to be a result of the insoluble fiber content in the algal meal. Insoluble fibers are

believed to have an effect on plasma cholesterol by modifying the absorption and metabolism of bile acids. This study supported previous work that noted no adverse or toxic effects to the animals that consumed *Nannochloropsis sp.*.

In an effort to determine alternative feeds to increase the yolk color and the EPA and DHA levels in poultry products, Nitsan et al. (1999) fed laying hens diets comparing whole *Nannochloropsis sp*., *Nannochloropsis* oil and mantur oil. The algal source contributed EPA (20:5) to the poultry diet, and the mantur oil contributed alpha-linolenic acid (LNA 18:3). Three experiments were carried out with six diets (1% *Nannochloropsis* meal, 1% *Nannochloropsis* meal + glucanase and pentinase, 0.3% *Nannochloropsis* lipid, 1% *Nannochloropsis* lipid, 1% mantur oil, basal diet). Experiment one compared three levels of *Nannochloropsis* biomass (0.1, 0.5 and 1.0%) and the effect on ω3 levels and color in the egg yolks. Experiment 2 studied the effect of carbohydrate-hydrolyzing enzymes on the digestibility of the algal biomass (fed at 0.1% and 1.0%), and the subsequent effect on yolk color. The hens were divided into 8 groups, 4 were fed 0.1% *Nannochloropsis* biomass and 4 were fed 1.0% *Nannochloropsis* biomass. The groups of hens were then fed diets with no added enzymes, added cellulose, added glucanase, and added cellulose and glucanase. The third experiment fed 35 birds the six prepared diets (1% *Nannochloropsis* meal, 1% *Nannochloropsis* meal + glucanase and pentinase, 0.3% *Nannochloropsis* lipid, 1% *Nannochloropsis* lipid, 1% mantur oil, basal diet), and the eggs collected, however the birds were sacrificed at the end of this experiment and the plasma, liver and thigh muscles were collected and analyzed for fatty acid composition. Yolk color was measured with a Hoffman-La Roche color fan, and in experiment one, the increased yolk color was found to be dose dependent on the algae level in the diet. In experiment two, greater color scores (~90% greater) were seen with the greater algae level as well (1.0% versus 0.1%). The addition of both cellulose and glucanase increased the rate of increased coloration during the first

ten days of feeding, compared to maximum coloration at day 26 with no added enzymes. Using either of the enzymes alone resulted in an intermediate increased rate of coloration (between combined enzymes and no added enzymes). The third experiment resulted in similar results, with the 1.0% *Nannochloropsis* lipid addition having the most significant effect on yolk color. The addition of mantur oil did not increase the yolk color, and was comparable to the basal diet. The second and more emphasized aspect of this study was on the increased ω3 fatty acid levels in the yolk as well as various tissues *(P*lasma, liver, thigh muscle). The addition of 0.1% or 0.5% *Nannochloropsis* (experiment one) into the poultry diet increased the DHA level in the yolk; however the addition of 1% *Nannochloropsis* did result in an increased DHA level (~25% over basal value). A similar increase in DHA level was seen in experiment two, from the addition of 1% *Nannochloropsis* supplemented with both cellulose and glucanase (~35% increase). Experiment three showed the biggest change in total fatty acid composition in the groups fed 1% *Nannochloropsis* oil or 1% mantur oil. Both of these groups displayed the lowest ω6 (LA + AA) levels, and 2-3 fold greater ω (LNA + EPA+DHA) versus control. The levels of the different ω3 fatty acids in the yolk, plasma, liver and thigh muscle were affected by the dietary source of the ω3 fatty acids. The yolks from hens fed *Nannochloropsis* biomass or lipid had only measurable levels of DHA, and the yolks from hens fed mantur oil had measurable ω3 levels that were 50% DHA and 50% LNA. EPA was not detected in any of the experimental group's yolks. The plasma from hens fed *Nannochloropsis* biomass or lipid had similar levels of 18:3 to the control hens, but much decreased levels than the hens fed mantur oil. All of the groups had low levels of EPA with the exception of the group fed 1.0% *Nannochloropsis* lipid, which had the highest plasma EPA level. DHA levels were increased in the plasma of hens fed either 1.0% *Nannochloropsis* lipid or 1.0% mantur oil as compared to the other groups. The livers from the hens of all the groups had very low/undetectable levels of EPA. The livers from the

Nannochloropsis fed hens had ~14-30% LNA and 86-70% DHA compared to 57:43 LNA/DHA in the mantur oil fed group. The thigh muscle analysis revealed only LNA as the ω3 source, with the mantur oil group possessing the highest LNA level. This study was able to provide support for the use of marine algae as a way to enrich the level of ω3 fatty acids and a way to manipulate the overall ω3 levels in poultry products. Additionally, no adverse health effects were noted by the researchers.

As mentioned previously, there is a paucity of data on feeding *Nannochloropsis* sp. algae extract, oil or whole biomass. In an effort to understand the nutritional value of *Nannochloropsis* oc. as a potential feedstuffs, Archibeque et al. (2009) compared the nutrient profiles of *Nannochloropsis* biomass, *Nannochloropsis* meal (lipid extracted), soybean meal, and steam flaked corn. The fiber composition of *Nannochloropsis* meal differed from soybean meal, and steam flaked corn with a greater ADF (6.64% vs. 5.89% and 2.92%), NDF (25.12% vs. 11.45% and 9.59%). The N fractions (%CP) was also noticeably different, as the algal meal had a much greater level of B_3 fraction (63.52%), as compared to 1.82% (soybean meal) and 11.92% (steam flaked corn). TDN was comparable at 79.04% (algal meal), and the testing done for this publication had a non-detectable level of fatty acids. This nutrient analysis offered support for the further exploration of utilizing *Nannochloropsis oculata* meal as a source of protein, and possibly minerals for livestock feed

***Schizochytrium* sp.**

The golden algal species *Schizochytrium* sp, is in the same kingdom and phylum as *Nannochloropsis* sp., yet differs in its fatty acid composition as well as color. This alga offers high levels of DHA as well as carotenoids, and has been researched to a greater degree than the microalgae *Nannochloropsis* sp. This is a result of the commercialization of a *Schizochytrium*

strain known as DHA-Gold®. This specific DHA product has been developed by Martek Biosciences Corporation.

The research began in 2001, when Hammond et al. began a series of studies in Sprague-Dawley®™ rats and New Zealand white rabbits. Hammond et al. (2001, 2002) investigated the anti-toxicity of *Schizochytrium* in a series of four studies. This research covered the feeding of Sprague-Dawley®™ rats for 13 weeks and evaluating them for any signs of toxicity at dosages up to 4000 mg/kg/day. The second study reviewed the feeding of gestational rats and rabbits, at levels of 180, 600 and 1800 mg/kg/day. The third study evaluated any reproductive toxicity and the final study focused on mutagenicity. All of the results showed no clinical signs of toxicity. These studies were isolated to the whole algae, with the fat included Dahm's et al.(2011) also evaluated the safety of the oil only from *Schizochytrium*, and in a 90 days study with rats, no adverse effects were noted

In 2003, a study was conducted by Abril et al. (2003), to evaluate the safety and toxicity of *Schizochytrium* sp. in growing swine. This study was ground breaking, as traditionally fish oils have been fed to increase the PUFA content of pork products, however there have been organoleptic issues with marine oils. A high DHA alga could offer an alternative to the off flavor/smell contributed by fish products, and subsequently increase the PUFA content of the pork. Abril et al. (2003) fed 145 growing swine were for 120 days. The algae was introduced to one group at a level of 2.68 kg per pig for the entire 120 days, while four other groups were fed the algae (1.169 kg/pig, 3.391 kg/pig, 5.746 kg/pig) in a finishing diet (last 42 days of the growing cycle). The researchers noted that no significant differences were seen in body weights, food consumption, mortality, hematologic values, gross necropsy evaluation, organ weights or histology, between any of the groups. The only difference they mentioned observing in the algae fed swine was increased weight gain and feed conversion efficiency, related to treatment level.

Abril et al. (2003) suggested that these increases were consequential of the increased fat content with the greater levels of algae fed This study was able to show that *Schizochytrium* sp. could be fed to growing swine without any adverse effects.

In 2009, Herber-McNeill et al. evaluated feeding *Schizochytrium* sp. to laying hens and the effect on egg yolk color and consumer acceptability. Generally fish oil (menhaden oil) is used to increase ω3 levels; however it can have an adverse effect on flavor. The whole alga was used, and the hen diets were supplemented with 2.4% or 4.8% algae. After a four week feeding the egg yolk color and flavor (consumer acceptance) was evaluated The flavor of the eggs from the hens fed both levels of algae was acceptable, and not significantly different from the control group's eggs. The color of the eggs from the hens fed algae, were more red in color as compared to the control group, but the yellowness of the yolks was not influenced The result of this study was data that suggested feeding algae could increase the nutritional quality (increased ω3 levels), without altering the eggs acceptability by consumers.

Schizochytrium sp has been fed to dairy cows, and dairy sheep in order to determine if there is an increase in nutritional benefits in the resulting milk, as well any effect on rumen fermentation. Franklin et al. (1999) fed *Schizochytrium* sp. *(P*rotected against rumen fermentation and unprotected) to dairy cows and the results showed that the milk from the algae fed cows had greater levels of conjugated linoleic acid (CLA), DHA, as well as transvaccenic acid. The cows fed protected algae had greater levels of DHA in their milk as compared to the cows fed unprotected algae. The algal fed cows also produced milk that was decreased in total saturated fatty acids versus cows fed a control diet. Another study in dairy cows took place at the University of Ghent, in Belgium. Boeckaert et al. (2006) studied the effects of feeding dairy cows *Schizochytrium* and changes to the rumen protozoa population, and accumulation of biohydrogentation intermediates. The analysis of the rumen protozoal population revealed that

the algal fed cows had a significantly greater number of protozoa versus the control fed cows; however the absence of one specific protozoa (Isotricha prostoma) was noted in the rumen material from the algal fed cows. The data also shows that the rumen contents of the cows fed the algae had significantly decreased levels of C18:0, C18:1 t6t9, and CLA c9t11, but much greater levels of biohydrogenation intermediates, C18:1 t10t11, C18:1 c9, C18:2 t11c15, CLA t10c12, tCLA and C18:3 c9t11c15 in comparison to the control diet. The accumulation of biohydrogenation intermediates appeared to be associated with the disappearance of the rumen ciliate Isotricha prostoma. The hypothesis was then drawn that Isotricha prostoma had a role in the biohydrogenation of fatty acids in the rumen. In summary, by altering the rumen protozoa population with an algal source of DHA, there was an accumulation of biohydrogenation intermediates, but decreased levels of resulting CLA and transvaccenic acid

Algal effects on rumen fermentation and milk fatty acid composition was explored further by Boekaert et al. in 2008. Two experiments were conducted, and the first study fed dairy cows for 21 days, and one of the test diets included *Schizochytrium* sp., which was supplemented directly into the rumen via a fistula at a rate of 43 g/k of dry matter intake. The algal diet had no effect on rumen pH, yet the algal diet did increase the rate of biohydrogenation of linoleic acid to linolenic acid As a result the milk fatty acid composition of the algal fed cows had a greater level of CLA c9t11, CLA t9c11, C18:1 t10, C18:1 t11 and C22:6 ω3. A negative effect of the algal diet was a 45% decrease in dry matter intake, and milk yield The researchers noted that these results were impractical, and developed a second study. The second study fed dairy cows for 20 days, and included *Schizochytrium* sp. at a rate of 9.35 g/kg of total dry matter intake. This study included the algal biomass directly into the concentrate that the cow was eating, rather than being introduced directly into the rumen. The outcome of this study showed an increase in the rumen pH, and decreased SCFA concentrations. In study 2, the milk fatty acid

profile changed similarly to study 1, yet the milk fat percentage decreased from 47.9 g/kg to 22 g/kg in the algal group. The decrease in dry matter intake and milk yield was decreased than seen in the first study, only ~10%. These studies showed an adverse impact in dry matter intake and subsequently milk yield for cows supplemented with *Schizochytrium* biomass, yet no adverse health or toxicity effects on the cows were note.

The attention to increasing the nutritional value of dairy products (milk) has also included dairy products from sheep. In 2010, Toral et al. fed 50 Assaf ewes 3 different levels of *Schizochytrium* sp. supplemented into their diet. The ewes were in mid-lactation, and fed diets supplemented with sunfdecreased oil and either 0 g/kg, 8 g/kg, 15 g/kg, or 23 g/kg algae biomass. There was no significant difference between the groups on dry matter intake, or milk yield, and only a small reduction (NS) in milk protein content when the ewes were fed the algae. The largest change was seen in the milk fat content between the groups. In all of the groups fed the algae there was a reduced level of milk fat, which only reached 30% in the group fed the highest amount of algae. Although no negative health effects were seen in the ewes fed algae, there were performance effects as seen by the reduced milk fat and milk protein content in the algae fed groups. Supplementing dairy ewes with a sunfdecreased oil & algae combination did improve the nutritional quality of the milk produced, with increased levels of rumenic acid, vaccenic acid and DHA, as well as a reduced ω6:ω3 fatty acid ratio.

Summary of Algal Research

In summary, the research that has been done, albeit minimal, has shown no negative health consequences. The animals have continued to grow properly, as well as produce milk, eggs normally, and no toxicological effects were noted in any study. However, as mentioned, the focus on algae more recently has been on the use of the oil for biofuel, resulting in a high protein co-product. This co-product could be a valuable protein feed for livestock, and it is clear after

reviewing the studies that have been conducted, there is a need for further study of the extract residue from *Nannochloropsis* sp.. The attention to new, alternative sources of raw materials for biofuel has reached a point that makes the study of algal meal necessary. As the race increases for new and sustainable sources of biofuel, it is useful to look back and see how it all started, where it is today and where it is going for the future.

BIOFUEL: The Past – A History of Fermentation and Distillation

Biofuel is the term that is utilized to describe fuel that derives its energy from carbon fixation or the reduction of CO_2 to organic compounds by living organisms. Generally, fossil fuels are not considered "biofuels" as the carbon in fossil fuels has been out of the carbon cycle for an extended period of time. Biofuel is a general term that encompasses both bioethanol and biodiesel. Bioethanol is an alcohol that is produced through the fermentation of carbohydrates, most commonly from corn and sugar cane. Biodiesel is the fuel that is produced from vegetable oils and animal fats.

Although recently there has been considerable focus on finding and developing alternative fuel sources since 2008 when crude oil prices sky rocketed to over $100 USD per barrel, using biofuels, specifically bioethanol has a much longer history. The first production of ethanol *(P*ure alcohol) dates as far back as 9000 years ago, when humans learned to ferment sugars and produce an alcohol that they could consume for recreational purposes (National Geographic 2005). By the first century A.D, people in Greece and the Middle East had learned how to increase the level of alcohol in their fermented beverages through the process of distillation (Forbes 1970).

The first uses of fermentation and distillation were clearly for the production of alcoholic beverages, however over time it became obvious that ethanol could be used in other applications; such as automobile fuel.

Beginning in 1824, Samuel Morey, the creator of the world's first internal combustion engine, made an engine that ran on ethanol and turpentine. In 1862 a special tax was placed in industrial alcohol in order to pay for the Civil War, during this time ethanol was not considered the fuel of choice due to the added taxation. This tax was repealed in 1906, and ethanol was once again used widely as a fuel source in the U.S.A. The first Ford Model T™ was manufactured in 1906 and was capable of using ethanol, gasoline or kerosene. The use of ethanol once again became unfavorable during prohibition which ran from 1919-1933, when it was illegal to manufacture, sell or transport alcohol. Prohibition combined with the end of World War I, made gasoline the most popular fuel source in the U.S.A and other parts of the world In Brazil at this time, cars were only just being introduced, and they were fueled by sugar cane ethanol. In fact by 1943, Brazil mandated that all car fuel must be at least 50% ethanol. In the U.S.A. World War II caused another increase in the demand and use of ethanol, however as soon as the war was over, gasoline became cheap and readily available again. This era of a fossil fuel energized society continued to grow until the 1970's, then oil embargoes resulted in greater oil prices. In 1974 the U.S.A. enacted the Solar Energy Research, Development and Demonstration Act, and allocated funds to develop alternative fuel options. By 1992, the Energy Policy Act was enacted, and this made it mandatory for a certain number of cars to be made that can run on alternative "flex" fuels. This Act required that some cars must be able to run on fuel that is at least 85% ethanol (E85).

Currently it is estimated that just over 40% of the world's corn is grown in the U.S.A., and as a result, the U.S.A. is also the world's largest producer of corn ethanol. Between 1979 and 1986, domestic production of ethanol rose from 20 million U.S. liquid gallons (75 million liters) to over 750 million U.S. liquid gallons (2.84 billion liters). The driving factor of this impressive increase was the Energy Tax Act of 1978, which created subsidies in the form of tax credits for

corn ethanol manufacturers. The U.S.A. followed this Act with the Energy Policy Act of 2005, which mandated that at least 7.5 billion gallons of ethanol must be consumed annually by 2012. In 2007, it was mandated through this Act that the annual consumption of corn ethanol by 2015 was to be increased to 15 billion gallons. It was also in 2007 that the Energy Independence and Security Act went into place to support the increased production of corn ethanol. This was done in an effort to both reduce greenhouse gas emissions as well as improve the economy in rural America (USDA 2010). The goal set forth was to use at least 36 billion gallons of bio-based fuels by 2022. In 2009, the U.S.A. produced just over 10 billion gallons of corn ethanol, and 2010 production was expected to exceed 12 billion gallons. Looking into the future, it was included in the mandate that 15 billion gallons of the 36 billion gallons could come from current biofuel products (corn ethanol), however 16 billion gallons must come from advanced cellulosic materials (other feedstuffs). This has opened the door for other biofuel options that are being explored in great detail around the world

With all of the corn ethanol produced in the U.S.A., it is important to look at the co-products of ethanol manufacturing. Since 1979 the amount of corn ethanol produced has increased from 20 million gallons to over 12 billion gallons by 2012. This massive increase has simultaneously increased the amount of ethanol co-product feed; distillers grains, by equally impressive proportions.

BIOFUEL: The Present – Co-Products of Biofuel Production in Animal Feed

Although the most common feedstuffs used in livestock rations are soybean meal, corn gluten feed, and steam flaked corn, and cracked corn, agricultural co-products have long had a place in cattle, dairy, swine and poultry diets. Feeding animals the co-products from corn fermentation and distillation goes as far back as 1900, when dried distillers grains were compared to oats (Henry, 1900). By 1945 the Distillers Feed Research Council was formed with the

specific task of expanding their knowledge of the nutrient composition of distiller's grains, and their applications. The Distillers Feed Research Council was later named the Distillers Grains Technology Council in 1997. The use of ethanol co-products in animal feed was extensively researched in the 1970-19080s. Research has continued as ethanol processing changes, and other feedstuffs are utilized, i.e. sorghum and wheat. Klopfenstein et al.(2008) reviewed the use of distiller's by-products in feeding beef cattle, and its benefits in replacing corn in a feedlot ration. They were able to outline the importance of utilizing the distiller's by-products and the improved ADG and F:G that can be achieved by feeding wet or dry distillers grains. Schingoethe et al. (2009) published similar data for the dairy industry. These researchers reported that similar performance was seen in lactating cows when fed wet or dry distillers co-products, due to the high level of RUP protein, and energy. The co-products of the ethanol industry have shown favorable results in ruminant diets and are common place now in beef and dairy diets a like. However, it has been shown through research that non-ruminant animals can also consume and benefit from distillers co-products. Stein et al. (2009) reviewed the use of dried distillers grain solubles in swine feed, which has been studied for more than 4 decades. The results of the studies that have been done, have shown that growing pigs at all phases of production can be fed feed rations with up to 30% DDGS, and lactating and gestating sows can tolerate diets including 30-50% DDGS without negatively affecting performance. It is clear that the role distiller's grains have in livestock feed is an important one, for the U.S. agricultural economy, and the U.S. economy in general. Because of this economic importance, ethanol co-products remain a pivotal point in the discussion of the increased ethanol production in the U.S.A..

A 56 pound bushel of corn can yield approximately 17 pounds of distiller's grains (dry milling) and 2.8 gallons of ethanol (RFA 2008). In 2007/2008 2.5 billion bushels of corn were processed into 19.3 million MT of distiller's grains. This marked a ten-fold increase over the last

decade. The traditional outlet for distiller's grains has been the livestock feed market. Initially the distiller's grains were fed to beef and dairy cattle. In recent more recent years the distillers grains are being fed to swine and poultry. In the December 2010 the USDA publication, Market Issues and Prospects for U.S. Distillers Grains: Supply, Use and Price Relationship report addressed the concerns of the agricultural community that the production of corn ethanol will produce an excessive amount of co-product (estimated to be 38 million metric tons in 2009/2010), much more than can be consumed by the livestock feed industry. This report went on to outline the potential usage of distillers grains in livestock feed systems. The market research referenced suggests that the projected need for distillers grains as livestock feed could potentially exceed 46,000,000 MT, meaning that production of distillers grains will not exceed the potential feed market for them (Table 1.1).

While it is clear that corn ethanol plays an important role in the feeding of livestock as well as supplying the nation with a non-fossil fuel option, the USDA report did outline the need for other "alternatives" to corn ethanol. Currently there are other biofuel options being explored, and with those biofuels there are new co-products that are becoming available for use in livestock feed As stated above, the potential usage of distiller's grains could exceed the actual production, this could be a good opportunity for other feed supplements to be utilized in livestock feeds.

BIOFUEL: The Future – Algae?

As other grains are being processed into ethanol products, there has been resurgence in the consideration of other plant-like materials, algae for example. It is interesting to note that between 1978 and 1996 the Department of Energy in the U.S. was funding a large project to develop a biofuel program using microalgae as the source of oil. This focus on alternative oil sources was in reaction to climbing crude oil prices at the time (>$80/barrel), however by the early 1990's crude oil prices had dropped back down ($20-30/barrel) and algae was no longer an

economical fuel source to explore. This perspective changed when in 2008 crude oil prices hit a historical high of >$100/barrel. Algae are being researched more and more as an alternative to both fossil fuel as well as corn ethanol.

Today, a basic search of "algae biofuel" on the internet will yield a minimum of 1.8 million topics. The interest in algae as a potential material for biofuel has grown immensely in Europe, with an algae research center opening in The Netherlands in June of 2011. The interest in algae has grown from the relatively large oil content of some algal species (>70% on a dry weight). The potential high oil yield per acre combined with the relative ease and minimal need for arable land makes algae a very appealing raw material for biofuel. Cristi et al. (2007) reported that microalgae appeared to be the only potential source of biofuel that could effectively replace fossil fuels. Cristi et al. (2007) compared the land area needed, and oil yield from common oil crops to that of microalgae. In order to meet 50% of the fuel needed for transportation in the U.S.A., 1540 M hectare of corn or 594 M hectares of soybeans, or 4.5 M hectares of 30% oil microalgae would be needed. The oil yield per hectare (L/ha) is estimated to be 172 L/ha for corn, 446 L/ha for soybeans and 58,700 L/ha of 30% oil microalgae (Cristi et al. 2007). Several species of microalgae have been explored for their potential use in biofuel production, and a couple of species in particular stand out: *Nannochloropsis* sp., *Neochloris oleobundans* (Gouveia et al. 2009). Both of these algal species have >28.7-29% oil by biomass weight. This oil content can be increased (by 50%) by growing the algae under nitrogen short conditions, as algae can be grown in reactors, under environmental conditions that would be un-favorable to traditional oil crops. The ramification of this is that the amount of land and type of land needed to produce large amounts of oil is very different than the land needed to grow food crops. In April of 2006, Solix Biofuels in Fort Collins, CO developed a reactor that could be

operated in conjunction with an existing power station (Singh et al. 2011). The high oil content and reactor technology make algae a promising, renewable raw material for biofuel.

As with corn ethanol, there is a potentially useful co-product that results from the manufacturing of algae oil; algal meal. The biomass that remains after the oil is extracted is a high protein, mineral rich feedstuffs that is being researched for its usefulness in livestock feed. A comparison of algal meal from *Nannochloropsis oculata*, to other commonly fed oil crops and an ethanol co-product is shown in Table 1.2. Although algal meal has not been fed to livestock in any great volume, the potential is there for a beneficial protein feedstuffs that could be used as a replacement for soybean meal, corn, and/or DDGS in feedlot, dairy, swine or ovine rations. Whole algae (different species) have been fed to beef cattle, dairy cattle, and poultry throughout the years, and currently work is being conducted on the safety and efficacy of the residual meal (oil extracted).

The TDN % and the DE (Mcal/kg) of *Nannochloropsis oculata* are very comparable to soybean meal and corn gluten feed Having a useful co-product makes the utilization of algae in livestock feed more appealing, as the co-product will allow the cost of the oil to be controlled As mentioned in the previous section, the potential use of DDGS in livestock feed is greater than the estimated projected production. If algal meal can be formulated into rations then it could provide an alternative to soybean meal and corn gluten feed, and possibly DDGS.

Final Summation

In 2008, 88% of the world's fuel needs was met primarily by fossil fuels (Singh et al. 2011). It is widely accepted now that new, alternative fuels must be used in larger amounts in order to meet the ever growing demand for energy. In the U.S.A. corn ethanol is meeting some of the growing demand for energy sources, however corn ethanol cannot meet the entire demand The use of corn (and other crops) as a source of fuel is directly competing with traditional

livestock feeds, as well as the human food chain. The co-products from ethanol production can be used in animal feeds, but there are maximum levels that the animals can consume and remain healthy and productive. The feeding limitations, combined with the competition between ethanol manufacturers and livestock producers is driving the cost of grains greater, resulting in a greater priced food supply for the population.

An alternative to both of these challenges is to look beyond traditional oil crops for biofuel raw material, and the utilization of the co-products of alternative biofuel production. Algae offer a possible solution to both issues and should be researched fully to understand its full potential.

Table 1.1. Estimates of Potential Annual DDGS Consumption, By Livestock Class

LIVESTOCK CLASS	AVERAGE 2000-2004 THOUSANDS OF METRIC TONS
Beef cattle	30,863
Beef Cows	7,793
Cattle on Feed	14,266
Other Cattle	8,804
Dairy Cattle	6,524
Dairy Cows	6,524
Swine	3,752
Breeding Swine	921
Market Swine	2,831
Poultry	5,606
Broilers	3,567
Layers	1,458
Pullets	133
Turkeys	686
TOTAL	46,744

Sources: Dhuyvetter et al.; 2005, Berger and Good, 2007; Dooley, 2008; Fox, 2008.

Table 1.2. Livestock Feedstuffs Comparison

FEED	IFN	DM %	DE Mcal/kg	CRUDE PROTEIN %	EE %	NDF %	ADF %	TDN %
Soybean Meal	5-20-637	90.9	3.7	51.8	1.67	10.3	7	84
Corn Gluten Feed	5-28-243	90	3.53	23.8	3.91	36.2	12.7	80
Corn Grain-Cracked	4-20-698	90	3.92	9.8	4.06	10.8	3.3	90
DDGS	5-28-236	90.3	3.88	30.4	10.7	46	21.3	90
Algal Meal (*Nanno. sp.*)	NA	90.4	3.60	31.3	8.9	29.6	16.1	81

Sources: NRC Nutrient Requirements of Beef Cattle, Update 2000.
SOLIX Biofuels - Algal Meal 2009.

LITERATURE CITED

Abril, R., J. Garrett, S. Zeller, W. Sander. 2003. Safety Assessment of DHA-rich Microalgae from *Scizochytrium* sp. Part V: target Animal Safety/Toxicity Study in Growing Swine. Reg. Tox. Pharm. 37:73-82.

Archibeque, S.A., A. Ettinger, B.D Willson. 2009. *Nannochloropsis oculata* As A Source For Animal Feed Acta. Agron. Hung. 57:245-248.

Boeckaert, C., N. Boon, I.Z. Abdulsudi, W. Verstraete, V. Fievez. 2006. Accumulation of Biohydrogenation Intermediates and Changes in the Rumen Protozoal Population After Micro Algae Feeding to Dairy Cattle. Comm. Appl. Biol. Sci. Ghent University. 71:83-86.

Boeckaert, C., B. Vlaeminck, J. Dijkstra, A. Issa-Zacharia, T. Van Nespen, W. Van Straalen, V. Fievez. 2008. Effect of Dietary Starch or Micro Algae Supplementation on Rumen Fermentation and Milk Fatty Acid Composition of Dairy Cows. J. Dairy Sci. 91:4714-4727.

Ciferri, O.. 1983. Spriulina, the Edible Microorganism. Microbiological Rev. 47.

Cole, K., R. Sheath. Biology of the Red Algae. 1990. Cambridge University Press 2:221-258.

Cristi, Y.. 2007. Review Paper: Biodiesel from Microalgae. Bio. Tech. Adv. 25:294-306.

Forbes, R.J.. 1970. A Short History of the Art of Distillation: From the Beginning up to the Death of Cellier Blumenthal. BILL., ISBN 9789004006171. 1970.

Dahms, I.F., P.A. Marone, E. Bailey-Hall, A.S. Ryan. 2011. Safety Evaluation of Algal Oil from *Schizochytrium* sp. Food and Chem. Tox. 49:70-77.

Franklin, S., K. Martin, R. Baer, D Schingoethe, A. Hippen. 1999. Dietary Marine Algal (Schizochytrium sp.) Increases Concentrations of Conjugated Linoleic, Docosohexaenoic and Transvaccenic Acids In Milk of Dairy Cows. J. Nutr. 129:2048-2052.

Gouveia, L., A.C. Oliveira. 2009. Microalgae as a Raw Material for Biofuels Production. J. Ind Microbiol. Biotech.. 36:269-274.

Hammond, B.G., DA. Mayhew, M.W. Naylor, F.A. Ruecker, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from *Schizochytrium* sp.: I. Subchronic Rat Feeding Study. Reg. Tox. and Pharm. 33:192-204.

Hammond, B.G., DA. Mayhew, J.F. Holson, M.DNemec, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from *Schizochytrium* sp.: II. Developmental Toxicity Evaluation in Rats and Rabbits. Reg. Tox. and Pharm. 33:205-217.

Hammond, B.G., DA. Mayhew, K. Robinson, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from *Schizochytrium* sp. III. Single Generation Rat Reproduction Study. Reg. Tox. and Pharm. 33:356-362.

Hammond, B.G., DA. Mayhew, L.D Kier, R.W. Mast, W.J. Sander. 2002. Safety Assessment of DHA Rich Microalgae from *Schizochytrium* sp. IV. Mutagentcity Studies. Reg. Tox. And Pharm. 35:255-265.

Herber-McNeill, S.M., M.E. Van Elswyki. 1998. Dietary Marine Algae Maintains Egg Consumer Acceptability While Enhancing Yolk Color. Poultry Science 77:493-496.

Henry, W.A. 1900. Dried Distillery Grains Compared with Oats. Feed and Feeding 2nd Edition 421. Morrison Publishing Co.

Hibberd, DJ.. 1981. Notes on the Taxonomy and Nomenclature of the Algal Classes Eustigmatophyceae and Tribophyceae. Bot. J. Linnean Soc. 82:93-99. 1981.

Klopfenstein, T.J., G.E. Erickson, V.R. Bremer. 2008. Board Invited Review: Use of Distillers By-Products in the Beef Cattle Feeding Industry. J. Anim. Sci. 86:1223-1231.
Lavy, A., Y. Neveh, R. Coleman, S. Mokady, M. Werman. 2003. Dietary Dunaliella bardawil, a β-carotene Rich Algae, Protects Against Acetic Acid Induced Small Bowel Inflammation in Rats. Inflammatory Bowel Disease 9(6): 372-379.
Lee, H., M. Kim. 2009. Effect of Chlorella vulgaris on Glucose Metabolism in Wistar Rats Fed High Fat Diet. J.Med Food 12(5):1029-1037.
Markovits, A., R. Conejeros, L. Lopez,M. Lutz. 1992. Evaluation of marine microalga *Nannochloropsis* sp as a potential dietary supplement. Chemical, nutritional and short term toxicological evaluation in rats. Nutr. Res. 12, 1273-1284.
Mokady, S. 1995. A Marine Unicellular Alga in Diets of Pregnant and Lactating Rats as a Source of ω3Fatty Acids for the Developing Brain of their Progeny. J. Sci. Food Agric.. 68:133-139.
Mokady, S., A. Ben-Amotz. 1991. Dietary Lipid Level and the Availability of β-carotene of *Dunaliella bardawili* in Rats. Nutrition & Cancer, 15:47-52.
Murthy, K.N., A. Vanitha, J. Rajesha, M. Swamy, P.R. Sowmya, G. Ravishankar. 2005. In Vivo Antioxidant Activity of Carotenoids from Dunaliella salina, a Green Microalgae. Life Sciences 76:1381-1390.
Nitsan, Z., S. Mokady, A. Sukenik. 1999. Enrichment of Poultry Products with ω3 Fatty Acids by Dietary Supplementation with Alga *Nannochloropsis* and Mantur Oil. J. Agric. Food Chem.. 47, 5127-5132.
Roach, J. 2005. 9000 Year-Old Beer Re-Created From Chinese Recipe. National Geographic News. www.news/nationalgeographic.com/news/2005/07/0718.
Renewable Fuels Association (RFA). 2008. Feeding the Future: The role of the U.S. Ethanol Industry in Food and Feed Production. Washington DC..
Scingoethe, DJ., K.F. Kalscheur, A.R. Hippen, A.D Garcia. 2003. Invited Review: The Use of Distillers Products in Dairy Cattle Diets. J. Dairy Sci. 92:5802-5813.
Sellner, K.G., G.J. Doucette, G.J. Kirkpatrick. 2003. Harmful Algal Blooms: Causes, Impacts, and Detection. J. of Indust. Microb. and Biotech.. 30:383-406.
Singh, A., P.S. Nigam, J.D Murphy. 2011. Renewable Fuels from Algae: An Answer to Debateable Land Based Fuels. Biores. Tech. 102:10-16.
Stein, H.H., G.C. Shurso. 2009. Board Invited Review: The Use and Application of Distillers Dried Grains with Solubles in Swine Diets. J. Anim. Sci. 87:1292-1303.
Sukenik, A., T. Hidehiro, S. Mokady. 1994. Dietary lipids from marine unicellular algae enhance the amount of liver and blood omega-3 fatty acids in rats. Ann. Nutr.Metab, 38:85-96.
Werman, M., A. Sukenik, S. Mokady. 2003. Effects of the marine unicellular alga *Nannochloropsis* sp. to reduce the plasma and liver cholesterol levels in male rats fed on diets with cholesterol. Biosci. Biotechnol. Biochem., 67 (10), 2266-2268.
A USDA Regional Roadmap to Meeting the Biofuels Goal of the Renewable Fuels Standards by 2022. USDA Biofuels Strategic Production Report. 2010.
Villar, R., M.R. Laguna, I. Cadavid, J.M. Calleja. 1994. Effects of Aqueous Extracts of Six Marine Microalgae on Smooth Muscle Contraction in Rat Duodenum and Vas Deferens. Planta Med 60:521-526.

Chapter 2

Nannochloropsis oculata algal meal as a safe and nutritionally adequate crude protein supplement for young growing rats[1]

INTRODUCTION

The use of algae oil as a source of biofuel has been researched for more than a decade however it has only been in more recent years that the consideration of algae oil for biofuel has become much more interesting. Certain species of algae can produce upwards of 5000 gallons of potential biofuel per acre of land, versus 18 gallons per ace of corn and 48 gallons per acre of soybeans (Sheehan et al. 1998). One of these high oil producing algal species in the microalgae *Nannochloropsis oculata. Nannochloropsis o.* can produce 40-45% oil, as well as a high protein algal meal that is the by-product of the oil extraction process. Archibeque et al.(2009) compared the chemical composition on a dry matter basis of the algal meal from *Nannochloropsis oculata* to that of soybean meal (SBM), and steam flaked corn (SFC), two common grains fed to commercial livestock as protein sources. The total crude protein (CP) content of the algal meal was 35.28%, as compared to 51.55% in the SBM and 8.86% in the SFC, making this algal meal an adequate protein supplement for animal diets, in the same manner SBM and SFC is used today. The soluble CP in the algal meal was comparable to the SBM (20.32% and 20.07% respectively). The B3 CP fraction was greater in the algal meal (63.52%), versus 1.82% in the SBM and 11.92% in the SFC. This composition suggests that commercial livestock could utilize the algal meal as a valuable source of CP when added to their feed. The amount of research

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conducted with this material is minimal and includes previous studies that have fed whole *Nannochloropsis oculata* to rats and chickens. The published data suggests there were no harmful or negative effects, even indicating potential health benefits from the high eicosapentanoic acid (EPA) levels. Therefore the objective of this study was to conduct a 36 d feeding trial to more fully evaluate the acceptability, digestibility and nutrient retention of a diet that included 10% algal meal *Nannochloropsis oculata* as compared to a diet with no algal meal.

MATERIALS AND METHODS

This experiment was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Algal Meal

The algal meal that was utilized in this study was supplied by SOLIX Biofuels Inc., Fort Collins, CO. The algae had been collected as a wet paste after centrifugation, dried at 100+/-5°C for ~24 hours. The remaining dried algal mass was then broken into smaller pieces and transferred into filter thimbles for a 24 hr. Soxhlet extraction in hexane. Post extraction the algal meal was then dried for 6-24 hrs at 100+/-5°C, thereby disrupting the cell wall to potentially increase digestibility. The resulting algal meal was then transferred to the Colorado State University Animal Sciences Department to be ground to a fine power using a Wiley Lab Mill model #4 and submitted for nutrient and toxic mineral analysis. The nutrient results for the algal meal are presented in Table 2.1. These results were shared with Harlan Laboratory (Madison, WI) and utilized to manufacture the Algal diet.

Animals and Treatments

A total of 24 Sprague-Dawley®™ 8 week old male rats were obtained from Harlan Laboratories (Madison, WI). The rats were received into the Colorado State University Laboratory Animal Resource center and immediately started on Harlan Teklad 22/5 Rodent diet.

The rats were held under standard laboratory conditions with a room temperature of 21^{0}C, 37-45% humidity and a 12/12 light/dark cycle in IACUC approved solitary cage measuring 10.5" wide, 19" long and 8" high. After a seven day acclimation period, the rats were randomly blocked according to body weight into two separate treatment groups, Control (**CON**) and Algal (**ALG**), and started on the diets specifically formulated for this experiment. The CON diet and the ALG diets were both formulated to be iso-nitrogenous, iso-caloric, and to meet all of the nutritional needs for a young, growing rat (NRC of Laboratory Animals, 4th Revised Edition, 1995), Table 2.2. Twelve rats were fed the CON diet and twelve rats were fed the ALG diet which incorporated 10% algal meal (sp *Nannochloropsis oculata*). The CON group weighed an average of 291+/-8 g initially and an average of 373+/-24g on d36, while the ALG group weighed an average of 291+/-15g, and an average of 377+/-20g on d36 (data not shown). Overall, the CON group gained 81.27 g throughout the study, a 27.86% increase in total BW, and the ALG group gained 85.25 g throughout the study, a 29.18% increase in BW. The ADG at the end of the study was 2.32 g/d (CON), and 2.44 g/d (ALG) (data not shown). Fresh water was made available at all times. Coprophagy was not prevented in order to encourage normal eating behavior.

Fatty Acid Composition

The fatty acid composition of the rat feed is shown in Table 2.3. The fatty acid profile was determined via gas chromatography using a Hewlett Packard (Avondale, PA) Model 6890 series II gas chromatograph fixed with a series 7683 injector and flame ionization detector. The instrument was equipped with a 100-m x 0.25-mm (id) fused silica capillary column (SP-2560 Supelco Inc. Bellefonte, PA). Methyl ester derivatives of fatty acids were prepared using a combination of $NaOCH_3$ followed by HCl/methanol as described by Kramer et al. (1997). Fatty acid methyl ester preparations were injected using the split mode. The carrier gas was helium, and

the split ratio was 100:1 at 180°C. The oven temperature was programmed from an initial temperature of 140°C (0 min) to a final temperature of 225°C at the rate of 2.8^{o}C/min. The final temperature was held for 18 min. Chromatograms were recorded with a computing integrator (Agilent Technologies, Palo Alto, CA). Standard fatty acid methyl ester mixtures were used to calibrate the gas chromatograph system using reference standards KEL-FIM-FAME-5 (Matreya Inc., PA). Identification of the fatty acids was made by comparing the relative retention times of fatty acid methyl ester peaks from samples with those of standards.

Chemical Analysis

Samples of the algal meal, diet, feces and rat samples were analyzed for DM, crude fat and ash using AOAC 2005 methods. The lipid content of the rats was determined using acid hydrolysis (AHF) (AOAC 2005). Crude protein was determined using the Kjeldahl AOAC 2005 method, and calculated from total N values (N x 6.25). NDF and ADF (Goering and Van Soest, 1970) were determined using a modified Van Soest using fiber bag technology (Ankom 200 and Daisy II Incubator, Ankom Technology Corp., Macedon, NY). Urine nitrogen was quantified using peroxysulfuric acid as described by Hach et al. (1985). Mineral analysis was conducted by drying the feed and fecal samples at 75° C overnight, and digesting the samples at 95° C with nitric acid at a 1 ml nitric acid:100 mg sample ratio. After digestion 5 ppm Yttrium was used as an internal standard, and the samples were diluted with water to a final volume of 25 mL (50x dilution). One mL of the digested and diluted sample was further diluted to 1:10 with a 20% nitric acid and 5 ppm Yttrium solution (500x final dilution). The final samples were then run on a Varian radial inductively coupled plasma (ICP) atomic emission spectrometer. The whole rat minerals were measured using the same method as for the feed and feces, however the final dilution was 50x, and 1 ppm of Yttrium was used as an internal standard and 500 ppm Cesium was used as and ionization quencher. Urine mineral content was quantified by digesting the

urine in 1 mL nitric acid:1 mL sample, and dried at 95° C overnight. After digestion and drying, 1 ppm of Yttrium was used as an internal standard, and 500 ppm Cesium was used as an ionization quencher. The samples were diluted with water to a final volume of 25 mL (50x dilution) and run on a Varian radial (ICP) atomic emission spectrometer.

Blood Metabolites

On d 0 and d 21 approximately 0.50 mL of blood was drawn with a syringe via a tail puncture and collected in microtainers treated with 15% EDTA K2. The samples were chilled during collection, and a sample was pulled from the microtainer and analyzed using single use ISTAT cartridges EC8+ and CG8+ which were then run through a portable ISTAT analyzer. pH, Total CO_2 (TCO_2), Partial Pressure O_2, Saturated O_2 (SO_2), Partial Pressure CO_2, HCO_3, Base Excess (BE), Sodium (Na), Chloride (Cl), Hematocrit (Hct), Hemoglobin (Hb), and Glucose were reported The remaining blood was then transferred into microtainers treated with NaFl K_2Ox . These samples were chilled on ice, and within 2 hours of collection the serum was separated by centrifugation (672 x g for 10 min. at 21° C). The serum was removed and frozen at -20° C for later analysis. On d 36 blood was drawn via a fatal heart stick following the iso-flourene anesthesia. At this time the blood was analyzed using ISTAT cartridges and portable analyzer (see above). Approximately 10 mL of blood from each rat was collected into microtainers treated with 15% EDTA K3. The blood was chilled on ice during collection and serum was separated by centrifugation (1512 x g. for 10 min. at 21° C) within 6 hours of collection. The serum was removed and frozen at -20^{0} C for later analysis. The serum from all three blood draws was thawed and analyzed for serum urea nitrogen content (SUN) using the Bio-Assay System Quanti-Chrom™ Urea Assay Kit (DIUR-500).

Nutrient Balance Trial

From d 21 to d 28 a balance trial was conducted and the feed intake, water intake, fecal and urinary output were measured for each rat every 24 hours. The rats were housed in IACUC approved solitary metabolism cages; there was no change to the environmental conditions. The CON group had cages designed with the feed bowl inside the cage and the ALG group had the same size cages, with the feed bowl located within an attached holder. Remaining feed and water was weighed every 24 h, and recorded. Feed and water were both offered ad libitum. Urine from each rat was collected every 24 h into a 15 mL conical tube containing 100 µg of 6NHCl to maintain a pH of less than 3.0, and prevent volatilization of urinary N. Each day's urine was combined into a 50 mL conical tube *(P*er rat) and frozen at -20^0 C. The feces from each rat was collected on a screen under the cage, collected and weighed every 24 h, stored in a plastic bag and frozen at -20^0 C. Urine urea nitrogen was determined using the Bio-Assay System Quanti-Chrom™ Urea Assay Kit (DIUR-500).

Bomb Calorimetry

Gross energy (GE) of the rat diets was determined using a Parr 1231 bomb calorimeter, utilizing 2418.5915 MJ as the energy equivalent for the bomb and the sample container. The energy calculation was standardized using benzoic acid tablets (26.953 MJ/kg each), and for every ten samples a standard was run to ensure consistency. The wire used to ignite the sample was Parr No. 45C10, with a standard energy of 2.3 calories/cm, and ten cm were used per test.

Euthanasia and Organ Evaluation

The rats were humanely anesthetized on d 36 via iso-flourene gas and exsanguination via a fatal heart stick. The bodies were then opened from sternum to pelvis and the complete digestive tract was removed. The small intestine, large intestine, stomach and cecum were thoroughly cleaned with a 0.9% saline solution. The livers, kidneys, lungs, heart, spleen were

removed and weighed on all 24 rats. The brains were removed and weighed from 4 rats from each group.

Freeze Drying and Whole Body Composition

The rats were frozen at -20^0 C and were sectioned into thirds for ease of handling. Each rat was then ground using a Robot Coupe Blixer 6V blender and liquid nitrogen. After grinding, the rats were weighed and then placed in separate 9" tins and placed in a Lab Conco Freeze Dryer System. The temperature in the condenser was -40°- -44° C, and the temperature in the sample chamber was 15°-20° C, under a vacuum of 1.33×10^{-3} mtorr. The rats were weighed two to three times per week until there was no weight change for a 24 h. period of time. The freeze dried material for each rat was individually ground through a 2 mm screen using a Wiley Lab Mill, model #4. The final material was then homogenized and submitted for chemical analysis.

Calculations

Calculations for apparent digestibility and nutrient retention were made using the following formulas:

Apparent Digestibility = (Nutrient Intake – Nutrient in Feces)/Nutrient Intake X 100

Nutrient Retention = (Nutrient Intake (g) – Nutrient in Feces (g) – Nutrient in Urine(g))

Statistical Analysis

Data for growth, blood parameters, digestibility, intake, nutrient retention, organ weights and body composition were analyzed using PROC MIXED procedure (SAS Institute Inc., Cary, NC) for repeated measures. The experimental unit was the individual rat, the fixed effect was treatment, and the random effect was date. Differences between treatments were considered statistically significant if $P \leq 0.05$ and trending towards significance if $P \leq 0.10$.

RESULTS

BW and ADG

The rats stayed healthy throughout the study, and were weighed on d0 and every 7 days for the entirety of the study. There was no significant effect from the algal treatment on ADG ($P=0.57$), however there was a Treatment*Date effect ($P<0.01$) as a result of a significant difference between the groups on d 28 and d 36 ($P<.01$), all other dates showed no difference between the groups ($P>0.50$). There was a noticeable weight loss in the ALG group during the 7 day balance trial, however after the trial, the animals were returned to their normal housing, and the ALG group gained weight and their feed intake returned to normal. Overall for the complete study the ALG diet had no significant effect on BW ($P=0.20$) (Figure 2.1), however there was a Treatment*Date interaction effect ($P<0.01$) as a result of a significant difference between the groups at d 28 ($P<0.01$).

Nutrient Intake, Digestibility, Retention and Excretion: DM, Crude Fat, ADF, NDF, Ash

During the 7 d balance trial the ALG group consumed less feed as compared to the CON group ($P<0.01$), yet maintained their water consumption ($P=0.62$). Although the water consumption was similar, the ALG group produced more urine ($P<0.01$), and more feces ($P<0.01$) than the CON rats, Table 2.4 .

The results for the nutrient intake, excretion, retention and digestibility are shown in Table 2.5. The average intake of DM, ADF, NDF and ash were affected by the ALG diet ($P\leq0.04$). These data are logical, as much less feed was consumed by the ALG group. While the intake of crude fat ($P=0.05$) appears to indicate a possible trend for a difference related to the ALG treatment, these data make sense as the ALG diet was greater in crude fat. The average excretion of crude fat was greater in the ALG group ($P<0.01$), while ADF, NDF and ash excretion was decreased in the ALG group ($P<0.01$). The retention of DM ($P<0.01$), crude fat

P=0.01), ADF (*P*=0.01), NDF (*P*=0.67) and ash (*P*=0.09) were all decreased in the ALG as compared to the CON group. Apparent digestibility of DM, crude fat and ADF was also decreased in the ALG rats (*P*<0.03), while there was no difference in the apparent digestibility of NDF and ash (*P*≥0.09).

Nutrient Intake, Digestibility, Retention and Excretion: N and P

The results for N and P intake, excretion, retention and apparent digestibility are shown in Table 2.6. There was decreased N and P consumed in the ALG group (*P*<0.01), these data are understandable, as less DM was consumed during the balance trial. There was more N excreted in the feces (*P*=0.07) and in the urine (*P*<0.01) of the ALG rats compared to the CON group. There was a difference in the amount of P excreted the urine and the feces between the CON and ALG rats (*P*<0.01). The amount of N retained in the two groups was negative, meaning they excreted more than they retained. The amount of N retained was less in the ALG group (*P*=0.02) as compared to the CON group. In contrast the level of P retained was greater in the ALG group (*P*=0.92). The apparent digestibility of the N was decreased in the ALG group (*P*<0.01), yet there was no difference in the apparent digestibility of P (*P*=0.56).

Urea Nitrogen

The amount of N excreted as UN is reported in Table 2.7. There was no difference in the level of UUN in both groups (*P*=0.40). The level of SUN was decreased in the ALG group (*P*=0.20). These data support the hypothesis that the protein from the algal meal would have no effect on UN levels.

Blood Metabolites

The blood metabolite results are presented in Table 2.8. The average pH of the ALG rats was 7.25, while the average pH of the CON rats was 7.28 (*P*=0.01). There was no difference in the blood levels of TCO_2, SO_2, HCO_3, BE, Na, Cl, Hct, Hb, and glucose (*P*>0.10). The difference

in the pH seems to be possibly related to the handling of the rats for the blood draws as opposed to a treatment effect, as the other blood metabolites were unaffected

Organ Evaluation

When the rats were euthanized at the completion of the study, each organ was removed from the animal and the weights recorded, these data are shown in Table 2.9. There were no differences seen in the blood, digestive tract, lungs, hearts, kidneys, spleens or brains between the two groups (P>0.07). There were visible differences in some of the ALG rat's livers, which included mottling and enlargement. The liver (P<0.01) weights were significantly different between the groups, with the ALG group's livers being heavier. The increased weight of the liver was also noted by Markovits et al. (1992) when whole *Nannochloropsis o.* was fed to rats. Markovits et al. (1992) noted that liver and kidney weights were not significantly different between control and 5% whole algae, but significantly larger when fed 10% whole algae. In our study the weights of the ALG group's kidneys were also heavier (P=0.07).

Whole Body Composition

Sixteen whole rats (sans blood) were analyzed to determine any effect of treatment on the whole body composition (Table 2.10). There were no differences seen (P>0.10) between the CON and ALG groups.

DISCUSSION

Rats are an easy to use, omnivorous, non-ruminant animal model. The algal meal utilized in this initial study was 14.8% ADF and 22.4% NDF, which are considerably greater levels than seen in traditional cereal crops such as soybean meal and steam flaked corn (Archibeque et al. 2009). With this fibrous material being utilized as a source of protein in the diet, the rat proved to be an adequate model for this study. Differences were seen in the nutrient digestibility of the CON and ALG groups in this study, which was expected for this type of

animal. Increased fiber levels in diets can lead to a more rapid transit time through the gastrointestinal tract, resulting in less time for microbial fermentation (Bach-Knudsen et al. 1983). Although rate of passage was not measured in this study, decreased digestibility of macro nutrients, specifically CP and crude fat were noted Past research has shown that the microflora in the rat's digestive tract has limited influence on the true protein digestibility when fed either conventional diets or diets with elevated natural fiber levels, and the capacity to digest the protein associated with the natural fiber is diminished (Bach-Knudsen et al. 1983). Similarly the reduced apparent digestibility of the crude fat in the diet could be a function of the fiber from the algal meal. Fiber can increase the excretion of fat in the feces by reducing TAG hydrolysis as cellulose can interfere with lipase activity (Gallaher et al. 1985). In this study the feces from the ALG rats had a greater fat content than the Control group (6.27% and 4.13% respectively). There has been minimal research in the area of feeding an algae meal as a protein supplement feed to animals. Sukenik et al. (1993) fed whole dried *Nannochloropsis oculata* at 5% and 10% of the basal diet, as well as the lipids (substituting part (2%) or all (5%) of the oil in the basal diet) extracted from *Nannochloropsis oculata* to rats. Their results from feeding whole *Nannochloropsis* o. was a decrease in the level of AA and a subsequent increase in the ω3 fatty acid levels in the liver and blood lipids of the rats. Feeding algal oil (*Nanno. sp.)* showed a reduction in both AA and linoleic acid levels in liver and blood lipids. Another study involved feeding whole *Nannochloropsis oculata* at 5% and 10% of the complete diet (Markovits et al. 1992). The data presented by Markovits et al. (1992) was similar to the results we saw in feeding algal meal (oil extracted), no toxicity, and no adverse effects on growth, or blood metabolites. The only difference of note by Markovits et al. (1992) was the increased level of HDL and total serum cholesterol in the rats that consumed the algal biomass, neither of which was measured in this study. Whole *Nannochloropsis* o. has also been fed to laying hens in an effort to increase

the level of ω3 fatty acids in eggs and meat (Nitsan et al. 1999), and to rats fed high cholesterol diets in an effort to decreased the levels of plasma and liver cholesterol (Werman et al. 2003).

The weight loss that was noticed during the 7 d balance trial is hypothesized to be related to the difference in the metabolism cages. The CON group had direct access to their pelleted feed, while the ALG group had to access their feed through an attached holder. At the end of the balance trial, the rats were returned to their normal housing and the ALG group's feed intake returned to normal within 24 h., and within 7 d they had gained weight. Similar data was reported by Markovits et al. (1992), with a decrease in feed intake at 3 weeks with rats fed the algal biomass. The final data reported no significant effect on growth or BW, and no further explanation was offered for the intake reduction.

In feeding this novel feedstuffs, it was interesting to see the apparent digestibility of P between the groups unaffected (P=0.56) by the algal treatment. Plants store their P as phytic acid, which renders the P unavailable to a monogastric animal, unless phytase is added to the diet (D'Mello et al. 2000). Our data also revealed that the amount of P excreted in the feces was significantly lower in the algal group (89.25 g/d vs. 110.20 g/d, P<0.01). These data in our study are encouraging, as it would appear that the algal meal does not have high levels of phytic acid, although we did not test the oil free meal for phytic acid. In fact, it would suggest that by the reduced excretion, and numerically improved retention (248.10 g/d vs. 196.80 g/d, P=0.92) that the P in the algal meal is possibly more available to the animal than in traditional grains.

Based on the results of this 36 d study, the algal meal from *Nannochloropsis oculata* had no measured deleterious effects, toxic or otherwise on the overall health of the young rats. Although there was a recorded weight loss during the study, the rats gained weight in a normal manner overall, and suffered from no diagnosed metabolic distress. The differences in digestibility and nutrient retention did not result in measurable nutritional deficiencies for the

animals. The algal meal from *Nannochloropsis* sp. is nutritionally adequate as a protein supplement, and should be further studied in commercial livestock in order to be approved as a feed.

Table 2.1. Nutrient composition (DM basis) of Algal Meal formulated into ALG diet fed to young, growing Sprague-Dawley™® rats

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	Ash, %	TDN, %	P, ppm
Algal Meal	33.4	ND	14.8	22.4	NA	80.0	2580.0

Table 2.2. Nutrient composition (DM basis) of CON and ALG diets fed to young, growing Sprague-Dawley™® rats

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	Ash, %	ME, kcal/g	P, ppm
CON[1]	22.2	6.9	7.3	16.7	6.46	1.44	7915.0
ALG[2]	21.6	7.9	6.4	18.0	6.79	1.51	7947.0

1.CON: Harlan Lab's 2018 (ground wheat, ground corn, wheat middlings, dehulled SBM, calcium carbonate, brewers dried yeast, vitamins, minerals) + an additional 5% wheat middlings & SBM to match increased CP from algal meal.
2.ALG: 2018 + 10% algal meal

Table 2.3. Fatty Acid Composition (DM basis) of CON and ALG diets fed to young, growing Sprague-Dawley™® rats

Item	C14:0 g/100g	C16:0 g/100g	C18:0 6/100g	C18:1 g/100g	C18:2 g/100g	C18:3 g/100g	C20:4 g/100g
CON[1]	0.03	1.29	0.35	1.19	2.93	0.22	0.33
ALG[2]	0.04	1.51	0.33	1.56	3.18	0.25	0.41

1.Control: Harlan Lab's 2018 (ground wheat, ground corn,wheat middlings,dehulled SBM, calcium carbonate, brewers dried yeast, vitamins, minerals) + an additional 5% wheat middlings & SBM to match increased CP from algal meal.
2.Algal: 2018 + 10% algal meal

Table 2.4. Balance Trial Summary for young, growing Sprague-Dawley™® Rats fed CON and ALG diets for 7 days

Response	CON	ALG	SE	P Value
Average Feed Intake, g/d	23.83	18.43	0.88	<0.01
Average Water Intake, mL/d	26.92	25.90	1.44	0.62
Average Urine Produced, mL/d	10.94	16.36	0.90	<0.01
Average Feces Produced, g/d	1.56	2.33	0.13	<0.01

Table 2.5. Nutrient Intake, Excretion, Retention and Digestibility Per Day for Young Sprague-Dawley™® Rats Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
OM				
Intake, g/d	20.28	15.63	0.75	<0.01
Excreted Feces, g/d	3.37	3.11	0.14	0.18
Retained, g/d	16.91	12.53	0.71	<0.01
Digestibility, %	83.12	79.91	0.84	0.01
DM				
Intake, g/d	21.68	16.77	0.80	<0.01
Excreted Feces, g/d	4.10	3.69	0.16	0.09
Retained, g/d	17.58	13.08	0.08	<0.01
Digestibility, %	80.83	77.73	0.95	0.03
Crude Fat				
Intake, g/d	1.50	1.32	0.06	0.05
Excreted Feces, g/d	0.17	0.23	0.01	<0.01
Retained, g/d	1.33	1.00	0.06	0.01
Digestibility, %	88.49	82.44	0.63	<0.01
ADF				
Intake, g/d	1.58	1.07	0.05	0.05
Excreted Feces, g/d	0.82	0.64	0.04	<0.01
Retained, g/d	0.76	0.43	0.06	0.01
Digestibility, %	47.45	40.36	3.16	<0.01
NDF				
Intake, g/d	3.59	3.02	0.15	0.01
Excreted Fecal, g/d	1.73	1.25	0.01	<0.01
Retained, g/d	1.86	1.77	0.14	0.67
Digestibility, %	51.60	58.28	2.63	0.09
Ash				
Intake, g/d	1.40	1.14	0.05	<0.01
Excreted Fecal, g/d	0.72	0.59	0.03	<0.01
Retained, g/d	0.68	0.55	0.05	0.09
Digestibility, %	47.64	47.91	2.48	0.94

Table 2.6. Nitrogen and Phosphorus Intake, Excretion, Retention and Digestibility Per Day for Young Sprague-Dawley™® Rats Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
N				
Intake, g/d	0.77	0.58	0.03	<0.01
Excreted Feces, g/d	0.14	0.16	0.01	0.07
Excreted Urine, g/d	0.29	0.37	0.02	0.01
Retained, g/d	-2.06	-3.41	0.02	0.02
Digestibility, %	81.62	72.94	0.71	<0.01
P				
Intake, mg/d	171.70	133.30	0.01	<0.01
Excreted Feces, mg/d	110.20	89.25	0.01	<0.01
Excreted Urine, mg/d	18.08	31.00	0.00	<0.01
Retained, mg/d	196.80	248.10	0.36	0.92
Digestibility, %	34.86	32.44	2.89	0.56

Table 2.7. Urea Nitrogen Data for Young Sprague-Dawley™® Rats Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
Urea Nitrogen, g/d	1.60	1.43	0.14	0.40
Serum Urea Nitrogen, mg/dL	25.78	22.36	1.82	0.20

Table2. 8. Effect of feeding CON and ALG diets to young, growing Sprague-Dawley™® rats on blood metabolites

				P Value		
Response	**CON**	**ALG**	**SE**	**Treatment**	**Date**	**Treatment*Date**
pH	7.28	7.25	0.01	0.01	<0.01	0.70
Total CO_2 (mMol/L)	22.97	23.96	0.46	0.14	<0.01	0.98
Partial Pressure O_2 (mmHg)	95.12	87.05	9.6	0.55	<0.01	0.78
Saturated O_2 (%)	89.64	86.83	1.36	0.16	<0.01	0.55
Partial Pressure CO_2 (mmHg)	46.57	51.83	1.37	0.01	<0.01	0.68
HCO_3 (mMol/L)	21.84	22.57	0.45	0.26	<0.01	0.96
BE(mMol/L)	-4.94	-4.75	0.53	0.79	<0.01	0.84
Na(mMol/L)	133.13	132.84	0.93	0.83	<0.01	0.86
Cl (mMol/L)	114.64	113.40	0.56	0.13	<0.01	0.37
Hematocrit (%PCV)	42.43	42.35	1.52	0.97	<0.01	0.35
Hemoglobin (g/dL)	14.49	14.26	0.52	0.76	<0.01	0.11
Glucose (mg/dL)	148.13	149.71	2.15	0.61	0.32	0.29
SUN(mg/dL)	25.78	22.36	1.83	0.20	<0.01	0.01

Table 2.9. Average Organ Weights of Young Sprague-Dawley™® Rats Fed CON and ALG Diets

Organ	CON	ALG	SE	P Value
Blood, g	10.19	10.52	0.63	0.71
Blood, % Live BW	2.73	2.78	0.16	0.80
Digestive Tract[1],g	12.02	13.06	0.53	0.18
Digestive Tract, % Live BW	3.22	3.46	0.12	0.18
Lungs, g	1.65	1.69	0.04	0.54
Lungs, % Live BW	0.44	0.45	0.01	0.80
Heart, g	1.21	1.25	0.04	0.43
Heart, % Live BW	0.32	0.33	0.01	0.57
Kidneys, g	2.42	2.55	0.05	0.07
Kidneys, % Live BW	0.65	0.68	0.12	0.17
Liver[2], g	11.86	13.59	0.27	<0.01
Liver, % Live BW	3.18	3.60	0.05	<0.01
Spleen, g	0.70	0.76	0.03	0.12
Spleen, %Live BW	0.19	0.20	0.01	0.19
Brain[2], g	1.79	1.72	0.03	0.11
Brain, % Live BW	0.48	0.47	0.01	0.42

[1] Digestive Tract includes stomach, small intestine, large intestine, cecum

[2]Average brain weight is on rats 15-18 (C) and 21-24 (A)

Table 2.10. Average Body Composition of young, growing Sprague-Dawley™® rats fed CON and ALG diets

Nutrient	CON	ALG	SE	P Value
OM, g	90.82	93.15	1.94	0.41
DM, g	102.35	105.39	2.15	0.33
N, g	11.77	11.65	1.27	0.68
EE, g	19.89	22.85	0.96	0.05
ADF, g	6.21	7.19	0.48	0.17
NDF, g	9.08	9.73	0.68	0.51
Ash, g	11.53	12.25	0.35	0.17
P, g	2.05	2.17	0.08	0.30

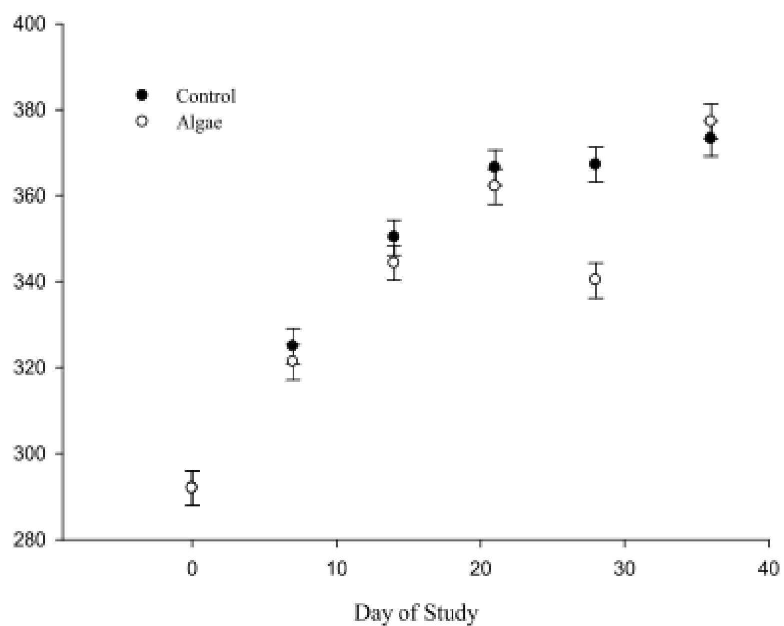

Figure 2.1. Body weight of growing Sprague-Dawley®™ rats fed CON and ALG diets

LITERATURE CITED

ALAT Training Manual. 2005. American Association for Lab Animal Sciences.

AOAC, 2005. Official Methods of Analysis. 18th ed Assoc. Off.Anal. Chem., Gaithersburg, MD.

Archibeque, S.L., A. Ettinger, B.D. Willson. 2009. *Nannochloropsis* oculata as a Source for Animal Feed Acta. Agronomica Hungarica 57:245-248.

Bach-Knudsen, K.E., J. Wolstrap, B.O. Eggum. 1983. The Influence of Dietary Fiber and Microbial Activity in the Digestive Tract on True Protein Digestibility and Biological Value in Rats. Tierphysiol, 49:173-180.

Cornell Sodium Borate-Sodium Phosphate Buffer Procedure. 1990. Incubated at ambient temperature. Cornell Nutrition Conference Proceedings. Pp. 85-86.

D'Mello, J.P.F., Officer, D.I.. Farm Animal Metabolism and Nutrition: Feed Enzymes. CABI Publishing, N.Y., N.Y. Pp. 405-426.

Gallaher, D, B.O. Schneeman. 1985. Effect of Dietary Cellulose on Site of Lipid Absorption. Am. J. Physiol. 249:G184-G191.

Goering, H.K., P.J. Van Soest. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC..

Hach, C.C., S.V. Brayton, A.L. Kopelove. 1985. Powerful Kjeldahl Nitrogen Method Using Peroxysulfuric Acd J. Agric. Food Chem. 33:1117-1122.

Markovits, A., R. Conejeros,L. Lopez, M. Lutz. 1992. Evaluation of Marine Microalga *Nannochloropsis* sp. As a Potential Dietary Supplement. Chemical, Nutritional and Short Term Toxicological Evaluation in Rats. Nutrition Research. 12:1273-1284.

Nitsan, A., S. Mokady, A. Sukenik. 1999. Enrichment of Poultry Products with Ω3 Fatty acids by Dietary Supplementation with the Algae *Nannochloropsis* and Mantur Oil. J. Agric. Food Chem. 47:5127-5132.

Sheehan, J., T. Dunahay, J. Benemann, P. Roessler. 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program-Bio-diesel from Algae, Closeout Report. NREL/TP-580-24 190.

Sukenik, A., H. Takahashi, S. Mokady. 1993. Dietary Lipids from Marine Unicellular Algae Enhance the Amount of Liver and Blood Fatty Acids in Rats. Ann. Nutr. Metab. 38:85-96.

Werman, M., A. Sukenik, S. Mokady. 2003. Effects of the Maine Unicellular Alga *Nannochloropsis* sp. to Reduce the Plasma and Liver Cholesterol Levels in Male Rats Fed on Diets with Cholesterol. Biosci. Biotechnol. Biochem. 67:2266-2268.

Villar, R., M.R. Laguna, I. Cadavid, J.M. Calleja. 1994. Effects of Aqueous Extracts of Six Marine Microalgae on Smooth Muscle Contraction in Rat Duodenum and Vas Deferens. Planta Med 60:521-526

Chapter 3

Nannochloropsis oculata: A safe microalgae as a source of minerals for young, growing rats[2]

INTRODUCTION

In 2010, the United States of America suffered one of the largest environmental disasters in its history, the Deepwater Horizon oil spill in the Gulf of Mexico. More than 4.5 million barrels worth of oil (53,000 barrels/day) ended up spilling into the ocean. It is environmental situations like this that have driven the US and the world to begin actively evaluating alternative biofuel sources. Oil rich microalgae, such as *Nannochloropsis* o. have come into the forefront as potential raw materials for the biofuel industry, due to its high oil yield (>35% DM basis). Although corn is a common biodiesel raw material, the focus on algae has increased due to its ability to grow on non-arable land, with minimal water as compared to corn and other traditional crops. Like corn ethanol, the production of algae oil produces a valuable co-product; algal meal. This meal is a high protein, mineral rich potential feedstuffs that could be fed to animals as a part of their normal ration. Archibeque et al. (2009) compared the chemical composition on a dry matter basis of the algal meal from *Nannochloropsis oculata* to that of soybean meal (SBM), and steam flaked corn (SFC), two common grains fed to commercial livestock as protein sources. The total CP content of the algal meal was 35.28%, as compared to 51.55% in the SBM and 8.86% in the SFC, making this algal meal an adequate protein supplement for animal diets, in the same manner SBM and SFC is used today. The amount of research conducted with oil extracted

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algal meal is minimal and includes previous studies that have fed whole *Nannochloropsis oculata* to rats and chickens. The published data suggests there were no harmful or negative effects, even indicating potential health benefits from the high EPA levels. The objective of this study was to conduct a 36 d feeding trial to more fully evaluate the safety of feeding *Nannochloropsis* o. to young rats in regards to mineral metabolism, organ histology and whole body composition.

MATERIALS AND METHODS

This experiment was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Algal Meal

The algal meal that was utilized in this study was supplied by SOLIX Biofuels Inc., Fort Collins, CO. The algae had been collected as a wet paste after centrifugation, dried at 100+/-5°C for ~24 hours. The remaining dried algal mass was then broken into smaller pieces and transferred into filter thimbles for a 24 hr. Soxhlet extraction in hexane. Post extraction the algal meal was then dried for 6-24 hrs at 100+/-5°C, thereby disrupting the cell wall to potentially increase digestibility. The resulting algal meal was then transferred to the Colorado State University Animal Sciences Department to be ground to a fine power using a Wiley Lab Mill model #4 and submitted for nutrient and toxic mineral analysis. The nutrient results for the algal meal are reported in Tables 3.1 and 3.2. These results were shared with Harlan Laboratory (Madison, WI) and utilized to manufacture the Algal diet.

Animals and Treatments

A total of 24 Sprague-Dawley®™ 8 week old male rats were obtained from Harlan Laboratories (Madison, WI). The rats were received into the Colorado State University Laboratory Animal Resource center and immediately started on Harlan Teklad 22/5 Rodent diet. The rats were held under standard laboratory conditions with a room temperature of 21°C, 37-

45% humidity and a 12/12 light/dark cycle in IACUC approved solitary cage measuring 10.5" wide, 19" long and 8" high. After a seven day acclimation period, the rats were randomly blocked according to body weight into two separate treatment groups, Control (**CON**) and Algal (**ALG**), and started on the diets specifically formulated for this experiment. The CON diet and the ALG diets were both formulated to be iso-nitrogenous, iso-caloric (Tables 3.3 and 3.4) and to meet all of the nutritional needs for a young, growing rat (NRC of Laboratory Animals, 4th Revised Edition, 1995). Twelve rats were fed a CON diet and twelve rats were fed an ALG diet which incorporated 10% algal meal (sp *Nannochloropsis oculata*). The CON group weighed an average of 291+/-8 g initially and an average of 373+/-24g on d36, while the ALG group weighed an average of 291+/-15g, and an average of 377+/-20g on d36. Overall, the CON group gained 81.27 g throughout the study, a 27.86% increase in total BW, and the ALG group gained 85.25 g throughout the study, a 29.18% increase in BW. Fresh water was made available at all times. Coprophagy was not prevented in order to encourage normal eating behavior.

Nutrient Balance Trial

From d 21 to 28 a balance trial was conducted and the feed intake, water intake, fecal and urinary output were measured for each rat every 24 hrs. The rats were housed in IACUC approved solitary metabolism cages; there was no change to the environmental conditions. Remaining feed and water was weighed every 24 h and recorded. Feed and water were both offered ad libitum. Urine from each rat was collected every 24 h into a 15 mL conical tube containing 100 µg of 6NHCl to maintain a pH of less than 3.0, and prevent volatilization of urinary N. Each day's urine was combined into a 50 mL conical tube *(P*er rat) and frozen at -20° C. The feces from each rat was collected on a screen under the cage, collected and weighed every 24 h, stored in a plastic bag and frozen at -20° C.

Chemical Analysis

Samples of the algal meal, diet, feces and rat samples were analyzed for DM, crude fat and ash using AOAC 2005 methods. The lipid content of the rats was determined using acid hydrolysis (AHF) (AOAC 2005). Crude protein was determined using the Kjeldahl AOAC 2005 method, and calculated from total N values (N x 6.25). NDF and ADF (Goering and Van Soest, 1970) were determined using a modified Van Soest using fiber bag technology (Ankom 200 and Daisy II Incubator, Ankom Technology Corp., Macedon, NY). Urine nitrogen was quantified using peroxysulfuric acid as described by Hach et al. (1985).

Mineral analysis was conducted by drying the feed and fecal samples at 75° C overnight, and digesting the samples at 95° C with nitric acid at a 1 ml nitric acid:100 mg sample ratio. After digestion 5 ppm Yttrium was used as an internal standard, and the samples were diluted with water to a final volume of 25 mL (50x dilution). One mL of the digested and diluted sample was further diluted to 1:10 with a 20% nitric acid and 5 ppm Yttrium solution (500x final dilution). The final samples were then run on a Varian radial inductively coupled plasma (ICP) atomic emission spectrometer. The whole rat minerals were measured using the same method as for the feed and feces, however the final dilution was 50x, and 1 ppm of Yttrium was used as an internal standard and 500 ppm Cesium was used as and ionization quencher. Urine mineral content was quantified by digesting the urine in 1 mL nitric acid:1mL sample, and dried at 95°C overnight. After digestion and drying, 1 ppm of Yttrium was used as an internal standard, and 500 ppm Cesium was used as an ionization quencher. The samples were diluted with water to a final volume of 25 mL (50x dilution) and run on a Varian radial (ICP) atomic emission spectrometer.

Euthanasia and Organ Evaluation

The rats were humanely anesthetized on d 36 via iso-flourene gas and exsanguination was via a fatal heart stick. The bodies were then opened from sternum to pelvis and the complete

digestive tract was removed. The small intestine, large intestine, stomach and cecum were thoroughly cleaned with a 0.9% saline solution. The livers, kidneys, lungs, heart, spleen were removed and weighed on all 24 rats. The brains were removed and weighed from 4 rats from each group.

Histology

Four animals from each group were randomly selected for histological evaluation. Samples for histological analysis were obtained from the large lobe of the liver, the spleen, and one kidney. Each sample was cut longitudinally from the organ and fixed in 10% neutral buffered formalin (NBF) for 24 h at 21°C. These tissues samples were then submitted to the Colorado State University VTH Diagnostic Laboratory for H&E stain slide preparation. Tissue samples taken from the heart, brain and abdominal skeletal muscle were fixed in 10% NBF and stored at 21°C for future testing.

Freeze Drying and Whole Body Composition

The rats were frozen at -20° C and each one was cut into thirds for ease of handling. Each rat was then ground using a Robot Coupe Blixer 6V blender and liquid nitrogen. After grinding, the rats were weighed and then placed in separate 9" tins and placed in a Lab Conco Freeze Dryer System. The temperature in the condenser was -40°- -44° C, and the temperature in the sample chamber was 15°-20° C, under a vacuum of 1.33 X 10^{-3} mtorr. The rats were weighed two to three times per week until there was no weight change for a 24 h period of time. The freeze dried material for each rat was individually ground through a 2 mm screen using a Wiley Lab Mill, model #4. The final material was then homogenized and submitted for chemical analysis.

Calculations

Calculations for apparent digestibility and nutrient retention were made using the following formulas:

Apparent Digestibility = (Nutrient Intake – Nutrient in Feces)/Nutrient Intake X 100

Nutrient Retention = (Nutrient Intake (g) – Nutrient in Feces (g) – Nutrient in Urine (g))

Statistical Analysis

Data for growth, digestibility, intake, nutrient retention, organ weights and body composition were analyzed using PROC MIXED procedure (SAS Institute Inc., Cary, NC) for repeated measures. The experimental unit was the individual rat, the fixed effect was treatment, and the random effect was date. Data for histology results were analyzed using the Fisher's Exact Test (SAS Institute Inc., Cary, NC), using the individual rabbit as the experimental unit and the Algal or Control diet as the fixed effect. Differences between treatments were considered statistically significant if $P \leq 0.05$ and trends if $P \leq 0.10$.

RESULTS

BW and ADG

All of the rats stayed healthy throughout the study, and were weighed on d0 and every 7 days for the entirety of the study. Overall for the complete study the ALG diet had no significant effect on BW *(P*=0.20) (Table 3.5), however there was a Treatment*Date interaction effect *(P*<0.01) as a result of a significant difference between the groups at d 28 *(P*<0.01).

Macro Mineral Intake, Excretion, Retention and Digestibility

There was less intake of Ca, K, Mg, S and P in the ALG group *(P*<0.05), while Na intake was greater *(P*<0.01) (Table 3.6). There was more Na in the ALG diet, therefore these data are justifiable. No difference was seen in the fecal excretion of K *(P*=0.33), yet differences were noted in the fecal excretion of Ca, Mg, Na, S and P *(P*<0.05). Ca excreted in the urine was

similar in both groups (*P*>0.20), while a difference was recorded in the urinary excretion of K, Mg, Na, S and P (*P*<0.05). Ca, K, Mg, and Na retention were all effected in the ALG group, and much decreased (*P*<0.01). The retention of P was greater for the ALG fed rats (*P*=0.92) suggesting that the P contributed by the algal meal was highly bioavailable. There was no difference in the apparent digestibility of Ca, K, Mg, Na and P (*P*>0.10). In contrast, the retention of S was different between the two groups (*P*<0.05). This digestibility data suggest that algal meal does not adversely affect the digestibility of macro minerals.

Trace Mineral Intake, Excretion, Retention and Digestibility

The metabolism of many trace minerals were also evaluated, Table 3.7. There were several minerals that were below detectable limits in the algal meal and subsequently undetected in the ALG diet, as well as the CON diet; Co, Mo, and Pb. The mineral Sb was found in the ALG diet, yet not in the algal meal itself, while there was no Sb detected in the CON diet. The intake of Al, Ba, Cr, Cu, Fe, and Mn were all decreased in the ALG group as compared to the Control group (*P*<0.05). In contrast, the intake of B was identical between the two groups (*P*>0.10). The fecal excretion of Al, B, Ba, Cr, Cu, Mn, and Zn were different between the groups (*P*<0.05). There was no difference between the groups in the fecal excretion of Fe (*P*>0.10). The level of B excreted in the urine was different between the groups (*P*<0.01), while there was no difference in the urinary excretion of Al, Ba, Cr, Cu, Fe, Mn, and Zn (*P*>0.10). Differences (*P*<0.01) were detected in the retention of B, while the retention of the other trace mineral remained unaffected Apparent digestibility was decreased in all of the trace minerals measured, yet the only significant difference was seen in the apparent digestibility of B (*P*<0.01). As expected in an omnivore the reduced capacity to handle fibrous diets appears to have resulted in the reduced digestibility of the trace minerals in the feed, this is in contrast to the improved digestibility seen with the macro minerals.

Hg and As were also analyzed for in the algal meal and diets and were found to be below the detection limits of the test methodology.

Whole Body Composition

The bodies of 16 whole rats (n=8/trt) were analyzed for macro and micro mineral levels, these data are presented in Table 3.8. There were no differences seen between the two groups (P>0.10), with the exception of Co (P=0.02).

Organs and Histology

The internal organs of each rat were visually evaluated after the animal was euthanized There was minimal to no obvious differences seen in the respiratory and circulatory organs (lungs, heart), digestive tract, or the spleen, kidneys or brain. There were visible differences in some of the ALG rat's livers, which included mottling and enlargement, (Table 3.9). Markovits et al. (1992) also reported a difference in the weight of the livers and kidneys in rats fed 10% whole *Nannochloropsis oculata.* The histology for the spleen, kidney and liver samples report noted no significant lesions or abnormalities in any of the samples (Table 3.10).

DISCUSSION

The data that has been published concerning diets manufactured with algal meal (oil extracted) is sparse. In the research that has been conducted, the marine algae *Nannochloropsis oculata* has been looked at specifically as a source of EPA, and the potential health benefits associated with the consumption of EPA. This is the first known study that has evaluated the oil free algal meal as a safe feedstuffs for animals. Our research has looked at the mineral composition of the algal meal, as well as the diets fed, excretory products produced and whole body composition.

Markovits et al. (1992) studied the effects of feeding whole *Nannochloropsis* sp. to young rats at both 5% and 10% inclusion levels. In their study, a mineral analysis of the algal

meal was reported. The Markovits et al. (1992) mineral data is different than ours, and showed much decreased levels of Na (0.31% v. 1.07%), K (0.06% v. 1.55%), and Fe (102 ppm v. 238 ppm). This could be attributed to the growth medium of the algae as well as the extraction method utilized for our study.

An algal species, *Schizochytrium* from the same phylum as *Nannochloropsis oculata,* (Heterokontophyta), has been evaluated for safety, and has been granted GRAS status by the FDA. Hammond et al. (2001, 2002) investigated the anti-toxicity of *Schizochytrium* in a series of four studies. This research covered the feeding of Sprague-Dawley®™ rats for 13 weeks and evaluating them for any signs of toxicity at dosages up to 4000 mg/kg/day. The second study reviewed the feeding of gestational rats and rabbits, at levels of 180, 600 and 1800 mg/kg/day. The third study evaluated any reproductive toxicity and the final study focused on mutagenicity. All of the results showed no clinical signs of toxicity. These studies were isolated to the whole algae, with the oil included Dahm's et al. (2011) also evaluated the safety of the oil only from *Schizochytrium*, and in a 90 days study with rats, no adverse effects were noted. Although there is no data with *Nannochloropsis oculata*, our data is in agreement with Hammond and Dahms, and does not suggest that the minerals contributed by the algal meal had any disadvantageous results in the animals.

The value of this co-product would be greatly increased if it could be utilized in a commercial feed ration for food animals (beef cattle or sheep). In reviewing the NRC guidelines for the tolerable levels of minerals, it appears that this algal meal could be included in ruminant feed at a level of at least 10%. Incorporating the algal meal into the feed at this level would allow the feed to remain below the maximum levels of Na (4.5% as NaCl), Ca (1.5%), P (0.70%), Mg (0.60%), S (0.30-0.50%), and K (2%).

Plants store their P as phytic acid, which renders the P unavailable to a monogastric animal, unless phytase is added to the diet (D'Mello et al. 2000). This is an important factor to consider when offering a novel feedstuffs (plant origin) to non-ruminant livestock. In this study, the apparent digestibility of P between the groups unaffected (P=0.56) by the algal treatment. Our data also revealed that the amount of P excreted in the feces was significantly lower in the algal group (89.25 g/d vs. 110.20 g/d, P<0.01). These data in our study are encouraging, as it would appear that the algal meal does not have high levels of phytic acid, although we did not test the oil free meal for phytic acid. These data would suggest that by the reduced excretion, and numerically improved retention (248.10 g/d vs. 196.80 g/d, P=0.92) that the P in the algal meal is possibly more available to the animal than in traditional grains.

Ours is the first study known to look at the whole body composition of animals fed *Nannochloropsis o.*, and we did not detect any levels of potentially toxic minerals in the bodies of the animals fed a diet with 10% algal meal. The effect of a high protein, mineral rich feed on organ histology has not been evaluated in studies concerning the feeding of algal meal (oil extracted), although Hammond (2001, 2002) reviewed organ histology with *Schizochytrium,* and mentioned no negative effects. Our study included histology for liver, kidney and spleen tissue, and the lack of any difference in organ histology between the two groups further supports the safety of the algal meal as a potential feedstuff.

Based on our study and subsequent mineral and tissue analysis, the algal meal from *Nannochloropsis oculata* is a safe, non-toxic potential feedstuffs for young growing rats. Further studies should be conducted to further understand the full usefulness of this type of biodiesel co-product.

Table 3.1. Nutrient composition (DM basis) of Algal used to formulate CON and ALG diets fed to young, growing Sprague-Dawley™® rats

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	TDN, %	P, ppm
Algal Meal	33.4	ND	14.8	22.4	80.0	2580.0

1.Control: Harlan Lab's 2018 (ground wheat, ground corn,wheat middlings,dehulled SBM, calcium carbonate, brewers dried yeast, vitamins, minerals) + an additional 5% wheat middlings & SBM to match increased CP from algal meal.
2.Algal: 2018 + 10% algal meal

Table 3.2. Mineral composition (DM basis) of Algal Meal used to formulate the CON and ALG diets fed to young, growing Sprague-Dawley™® rats

Item	Al, ppm	As, ppm	B, ppm	Ba, ppm	Ca, %	Cd, ppm	Co, ppm	Cr, ppm	Cu, ppm	Fe, ppm	Hg, ppm	K, %	Mg, %	Mn, ppm	Mo, ppm	Na, %	P, %	Pb, ppm	S, %	Sb, ppm	Se, ppm	Tl, ppm	Zn, ppm
Algal Meal	<5.0	<2.5	35.5	9.4	0.38	<0.3	<0.5	<1.0	24.2	338.0	<10.0	1.55	0.34	45.4	<1.0	1.07	0.26	<2.5	0.64	<5.0	<10.0	<12.5	53.1

Table 3.3. Nutrient composition (DM basis) of CON and ALG diets fed to young, growing Sprague-Dawley™® rats

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	Ash, %	ME, kcal/g	P, %
CON[1]	22.2	6.9	7.3	16.7	6.46	1.44	0.79
ALG[2]	21.6	7.9	6.4	18.0	6.79	1.51	0.79

1.CON: Harlan Lab's 2018 (ground wheat, ground corn,wheat middlings,dehulled SBM, calcium carbonate, brewers dried yeast, vitamins, minerals) + an additional 5% wheat middlings & SBM to match increased CP from algal meal.
2.ALG: 2018 + 10% algal meal

Table 3.4. Mineral composition (DM basis) of CON and ALG diets fed to young, growing Sprague-Dawley™® rats

Item	Al, ppm	As, ppm	B, ppm	Ba, ppm	Ca, %	Cd, ppm	Co, ppm	Cr, ppm	Cu, ppm	Fe, ppm	Hg, ppm	K, %	Mg, %	Mn, ppm	Mo, ppm	Na, %	P, %	Pb, ppm	S, %	Sb, ppm	Se, ppm	Tl, ppm	Zn, ppm
CON[1]	114.0	<2.5	6.9	8.2	0.92	<0.5	<1.0	4.76	15.6	199.0	<10.0	0.90	0.27	96.0	<1.0	0.20	0.79	<2.5	0.27	<5.0	<10.0	<12.5	76.0
ALG[2]	111.0	<2.5	9.2	7.7	0.97	<0.5	<1.0	4.9	16.4	223.0	<10.0	0.85	0.26	91.0	<1.0	0.33	0.79	<2.5	0.31	3100.0	<10.0	<12.5	73.0

1.CON: Harlan Lab's 2018 (ground wheat, ground corn,wheat middlings,dehulled SBM, calcium carbonate, brewers dried yeast, vitamins, minerals) + an additional 5% wheat middlings & SBM to match increased CP from algal meal.
2.ALG: 2018 + 10% algal mea

Table 3.5. Effect of feeding CON and ALG diets to young, growing Sprague-Dawley™® rats on BW and ADG

RESPONSE	P VALUE TREATMENT	DATE	TREATMENT*DATE
Weight, g	0.20	<0.01	<0.01
ADG, g	0.57	<0.01	<0.01

Table 3.6. Macro Mineral Intake, Excretion, Retention and Digestibility Per Day for Young Sprague-Dawley™® Rats Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
Ca				
Intake, mg/d	198.80	163.10	0.01	<0.01
Excreted Fecal, mg/d	150.00	130.20	0.01	0.02
Excreted Urine, mg/d	2.17	2.75	0.00	0.25
Retained, g/d	0.97	0.90	0.01	<0.01
Digestibility, %	23.46	19.31	3.50	0.41
K				
Intake, mg/d	195.40	142.30	0.01	<0.01
Excreted Fecal, mg/d	19.08	16.83	0.00	0.33
Excreted Urine, mg/d	88.92	125.30	0.01	<0.01
Retained, g/d	0.50	-0.02	0.05	<0.01
Digestibility, %	90.03	87.64	1.34	0.22
Mg				
Intake, mg/d	57.68	43.92	0.00	<0.01
Excreted Fecal, mg/d	39.00	29.58	0.00	<0.01
Excreted Urine, mg/d	4.83	8.83	0.00	0.04
Retained, g/d	0.35	0.33	0.13	0.01
Digestibility, %	30.93	32.00	3.18	0.81
Na				
Intake, mg/d	43.83	54.67	0.00	<0.01
Excreted Fecal, mg/d	8.58	11.00	0.00	0.03
Excreted Urine, mg/d	24.33	44.67	0.00	<0.01
Retained, g/d	0.30	0.04	0.05	<0.01
Digestibility, %	79.62	79.60	1.49	0.99
S				
Intake, mg/d	59.33	52.00	0.00	0.04
Excreted Fecal, mg/d	15.50	19.60	0.00	<0.01
Excreted Urine, mg/d	24.83	33.83	0.00	<0.01
Retained, g/d	0.43	0.08	0.05	<0.01
Digestibility, %	73.39	61.61	1.60	<0.01
P				
Intake, mg/d	171.70	133.30	0.01	<0.01
Excreted Fecal , mg/d	110.20	89.25	0.00	<0.01
Excreted Urine, mg/d	18.08	31.00	0.00	<0.01
Retained, mg/d	196.80	248.10	0.36	0.92
Digestibility, %	34.86	32.44	2.89	0.56

Table 3.7. Trace Mineral Intake, Excretion, Retention and Digestibility Per Day for Young Sprague-Dawley™® Rats Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
Al				
Intake, mg/d	2.50	1.88	0.00	<0.01
Excreted Fecal, mg/d	2.83	1.96	0.00	<0.01
Excreted Urine, µg/d	14.00	16.00	0.00	0.42
Retained, g/d	0.87	1.22	0.12	0.06
Digestibility, %	-15.91	-6.14	4.84	0.17
B				
Intake, mg/d	0.15	0.15	0.00	0.67
Excreted Fecal, µg/d	32.00	41.00	0.00	<0.01
Excreted Urine, µg/d	69.00	111.00	0.00	<0.01
Retained, mg/d	403.80	4.65	0.05	<0.01
Digestibility, %	78.29	72.56	1.43	<0.01
Ba				
Intake, mg/d	0.18	0.13	0.00	<0.01
Excreted Fecal, µg/d	158.00	122.00	0.00	<0.01
Excreted Urine, µg/d	0.13	0.18	0.00	0.24
Retained, g/d	1.18	1.04	0.19	0.60
Digestibility, %	10.06	4.64	4.09	0.36
Cr				
Intake, mg/d	0.10	0.08	0.00	<0.01
Excreted Fecal, µg/d	92.00	75.00	0.00	<0.01
Excreted Urine, µg/d	0.52	0.22	0.00	0.16
Retained, g/d	1.20	1.05	0.38	0.79
Digestibility, %	9.09	8.34	4.02	0.90
Cu				
Intake, mg/d	0.34	0.27	0.00	<0.01
Excreted Fecal, mg/d	0.32	0.28	0.00	0.03
Excreted Urine, µg/d	0.63	0.82	0.00	0.14
Retained, mg/d	51.30	4.40	0.23	0.83
Digestibility, %	4.31	-2.11	4.52	0.33
Fe				
Intake, mg/d	4.31	3.73	0.00	0.03
Excreted Fecal, mg/d	3.49	3.16	0.00	0.12
Excreted Urine, µg/d	63.00	49.00	0.00	0.40
Retained, mg/d	0.52	0.42	0.00	0.63
Digestibility, %	17.91	14.75	3.86	0.57
Mn				
Intake, mg/d	2.10	1.54	0.00	<0.01
Excreted Fecal, mg/d	1.92	1.47	0.00	<0.01
Excreted Urine, µg/d	13.00	20.00	0.00	0.21
Retained, mg/d	0.85	0.66	0.17	0.23
Digestibility, %	6.32	2.80	4.30	0.57
Zn				
Intake, mg/d	3.00	1.23	0.00	0.21
Excreted Fecal, mg/d	1.51	1.22	0.00	<0.01
Excreted Urine, µg/d	50.00	39.00	0.00	0.44
Retained, g/d	0.81	0.71	0.27	0.80
Digestibility, %	7.31	-0.65	4.14	0.19

Table 3.8. Average Body Composition (DM basis) of young, growing Sprague-Dawley™® rats fed CON and ALG diets

Nutrient	CON	ALG	SE	P Value
Al, mg	1.60	2.30	0.00	0.58
Ba, mg	0.10	0.10	0.00	1.00
Ca, g	3.33	3.55	0.15	0.33
Cd, mg	0.03	0.02	0.00	0.52
Co, mg	0.30	0.10	0.00	0.02
Cr, mg	0.10	0.10	0.00	0.33
Cu, mg	1.00	0.50	0.00	0.29
Fe, mg	9.00	10.00	0.00	0.25
K, g	0.94	0.96	0.02	0.59
Mg, g	0.12	0.13	0.00	0.23
Mn, mg	0.20	0.30	0.00	0.14
Mo, mg	0.02	0.02	0.00	0.33
Na, g	0.35	0.37	0.01	0.18
P, g	2.05	2.17	0.08	0.30
S, g	0.89	0.87	0.02	0.71
Zn, mg	9.00	9.00	0.00	0.63

Table 3.9. Average Organ Weights of Young Sprague-Dawley™® Rats Fed CON and ALG Diets

Organ	CON	ALG	SE	P Value
Blood, g	10.19	10.52	0.63	0.71
Blood, % Live BW	2.73	2.78	0.16	0.80
Digestive Tract[1],g	12.02	13.06	0.53	0.18
Digestive Tract, % Live BW	3.22	3.46	0.12	0.18
Lungs, g	1.65	1.69	0.04	0.54
Lungs, % Live BW	0.44	0.45	0.01	0.80
Heart, g	1.21	1.25	0.04	0.43
Heart, % Live BW	0.32	0.33	0.01	0.57
Kidneys, g	2.42	2.55	0.05	0.07
Kidneys, % Live BW	0.65	0.68	0.12	0.17
Liver[2], g	11.86	13.59	0.27	<0.01
Liver, % Live BW	3.18	3.60	0.05	<0.01
Spleen, g	0.70	0.76	0.03	0.12
Spleen, %Live BW	0.19	0.20	0.01	0.19
Brain[2], g	1.79	1.72	0.03	0.11
Brain, % Live BW	0.48	0.47	0.01	0.42

[1] Digestive Tract includes stomach, small intestine, large intestine, cecum
[2]Average brain weight is on rats 15-18 (CON) and 21-24 (ALG)

Table 3.10. Organ histology of young, growing Sprague-Dawley™® Rats Fed CON and ALG Diets

Organ	Histology	Presence/Absence	P Value
Spleen	Lymphoid Follicles	100%C/100%A	NA
Liver	Lymphocytes	50% C/25% A	1.00
Kidney	Protein	50% C/ 100% A	0.43

LITERATURE CITED

ALAT Training Manual. 2005. American Association for Lab Animal Sciences.

AOAC, 2005. Official Methods of Analysis. 18th ed Assoc. Off.Anal. Chem., Gaithersburg, MD

Archibeque, S.L., A. Ettinger, B.D. Willson. 2009. *Nannochloropsis* oculata as a Source for Animal Feed Acta. Agronomica Hungarica 57:245-248.

Dahms, I.F., P.A. Marone, P.A. E. Bailey-Hall, A.S. Ryan 2011. Safety Evaluation of Algal Oil from Schizochytrium sp.. Food and Chem. Tox. 49:70-77.

D'Mello, J.P.F., Officer, D.I.. Farm Animal Metabolism and Nutrition: Feed Enzymes. CABI Publishing, N.Y., N.Y. Pp. 405-426.

Hammond, B.G., D.A. Mayhew, M.W. Naylor, F.A. Ruecker, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp.: I. Subchronic Rat Feeding Study. Reg. Tox. and Pharm. 33:192-204.

Hammond, B.G., D.A. Mayhew, J.F. Holson, M.D.Nemec, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp.: II. Developmental Toxicity Evaluation in Rats and Rabbits. Reg. Tox. and Pharm. 33:205-217.

Hammond, B.G., D.A. Mayhew, K. Robinson, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp. III. Single Generation Rat Reproduction Study. Reg. Tox. and Pharm. 33:356-362.

Hammond, B.G., D.A. Mayhew, L.D. Kier, R.W. Mast, W.J. Sander. 2002. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp. IV. Mutagentcity Studies. . Reg. Tox. and Pharm. 35:255-265.

Markovits, A., R. Conejeros, L. Lopez, M. Lutz. 1992. Evaluation of Marine Microalga *Nannochloropsis* sp. As a Potential Dietary Supplement. Chemical, Nutritional and Short Term Toxicological Evaluation in Rats. Nutrition Research. 12:1273-1284.

National Research Council. Mineral Tolerance of Animals. 2nd revised edition.

Chapter 4

Feeding *Nannochloropsis oculata* meal to hind gut fermenting herbivores as a potential protein source did not significantly alter nutrient usage and had no adverse health effects[3]

INTRODUCTION

When crude oil prices hit an historical high of >$100/barrel in 2008, the race was on to develop alternative fuel options. Traditionally, corn ethanol has been the primary alternative fuel source utilized in the US, and in August of 2010, the US produced approximately 867,000 barrels (1134 million gallons) of corn ethanol (Mathews et al. 2009). Approximately 1 acre of land is necessary to produce 120 bushels of corn, which can produce 240 gallons of ethanol. Alternatively, some species of microalgae can yield up to 10,000 gallons of oil per acre (Net Recources). One of the high oil producing algal species is the microalgae *Nannochloropsis oculata*. *Nannochloropsis* o. can produce 40-45% oil, making it viable biofuel raw material. Like corn ethanol, the production of algae oil results in an algal meal that could be used as a livestock feedstuffs. Archibeque et al. (2009) compared the algal meal from *Nannochloropsis o.* to traditional livestock feedstuffs. On a dry matter basis, the total CP content of the algal meal was 35.28%, as compared to 51.55% in the SBM and 8.86% in the SFC. The soluble CP in the algal meal was comparable to the SBM (20.32% and 20.07% respectively). The B3 CP fraction was greater in the algal meal 63.52%, versus 1.82% in the SBM and 11.92% in the SFC. This composition suggests that commercial ruminant livestock could utilize the algal meal as a valuable source of CP when added to their feed. The amount of research conducted with this

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material is minimal and includes previous studies that have fed whole *Nannochloropsis oculata* to rats and chickens. Therefore the objective of this study was to conduct a 36 d feeding trial with a non-ruminant, hind-gut fermenting herbivore to more fully evaluate the acceptability, digestibility and nutrient retention of a diet that included 10% algal meal sp. *Nannochloropsis oculata* as compared to a diet with no algal meal.

MATERIALS AND METHODS

This experiment was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Algal Meal

The algal meal that was utilized in this study was supplied by SOLIX Biofuels Inc., Fort Collins, CO. A slurry was made from frozen algae, and the cells were lysed using high shear mixing. Hexane was added to the slurry, and the material was allowed to separate. The aqueous fraction was then re-extracted with hexane, and allowed to separate a second time. After this second separation, the aqueous portion was mixed with water and ethanol and evaporated at 100° C for ~24 hours. The resulting algal meal was then transferred to the Colorado State University Animal Sciences Department to be ground to a fine power using a Wiley Lab Mill model #4 and submitted for nutrient and toxic mineral analysis. The nutrient results for the algal meal are presented in Table 4.1. They were shared with Harlan Laboratory and utilized to manufacture the Algal diet.

Animals and Treatments

A total of 24 6-8 week old New Zealand White male rabbits were obtained from Western Oregon Rabbit Company. The rabbits were received into the Colorado State University Laboratory Animal Resource center and immediately started on Harlan Teklad 2031 Global High Fiber Rabbit diet. The rabbits were held under standard laboratory conditions with a room

temperature of 21°C, 37-45% humidity and a 12/12 light/dark cycle in IACUC approved solitary cage measuring 23.0” wide, 24.0” long and 15.0” high. After an eleven day acclimation period, the rabbits were randomly blocked according to body weight (BW) into two separate treatment groups, Control (**CON**) and Algal (**ALG**). In order to acclimate the rabbits to different diets, for 7 days, twelve rabbits were fed a 50/50 Control/2031 diet and twelve rabbits were fed a 50/50 Algal/2031 diet which incorporated 10% algal meal (sp *Nannochloropsis* oculata). On day 7, the rabbits were switched to 100% **CON** and 100% **ALG** diets. The CON diet and the ALG diet were formulated to be iso-nitrogenous, iso-caloric (Table 4.2) and to meet all of the nutritional needs for a young, growing rabbit (Nutrient Requirements of Rabbits, 2nd Revised Edition, 1977). The CON group weighed an average of 3.51+/-0.75 kg initially (d 1 of 100% CON diet) and an average of 4.17+/-1.33 kg on d36, while the ALG group weighed an average of 3.45 +/-0.72 kg (d 1 of 100% ALG diet), and an average of 3.99+/-0.92 kg on d36. Overall, the CON group gained 0.66 kg throughout the study, an 18.80 % increase in total BW, and the ALG group gained 0.54 kg throughout the study, a 15.65% increase in BW. The ADG at the end of the study was 14 g/d (CON), and 10 g/d (ALG) (Figure 4.1). Fresh water was made available at all times. Coprophagy was not prevented in order to encourage normal eating behavior.

Fatty Acid Composition

The fatty acid composition of the rabbit feed (Table 4.3) was determined via gas chromatography using a Hewlett Packard (Avondale, PA) Model 6890 series II gas chromatograph fixed with a series 7683 injector and flame ionization detector. The instrument was equipped with a 100-m x 0.25-mm (id) fused silica capillary column (SP-2560 Supelco Inc. Bellefonte, PA). Methyl ester derivatives of fatty acids were prepared using a combination of NaOCH3 followed by HCl/methanol as described by Kramer et al. (1997). Fatty acid methyl ester preparations were injected using the split mode. The carrier gas was helium, and the split ratio

was 100:1 at 180° C. The oven temperature was programmed from an initial temperature of 140° C (0 min) to a final temperature of 225° C at the rate of 2.8^{o} C/min. The final temperature was held for 18 min. Chromatograms were recorded with a computing integrator (Agilent Technologies, Palo Alto, CA). Standard fatty acid methyl ester mixtures were used to calibrate the gas chromatograph system using reference standards KEL-FIM-FAME-5 (Matreya Inc., PA). Identification of the fatty acids was made by comparing the relative retention times of fatty acid methyl ester peaks from samples with those of standards.

Chemical Analysis

Samples of the algal meal, diet, feces and rabbit samples were analyzed for DM, crude fat and ash using AOAC 2005 methods. The lipid content of the rabbits was determined using acid hydrolysis (AHF) (AOAC 2005). Crude protein was determined using the Kjeldahl AOAC 2005 method, and calculated from total N values (N x 6.25). Soluble protein was determined using sodium borate-sodium phosphate buffer solution. NDF, ADF and lignin (Goering and Van Soest, 1970) were determined using a modified Van Soest using fiber bag technology (Ankom 200 and Daisy II Incubator, Ankom Technology Corp., Macedon, NY). Urine nitrogen was quantified using peroxysulfuric acid as described by Hach et al. (1985).

Mineral analysis was conducted by drying the feed and fecal samples at 75^{0} C overnight, and digesting the samples at 95^{0} C with nitric acid at a 1 ml nitric acid:100 mg sample ratio. After digestion 5 ppm Yttrium was used as an internal standard, and the samples were diluted with water to a final volume of 25 mL (50x dilution). One mL of the digested and diluted sample was further diluted to 1:10 with a 20% nitric acid and 5 ppm Yttrium solution (500x final dilution). The final samples were then run on a Varian radial inductively coupled plasma (ICP) atomic emission spectrometer. The whole rabbit minerals were measured using the same method as for the feed and feces, however the final dilution was 50x, and 1 ppm of Yttrium was used as an

internal standard and 500 ppm Cesium was used as and ionization quencher. Urine mineral content was quantified by digesting the urine in 1 mL nitric acid:1mL sample, and dried at 95 ^{0}C overnight. After digestion and drying, 1 ppm of Yttrium was used as an internal standard, and 500 ppm Cesium was used as an ionization quencher. The samples were diluted with water to a final volume of 25 mL (50x dilution) and run on a Varian radial (ICP) atomic emission spectrometer.

Blood Metabolites

On d 0 and d 28 approximately 0.50 mL of blood was drawn with a syringe via a venous ear puncture and collected in microtainers treated with 15% EDTA K2. The samples were chilled during collection, and a sample was pulled from the microtainer and analyzed using single use ISTAT cartridges EC8+ and CG8+ which were then run through a portable ISTAT analyzer. pH, Total CO_2 (TCO_2), Partial Pressure O_2, Saturated O_2 (SO_2), Partial Pressure CO_2, HCO_3, Base Excess (BE), Sodium (Na), Chloride (Cl), Hematocrit (Hct), Hemoglobin (Hb), and Glucose were reported The remaining blood was then transferred into microtainers treated with NaFl K_2Ox . These samples were chilled on ice, and within 2 hours of collection the serum was separated by centrifugation (672 x g for 10 min. at 21° C). The serum was removed and frozen at -20° C for later analysis. On d 45 blood was drawn via a fatal heart stick, at which time the blood was analyzed using ISTAT cartridges and portable analyzer (see above). Approximately 115 mL of blood from each rabbit was collected into microtainers treated with 15% EDTA K3. The blood was chilled on ice during collection and serum was separated by centrifugation (1512 x g. for 10 min. at 21° C) within 6 hours of collection. The serum was removed and frozen at -20° C for later analysis.

Nutrient Balance Trial

From d 21 to 38 a balance trial was conducted and the feed intake, water intake, fecal and urinary output were measured for each rabbit every 24 hours. The rabbits were housed in their regular IACUC approved solitary cages, and there was no change to the environmental conditions. Remaining feed and water was weighed every 24 h, and recorded Feed and water were both offered ad libitum. Urine from each rabbit was collected every 24 h into a plastic bowl containing 1mL of 6NHCl to maintain a pH of less than 3.0, and prevent volatilization of urinary N. Each day's urine was combined into a large resalable plastic bag (per rabbit) and frozen at -20° C. The feces from each rabbit was collected on a screen under the cage, captured and weighed every 24 h, stored in a plastic bag and frozen at -20° C.

Euthanasia and Organ Evaluation

The rabbits were humanely anesthetized on d 45 via iso-flourene gas and exsanguination was via a fatal heart stick. The bodies were then opened from sternum to pelvis and the complete digestive tract was removed. The small intestine, large intestine, stomach and cecum were thoroughly cleaned with a 0.9% saline solution. The livers w/ gall bladders, kidneys, lungs, heart, and spleen were removed and weighed on all 24 rabbits. The brains were removed and weighed from 4 rabbits from each group.

Freeze Drying and Whole Body Composition

Sixteen whole rabbits (n=8/trt) were quartered for ease of handling and frozen at -20° C. Each rabbit was weighed and then placed in a separate 9x24" tins and placed in a Virtis Lyotrel Freeze Dryer System. The temperature in the condenser was -40°--44° C, and the temperature in the sample chamber was 30°-32° C, under a vacuum of 140 torr. The rabbits were weighed one to two times per week until there was no weight change for a 24 h period of time, and approximately 45% of the initial body mass was remaining. The freeze dried material for each rabbit was

individually ground through a 7 mm screen using a Weston #32 meat grinder. The final material was then homogenized and submitted for chemical analysis.

Bomb Calorimetry

Gross energy (GE) of the rabbit diets was determined using a Parr 1231 bomb calorimeter, utilizing 2418.5915 MJ as the energy equivalent for the bomb and the sample container. The energy calculation was standardized using benzoic acid tablets (26.953 MJ/kg each), and for every ten samples a standard was run to ensure consistency. The wire used to ignite the sample was Parr No. 45C10, with a standard energy of 2.3 calories/cm, and ten cm were used per test.

Urea Nitrogen

The urine from each rabbit was thawed and urine urea nitrogen (UUN) was determined using the Bio-Assay System Qaunti-Chrom™ Urea Assay Kit (DIUR-500). The serum from all three blood draws was thawed and analyzed for serum urea nitrogen content (SUN) using the Bio-Assay System Qaunti-Chrom™ Urea Assay Kit (DIUR-500).

Calculations

Calculations for apparent digestibility and nutrient absorption were made using the following formulas:

Apparent Digestibility = (Nutrient Intake – Nutrient in Feces)/Nutrient Intake X 100

Nutrient Retention = (Nutrient Intake (g) – Nutrient in Feces (g) – Nutrient in Urine(g))

Statistical Analysis

Data for growth, blood parameters, digestibility, intake, nutrient retention, organ weights and body composition were analyzed using PROC MIXED procedure (SAS Institute Inc., Cary, NC) for repeated measures. The experimental unit was the individual rabbit, the fixed effect was

treatment, and the random effect was date. Differences between treatments were considered statistically significant if $P \leq 0.05$ and trends if $P \leq 0.10$.

RESULTS

Body Weight and ADG

The rabbits were weighed on d 0 and then every 7 days after the study began. There was no significant effect from the algal treatment on overall BW (P=0.82) or ADG (P=0.50), (Table 4.4).

Nutrient Intake, Digestibility, Excretion, and Retention: DM, Crude Fat, ADF, NDF, Ash

During the 7 day nutrient balance trial, food and water was offered ad libitum, and coprophagy was not restricted Every 24 h, all food and water intake was measured, as well as all urine and fecal output (Table 4.5). The CON group consumed numerically more feed over the 7 days, than did the ALG group (P=0.19), while the ALG group consumed more water than the CON group (P=0.20) and subsequently the ALG group produced more urine (P=0.03).

The average nutrient intake, excretion, retention and digestibility for DM, crude fat, ADF, NDF and Ash data are summarized in Table 4.6. As the two diets were formulated to be iso-caloric and iso-nitrogenous, there was no difference seen in the nutrient intake, with the exception of ADF. This difference (P<0.01) was not unexpected as the ALG diet had a decreased level of ADF than the CON diet. The excretion of ADF and NDF were both less in the ALG group as compared to the CON, which is logical as less of these nutrients were consumed More crude fat was consumed by the ALG group (P=0.19), and subsequently more was excreted (P<0.01). There were no differences seen in the retention of DM, crude fat, NDF or ash. A decreased amount of ADF was retained in the ALG group (P<0.01), which is in line with the reduced intake of this nutrient. Apparent digestibility of DM, NDF and ash were greater for the ALG group ($P \leq 0.04$), while crude fat and ADF apparent digestibility was decreased ($P \leq 0.03$).

Nutrient Intake, Digestibility, Excretion, and Retention: N and P

The intake, digestibility, excretion and retention of N and P are shown in Table 4.7. The intake of both N and P were decreased in the ALG group, (*P*>0.10). There was no difference in the amount of N excreted in the feces or urine between the groups (*P*>0.10). A difference was noted in the amount of P excreted in the feces (*P*<0.01), with a decreased amount excreted in the ALG group. P was excreted in the urine similarly between the groups (*P*>0.10). A greater level of N was retained in the ALG group (*P*=0.03), and there was no difference in the amount of P retained between the groups (*P*=0.99). No difference was seen in the apparent digestibility of the N (*P*=0.74), while P digestibility was much greater in the ALG rabbits (*P*<0.01).

Blood Metabolites

No differences were seen between ALG and CON groups in pH, TCO_2, SO_2, HCO_3, BE, Na, Cl, Hct, Hb, glucose or SUN (*P*>0.10) (Table 4.8). These data are indicative of there being no physiological effect of feeding a diet with algal meal to growing animals. There was a date effect for each measured metabolite (*P*<0.01), yet no Treatment*Date effect (*P*>0.10).

Urea Nitrogen

The amount of N that was eliminated from the body in the form of urea N is reported in Table 4.9. The UUN level was decreased in the ALG fed rabbits (*P*=0.01). Subsequently, the amount of urea in the form of SUN was numerically greater in the ALG group (*P*=0.15). These data are consistent with the reduced amount of N that was excreted in the urine and feces for the ALG group, and the greater level of retained N as compared to the CON group.

Organ Evaluation

When the rabbits were sacrificed at the completion of the study, each organ was removed from the animal and the weights recorded, these data are shown in Table 4.10. There were no differences in the weights of the internal organs (*P*>0.10). In a previous study conducted feeding

the algal meal from *Nannochloropsis oculata* to young Sprague-Dawley®™ rats, a significant difference *(P*<0.01) was seen between the control and algal groups liver weights. In this study with rabbits, this difference was not seen, as the liver weights (gall bladder included) were similar between the groups *(P*=0.85).

Whole Body Composition

Sixteen whole rabbits (sans blood) were analyzed (Table 4.11), for DM, crude fat, ADF, NDF and ash. There were no differences seen *(P*>0.17) between the CON and ALG groups. Crude fat, ADF, NDF and ash levels were all numerically greater in the ALG group.

DISCUSSION

Nannochloropsis o. is a high protein feedstuff, however it is also a highly fibrous material. For this reason, it was of interest to determine the availability of the nutrients in this material to the animal in order to assess its functionality as a protein supplement. When developing this study, it was decided to utilize a metabolically unique animal, the non-ruminant herbivore, as this is an animal that can subsist primarily on a diet of fibrous material (Hintz et al. 1978). Although rabbits are approximately ½ as efficient as a true ruminant (bovine) (Slade et al. 1969) in fiber digestion, they do have a well-developed cecum, as well as a digesta separation mechanism in their colon (Hornicke et al. 1980) which is capable of the selective retention for both fluid and fine particles from their diet (Pickard et al. 1972). This capability does not increase fiber digestibility in the animal, however it allows a rabbit to quickly eliminate the hard to digest material from their digestive tract and thus maintain a high level of feed intake. Rabbits also practice coprophagy in order to maximize the utilization of high fiber diets (Carabano et al. 1988).

In the previous study with rats, we noticed the reduced excretion of P in the feces of the animals fed the algal diet (89.25 g/d vs. 110.20 g/d, *P*<0.01). In this current rabbit study, we also

noticed the reduced fecal excretion of P (0.67 g/d vs. 0.92 g/d, $P<0.01$) in the algal fed animals. The algal fed rabbits also exhibited an increased apparent digestibility of P (44.53% vs. 29.35%, $P<0.01$). These data are very encouraging, as plants generally store P as phytic acid, rendering it unavailable to a non-ruminant animal (D'Mello et al. 2000). Our data would suggest that the P contributed by the algal meal is potentially more bioavailable to the animal that the P offered by traditional grains. The reduced excretion of the P would also offer livestock producers an advantage with less P being excreted into the environment.

This was the first known study to offer rabbits an algal meal (oil extracted) as a feedstuff in a diet, although rats, cattle, swine and avian species have been fed algae (whole and extracted) in previous work (Sukenik et al. 1993, 2003) (Markovits et al. 1992) (Nitsan et al. 1992) (Villar et al. 1994), (Herber-McNeill et al. 1998), (Boeckaert et al. 2006, 2008), (Franklin et al. 1999), (Abril et al.2003). In all previous work reviewed, no adverse health effects in the animals fed the algae were noted. Similar results were seen in our study, additionally the New Zealand white rabbits exhibited normal growth and nutrient intake for the duration of the study. The measurable differences in apparent digestibility and nutrient retention did not result in discernible nutritional deficiencies for the animals. The algal meal from *Nannochloropsis* sp. can be considered as a nutritionally adequate protein supplement when included a 10% in a complete rabbit diet, and should be further studied in commercial livestock in order to be approved as a feedstuff.

Table 4.1. Nutrient composition (DM basis) of Algal Meal used to formulate the CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	Ash. %	TDN, %
Algal Meal	31.30	8.90	16.10	29.60	8.65	81

Table 4.2. Nutrient composition (DM basis) of Algal Meal and CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	Ash. %	GE, Mcal/kg
CON[1]	19.90	3.60	27.40	42.90	8.62	4.33
ALG[2]	19.70	4.20	23.70	42.60	8.76	4.37

1.CON: Harlan Lab's 2031 (alfalfa meal,soybean hulls,ground oats,wheat middlings, dehulled SBM,ground corn,dical P, cane molasses,salt, vitamins, minerals) + an additional wheat middlings,SBM, & soy oil to match increased CP & CF from algal meal.
2.ALG: Harlan Lab's 2031 + 10% algal meal

Table 4.3. Fatty Acid Composition (DM basis) of CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	C14:0 g/100g	C16:0 g/100g	C16:1 g/100g	C18:0 g/100g	C18:1 g/100g	C18:2 g/100g	C18:3 g/100g
CON[1]	0.04	0.93	0.02	0.17	0.70	0.97	0.50
ALG[2]	0.04	1.51	0.51	0.33	1.56	3.18	0.25

1.CON: Harlan Lab's 2031 (alfalfa meal,soybean hulls,ground oats,wheat middlings, dehulled SBM,ground corn,dical P, cane molasses,salt, vitamins, minerals) + an additional wheat middlings,SBM, & soy oil to match increased CP & CF from algal meal.
2.ALG: Harlan Lab's 2031 + 10% algal meal

Table 4.4. Effect of feeding CON and ALG diets to young, growing New Zealand White Rabbits on body weight and ADG

RESPONSE	P VALUE TREATMENT	DATE	TREATMENT*DATE
Weight, kg (all days)	0.82	<0.01	<0.01
Weight, kg d7*			0.68
Weight, kg d14**			<0.01
Weight, kg d21			0.67
Weight, kg d28			0.37
Weight, kg d35			0.42
Weight, kg d42			0.15
ADG, kg (all days)	0.50	<0.01	<0.01
ADG, kg d7*			0.92
ADG, kg d14**			<0.01
ADG, kg d21			<0.01
ADG, kg d28			0.56
ADG, kg d35			0.91
ADG, kg d42			0.44

*D7-13: ALG group fed 50/50 algal diet/control diet, CON group fed 100% control diet
**D14-42: ALG group fed 100% algal diet, CON group fed 100% control diet

Table 4.5. Balance Trial Summary for young, growing New Zealand White Rabbits fed CON and ALG diets for 7 days

Response	CON	ALG	SE	P Value
Average Feed Intake, g/d	216.59	200.75	8.25	0.19
Average Water Intake, mL/d	405.44	446.70	22.11	0.20
Average Urine Produced, mL/d	144.02	188.71	13.38	0.03
Average Feces Produced, g/d	126.13	121.35	8.38	0.69

Table 4.6. Nutrient Intake, Excretion, Retention and Digestibility Per Day for Young New Zealand White Rabbits Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
OM				
Intake, g/d	178.12	164.48	6.77	0.17
Excreted Fecal, g/d	73.20	65.40	3.51	0.13
Retained, g/d	104.92	99.08	3.41	0.24
Digestibility, %	59.04	60.45	0.59	0.11
DM				
Intake, g/d	194.93	180.27	7.42	0.18
Excreted Fecal, g/d	81.98	72.61	3.93	0.11
Retained, g/d	112.94	107.66	3.65	0.32
Digestibility, %	58.08	59.95	0.60	0.04
Crude Fat				
Intake, g/d	7.02	7.57	0.29	0.19
Excreted Fecal, g/d	1.08	1.64	0.13	<0.01
Retained, g/d	5.94	5.93	0.18	0.97
Digestibility, %	84.93	78.74	1.00	<0.01
ADF				
Intake, g/d	53.41	42.72	1.89	<0.01
Excreted Fecal, g/d	35.22	30.58	1.70	0.07
Retained, g/d	18.19	12.14	0.91	<0.01
Digestibility, %	34.28	28.44	1.80	0.03
NDF				
Intake, g/d	83.62	76.80	3.17	0.14
Excreted Fecal, g/d	51.32	43.36	2.65	0.05
Retained, g/d	32.30	33.44	1.18	0.50
Digestibility, %	38.98	43.85	1.43	0.03
Ash				
Intake, g/d	16.80	15.79	0.65	0.28
Excreted Fecal, g/d	8.78	7.21	0.43	0.02
Retained, g/d	8.02	8.58	0.26	0.14
Digestibility, %	47.91	54.70	0.92	<0.01

Table 4.7. Nitrogen and Phosphorus Intake, Excretion, Retention and Digestibility Per Day for Young New Zealand White Rabbits Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
N				
Intake, g/d	6.21	5.68	0.23	0.13
Excreted Fecal, g/d	1.98	1.84	0.11	0.40
Excreted Urine, g/d	2.43	2.11	0.19	0.25
Retained, g/d	0.03	0.17	0.42	0.03
Digestibility, %	68.35	67.99	0.74	0.74
P				
Intake, g/d	1.30	1.19	0.05	0.15
Excreted Fecal, g/d	0.92	0.67	0.05	<0.01
Excreted Urine, g/d	0.08	0.10	0.01	0.20
Retained, g/d	0.81	0.81	0.03	0.99
Digestibility, %	29.35	44.53	1.82	<0.01

Table 4.8. Effect of feeding CON and ALG diets to young, growing New Zealand White Rabbits on blood metabolites

				P Value		
Response	**CON**	**ALG**	**SE**	**Treatment**	**Date**	**Treatment*Date**
pH	7.27	7.31	0.02	0.11	<0.01	0.77
Total CO_2 (mMol/L)	23.41	24.69	0.61	0.15	<0.01	0.47
Partial Pressure O_2 (mmHg)	89.06	108.47	9.22	0.15	<0.01	0.18
Saturated O_2 (%)	91.95	93.44	1.15	0.37	0.05	0.77
Partial Pressure CO_2 (mmHg)	49.82	47.41	1.61	0.30	<0.01	0.90
HCO_3 (mMol/L)	21.88	23.29	0.60	0.11	<0.01	0.39
BE(mMol/L)	-4.95	-2.85	0.86	0.09	<0.01	0.48
Na(mMol/L)	139.62	140.34	1.42	0.72	<0.01	0.29
Cl (mMol/L)	109.68	109.51	0.52	0.81	<0.01	0.80
Hematocrit (%PCV)	37.31	36.19	0.54	0.16	<0.01	0.60
Hemoglobin (g/dL)	12.58	12.31	0.16	0.24	<0.01	0.96
Glucose (mg/dL)	130.53	130.72	2.57	0.96	<0.01	0.95
SUN(mg/dL)	9.53	10.94	0.68	0.15	<0.01	0.30

Table 4.9. Urea Nitrogen Data for Young New Zealand White Rabbits Fed CON and ALG Diets

Response	Control	Algal	SE	P Value
UUN, mL/d	1.66	1.06	0.15	0.01
SUN, mg/dL	9.52	10.42	0.68	0.15

Table 4.10. Average Organ Weights of Young New Zealand White Rabbits Fed CON and ALG Diets

Organ	CON	ALG	SE	P Value
Blood, g	117.15	125.95	5.77	0.29
Blood, % Live BW	2.85	3.16	0.15	0.16
Digestive Tract[1],g	204.76	197.10	5.71	0.35
Digestive Tract, % Live BW	4.90	4.95	0.08	0.70
Lungs, g	13.64	12.82	0.43	0.18
Lungs, % Live BW	0.33	0.32	0.01	0.64
Heart, g	9.45	9.09	0.41	0.55
Heart, % Live BW	0.23	0.23	0.01	0.97
Kidneys, g	21.11	20.65	0.73	0.66
Kidneys, % Live BW	0.51	0.52	0.01	0.51
Liver[2], g	132.67	129.66	11.11	0.85
Liver, % Live BW	3.17	3.24	0.25	0.85
Spleen, g	1.53	1.70	0.09	0.19
Spleen, % Live BW	0.04	0.04	0.00	0.05
Brain[3], g	9.05	9.29	0.31	0.60
Brain, % Live BW	0.24	0.24	0.01	0.62

[1] Digestive Tract includes stomach, small intestine, large intestine, cecum
[2]Includes gall bladder
[3]Average brain weight is on rabbits 17,19,21,23 (C) and 10,20,22,24 (A)

Table 4.11. Average Body Composition of young, growing New Zealand White Rabbits fed CON and ALG diets

Nutrient	CON	ALG	SE	P Value
OM, kg	1.56	1.42	60.60	0.17
DM, kg	1.70	1.58	61.86	0.19
N, kg	0.85	0.87	24.67	0.66
Crude Fat, kg	0.72	0.62	54.01	0.22
ADF, kg	0.17	0.15	13.70	0.45
NDF, kg	0.30	0.27	20.64	0.44
Ash, kg	0.15	0.15	4.67	0.82

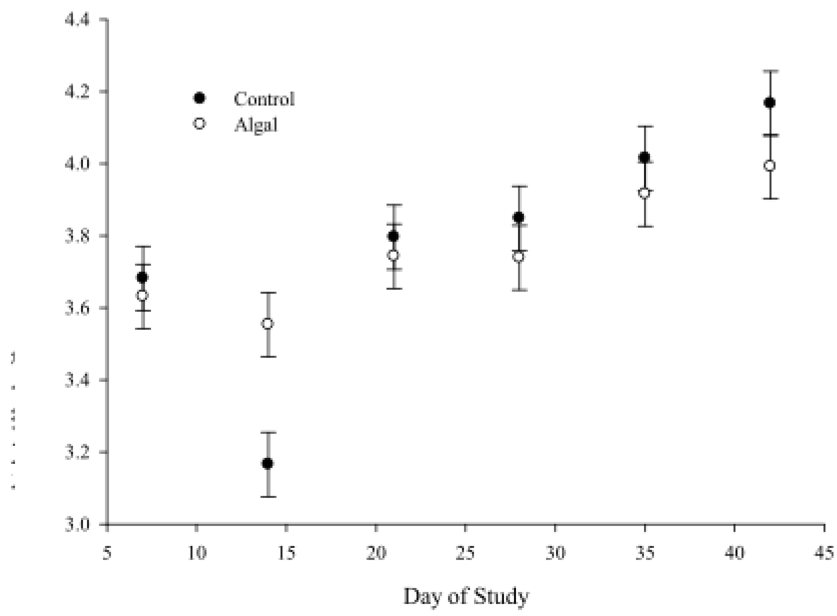

Figure 4.1. Body weight of growing New Zealand White Rabbits fed CON and ALG diets

LITERATURE CITED

Abril, R., J. Garrett, S. Zeller, W. Sander, R.W. Mast. 2003. Safety Assessment of DHA-rich Microalgae from Schizochytrium sp. Part V: Target Animal Safety/Toxicity Study in Growing Swine. Reg. Toxic. Pharm.37:73-82.

ALAT Training Manual. 2005. American Association for Lab Animal Sciences.

AOAC, 2005. Official Methods of Analysis. 18th ed Assoc. Off.Anal. Chem., Gaithersburg, MD.

Archibeque, S.L., A. Ettinger, B.D. Willson. 2009. *Nannochloropsis* oculata as a Source for Animal Feed Acta. Agronomica Hungarica 57:245-248.

Boeckaert, C., N. Boon, I.Z. Abdulsudi, W. Verstraete, V. Fievez. 2006. Accumulation of Biohydrogenation Intermediates and Changes in the Rumen Protozoal Population After Micro Algae Feeding to Dairy Cattle. Comm. Appl. Biol. Sci. Ghent University. 71:83-86.

Boeckaert, C., B. Vlaeminck, J. Dijkstra, A. Issa-Zacharia, T. Van Nespen, W. Van Straalen, V. Fievez. 2008. Effect of Dietary Starch or Micro Algae Supplementation onRumen Fermentation and Milk Fatty Acid Composition of Dairy Cows. J. Dairy Sci. 91:4714-4727.

Cornell Sodium Borate-Sodium Phosphate Buffer Procedure. 1990. Incubated at ambient temperature. Cornell Nutrition Conference Proceedings. Pp.85-86.

D'Mello, J.P.F., Officer, D.I.. Farm Animal Metabolism and Nutrition: Feed Enzymes. CABI Publishing, N.Y., N.Y. Pp. 405-426.

Franklin, S., K. Martin, R. Baer, D. Schingoethe, A. Hippen. 1999. Dietary Marine Algae (Schizochytrium sp.) Increases Concentrations of Conjugated Linoleic, Docosohexaenoic and Transvaccenic Acids In Milk of Dairy Cows. J. Nutr. 129:2048-2052.

Goering, H.K.,P.J. Van Soest. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC.

Hach, C.C., S.V. Brayton, A.L. Kopelove. 1985. Powerful Kjeldahl Nitrogen Method Using Peroxysulfuric Acd J. Agric. Food Chem. 33 (6) 1117-1122.

Herber-McNeill, S.M., M.E. Van Elswyki. 1998. Dietary Marine Algae Maintains Egg Consumer Acceptability While Enhancing Yolk Color. Poultry Science 77:493-496.

Hornicke, H., G. Bjonhag. 1980. Coprophage and Related Strategies for Digesta Utilization. Digestive Physiology and Metabolism in Ruminants. 707-730.

Hintz, H.F., H.F. Schryver, C.E. Stevens. 1978. Digestion and Absorption in the Hindgut of the Nonuminant Herbivores. J. An. SCi. (46)6:1803-1807.

Markovits, A., R. Conejeros, L. Lopez, M. Lutz. 1992. Evaluation of Marine Microalga *Nannochloropsis* sp. As A Potential Dietary Supplement Chemical, Nutritional and Short Term Toxicological Evaluation in Rats. Nutr. Res. (12):1273-1284.

Mathews, K., M. McConnell. 2009. Ethanol Co-Product Use in U.S. Cattle Feeding – Lessons and Considerations. www.ers.usda.gov/Publications/FDS/2009/04Apr/FDS09D01.

Nitsan, A., S. Mokady, A. Sukenik. 1999. Enrichment of Poulry Products with Ω3 Fatty acids by Dietary Supplementation with the Algae *Nannochloropsis* and Mantur Oil. J. Agric. Food Chem. 47:5127-5132.

NRC for Domestic Animals, 2nd Revised Edition. 1997

Net Resources International. Hydrocarbons Technology.

Pickard, DW., C.E. Stevens. 1972. Digesta Flow Through the Rabbit Large Intestine. Am. J. Physiol. 222:1161-1166.

Sakaguchi, E., K. Kaizu, M. Nakamichi. 1992. Fiber Digestion and Digesta Retention from Different Physical Forms of the Feed in the Rabbit. Comp. Biochem. Physiol. 3:559-563.

Sheehan, J.,T. Dunahay, J. Benemann, P. Roessler. 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program-Bio-diesel from Algae, Closeout Report. NREL/TP-580-24 190.

Slade, L.M., H.F. Hintz. 1969. Comparison of Digestion in Horses, Ponies, Rabbits and Guinea Pigs. J. Anim. Sci. 28-842.

Sukenik, A., H. Takahashi, S. Mokady. 1993. Dietary Lipids from Marine Unicellular Algae Enhance the Amount of Liver and Blood Fatty Acids in Rats. Ann. Nutr. Metab. 38:85-96.

Werman, M., A. Sukenik, S. Mokady. 2003. Effects of the Maine Unicellular Alga *Nannochloropsis* sp. to Reduce the Plasma and Liver Cholesterol Levels in Male Rats Fed on Diets with Cholesterol. Biosci. Biotechnol. Biochem. 67:2266-2268.

Villar, R., M.R. Laguna, I. Cadavid, J.M. Calleja. 1994. Effects of Aqueous Extracts of Six Marine Microalgae on Smooth Muscle Contraction in Rat Duodenum and Vas Deferens. Planta Med 60:521-526.

Chapter 5

Nannochloropsis oculata: A safe microalgae as a source of minerals for young, growing rabbits[4]

INTRODUCTION

In the U.S.A, corn is the largest source of raw material for biodiesel in the United States of America. One bushel of corn can produce ~2.8 gallons of ethanol, and in the last decade the demand for corn ethanol has increased more than 10 fold. In 2008, ~2.5 million bushels of corn were processed into ethanol. The demand for corn ethanol is expected to be over 12 billion gallons in 2012. Although the use of corn as a raw material for biodiesel has resulted in a useful co-product, distiller's grains, the land, water and other resources that are necessary to grown corn has spurred research into other fuel alternatives. Today, a main focus of biofuel research is microalgae, as algae can grow on land that cannot support traditional crops, and requires very little water. There are several high oil producing microalgae species, *Nannochloropsis oculata* being one of them. This specific alga can produce upwards of 30% oil (DM basis), that can be extracted and used as a biofuel, and an alga such as *Nannochloropsis* sp. could possibly produce more than 5000 gallons of oil per acre of land When compared to the 18 gallons of ethanol per acre of corn, algae becomes much more interesting. The production of algae oil also results in a valuable co-product, a high protein, mineral rich algal meal. This meal is upwards of 35% CP (DM), and could potentially be used in livestock diets as a source of protein. While the research into utilizing algal meal (oil extracted) is minimal, the research that has been conducted suggests

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that animals fed whole *Nannochloropsis oculata* have not been adversely affected. The objective of this study was to conduct a 36 d feeding trial to more fully evaluate the safety of feeding *Nannochloropsis* o. to young rabbits in regards to mineral metabolism, organ histology and whole body composition.

MATERIALS AND METHODS

This experiment was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Algal Meal

The algal meal that was utilized in this study was supplied by SOLIX Biofuels Inc., Fort Collins, CO. A slurry was made from frozen algae, and the cells were lysed using high shear mixing. Hexane was added to the slurry, and the material was allowed to separate. The aqueous fraction was then re-extracted with hexane, and allowed to separate a second time. After this second separation, the aqueous portion was mixed with water and ethanol and evaporated at 100° C for ~24 hours. The resulting algal meal was then transferred to the Colorado State University Animal Sciences Department to be ground to a fine power using a Wiley Lab Mill model #4 and submitted for nutrient and toxic mineral analysis. The nutrient results (Tables 5.1 and 5.2) were shared with Harlan Laboratory and utilized to manufacture the Algal diet.

Animals and Treatments

A total of 24 6-8 week old New Zealand White male rabbits were obtained from Western Oregon Rabbit Company. The rabbits were received into the Colorado State University Laboratory Animal Resource center and immediately started on Harlan Teklad 2031 Global High Fiber Rabbit diet. The rabbits were held under standard laboratory conditions with a room temperature of 21°C, 37-45% humidity and a 12/12 light/dark cycle in IACUC approved solitary cage measuring 23.0” wide, 24.0” long and 15.0” high. After an eleven day acclimation period,

the rabbits were randomly blocked according to body weight into two separate treatment groups, Control and Algal, and the groups were then was stepped up onto the diets specifically formulated for this experiment. In order to acclimate the rabbits to different diets, for 7 days, twelve rabbits were fed a 50/50 Control/2031 diet and twelve rabbits were fed a 50/50 Algal/2031 diet which incorporated 10% algal meal (sp *Nannochloropsis oculata*). On day 7, the rabbits were switched to 100% Control (**CON**) and 100% Algal (**ALG**) diets. The CON diet and the ALG diet were formulated to be iso-nitrogenous, iso-caloric (Tables 5.3 and 5.4) and to meet all of the nutritional needs for a young, growing rabbit (Nutrient Requirements of Rabbits, 2nd Revised Edition, 1977). The rabbits were weighed on d0 and then every 7 days after the study began. The CON group weighed an average of 3.51+/-0.75 kg initially (d 1 of 100% CON diet) and an average of 4.17+/-1.33 kg on d36, while the ALG group weighed an average of 3.45 +/-0.72 kg (d 1 of 100% ALG diet), and an average of 3.99+/-0.92 kg on d36. Overall, the CON group gained 0.66 kg throughout the study, an 18.80 % increase in total BW, and the ALG group gained 0.54 kg throughout the study, a 15.65% increase in BW. The ADG at the end of the study was 14 g/d (CON), and 10 g/d (ALG). Fresh water was made available at all times. Coprophagy was not prevented in order to encourage normal eating behavior.

Chemical Analysis

Samples of the algal meal, diet, feces and rabbit samples were analyzed for DM, crude fat and ash using AOAC 2005 methods. The lipid content of the rabbits was determined using acid hydrolysis (AHF) (AOAC 2005). Crude protein was determined using the Kjeldahl AOAC 2005 method, and calculated from total N values (N x 6.25). Soluble protein was determined using sodium borate-sodium phosphate buffer solution. NDF, ADF and lignin (Goering and Van Soest, 1970) were determined using a modified Van Soest using fiber bag technology (Ankom

200 and Daisy II Incubator, Ankom Technology Corp., Macedon, NY). Urine nitrogen was quantified using peroxysulfuric acid as described by Hach et al. (1985).

Mineral analysis was conducted by drying the feed and fecal samples at 75° C overnight, and digesting the samples at 95° C with nitric acid at a 1 ml nitric acid:100 mg sample ratio. After digestion 5 ppm Yttrium was used as an internal standard, and the samples were diluted with water to a final volume of 25 mL (50x dilution). One mL of the digested and diluted sample was further diluted to 1:10 with a 20% nitric acid and 5 ppm Yttrium solution (500x final dilution). The final samples were then run on a Varian radial inductively coupled plasma (ICP) atomic emission spectrometer. The whole rabbit minerals were measured using the same method as for the feed and feces, however the final dilution was 50x, and 1 ppm of Yttrium was used as an internal standard and 500 ppm Cesium was used as and ionization quencher. Urine mineral content was quantified by digesting the urine in 1 mL nitric acid:1mL sample, and dried at 95°C overnight. After digestion and drying, 1 ppm of Yttrium was used as an internal standard, and 500 ppm Cesium was used as an ionization quencher. The samples were diluted with water to a final volume of 25 mL (50x dilution) and run on a Varian radial (ICP) atomic emission spectrometer.

Euthanasia and Organ Evaluation

The rabbits were humanely anesthetized on d 45 via iso-flourene gas and exsanguination was via a fatal heart stick. The bodies were then opened from sternum to pelvis and the complete digestive tract was removed. The small intestine, large intestine, stomach and cecum were thoroughly cleaned with a 0.9% saline solution. The livers w/gall bladders, kidneys, lungs, heart, spleen were removed and weighed on all 24 rabbits. The brains were removed and weighed from 4 rabbits from each group.

Histology

Samples for histological analysis were obtained from the large lobe of the liver, the spleen, and one kidney. Each sample was cut longitudinally from the organ and fixed in 10% neutral buffered formalin (NBF) for 24 h at 21^0C. These tissues samples were then submitted to the Colorado State University VTH Diagnostic Laboratory for H&E stain slide preparation. Tissue samples taken from the heart, brain and abdominal skeletal muscle were fixed in 10% NBF and stored at 21^0C for future testing.

Freeze Drying and Whole Body Composition

Sixteen whole rabbits (n=8/trt) (4 from each group had histological samples taken) were quartered for ease of handling and frozen at -20° C. Each rabbit was weighed and then placed in a separate 9x24" tins and placed in a Virtis Lyotrel Freeze Dryer System. The temperature in the condenser was -40°--44° C, and the temperature in the sample chamber was 30°-32° C, under a vacuum of 140 torr. The rabbits were weighed one to two times per week until there was no weight change for a 24 h period of time. The freeze dried material for each rabbit was individually ground through a 7 mm screen using a Weston #32 meat grinder. The final material was then homogenized and submitted for chemical analysis.

Calculations

Calculations for apparent digestibility and nutrient retention were made using the following formulas:

Apparent Digestibility = (Nutrient Intake – Nutrient in Feces)/Nutrient Intake X 100

Nutrient Retention = (Nutrient Intake (g) – Nutrient in Feces (g) – Nutrient in Urine (g))

Statistical Analysis

Data for growth, digestibility, intake, nutrient retention, organ weights and body composition were analyzed using PROC MIXED procedure (SAS Institute Inc., Cary, NC) for

repeated measures. The experimental unit was the individual rabbit, the fixed effect was treatment, and the random effect was date. Data for histology results were analyzed using the Fisher's Exact Test (SAS Institute Inc., Cary, NC), using the individual rabbit as the experimental unit and the Algal or Control diet as the fixed effect. Differences between treatments were considered statistically significant if $P \leq 0.05$ and trends if $P \leq 0.10$.

RESULTS

BW and ADG

All of the rabbits remained healthy for the duration of the study, and were weighed every 7 days for the duration of the study. There was no significant effect from the ALG treatment on overall BW (P=0.82) or ADG (P=0.50), (Table 5.5).

Macro Mineral Intake, Excretion, Retention and Digestibility

Macro mineral analyses were conducted on the feed, feces and urine these data are shown in Table 5.6. There was no difference in the intake of Ca, K, Mg, S and P (P>0.10). The intake of Na was greater in the ALG group (P<0.01). The concentration of Na was greater in the ALG diet, so the increased intake is explained. The fecal excretion of Ca and P was decreased in the ALG group (P<0.01), in contrast the fecal excretion of Na and Mg were greater (P<0.05). The urinary excretion of Ca, K, Mg, Na, S and P were unaffected (P>0.10) by the treatment. There was no difference in the retention of the macro minerals analyzed (P>0.10). The apparent digestibility of Ca, K, Mg and P were all improved in the ALG group over the CON group ($P \leq 0.06$), while S digestibility was unaffected (P<0.10). These data suggest that the algal meal addition to a diet does not negatively affect the apparent digestibility of macro minerals.

Trace Mineral Intake, Excretion, Retention and Digestibility

The metabolism of trace minerals is shown in Tables 5.7 and 5.8. There was a difference in the intake of Co and Zn the levels were greater in the ALG group (P<0.01). There were no

other intake differences noted (*P*>0.10). The fecal excretion of Al, Ba and Mn were reduced in the ALG group (*P*<0.05). There were no differences seen in the fecal excretion of B, Co, Cr, Cu, Fe, and Zn (*P*>0.10). No Mo was detected in the feces of either group (<1 ppm). Urinary excretion of Al, B, Ba, Co, Cu, Mn, Mo, and Zn were unaltered by the algal treatment (*P*>0.05). The amount of Cr and Fe excreted in the urine of the ALG group was greater than the CON group (*P*<0.05). There was also no difference in the retention of the trace minerals analyzed (*P*>0.10). In contrast to the macro mineral apparent digestibility data, there were differences seen in the trace mineral apparent digestibility (*P*≤0.02). With the exception of Zn (*P*>0.10), the apparent digestibility of the other trace minerals was improved in the ALG group over the CON group. These data suggest that the minerals in the ALG diet were more bioavailable than the minerals in the CON diet.

Cd was not detected in the algal meal or the ALG and CON diets, however it was detected in the feces of both groups (*P*>0.10) (data not shown). Hg and As were also analyzed for in the algal meal and ALG and CON diets, and were found to be below the detection limits of the test methodology.

Whole Body Composition

The bodies of 16 whole rabbits (n=8/trt)), were analyzed for macro and trace mineral levels, these data are presented in Table 5.9. No differences were noted in the levels of the minerals analyzed (*P*>0.10), with the exception of Al (*P*<0.05).

Organs and Histology

The internal organs of each rabbit were visually evaluated after the animal was euthanized There was minimal to no obvious differences seen in the respiratory and circulatory organs (lungs, heart) or the spleen, kidneys or brain. The weight of the blood, digestive tract, lungs, kidneys, spleen and liver were recorded for each animal (Table 5.10). There were no

significant *(P*>0.10) differences in the weight of any of the organs. The histology for the spleen, kidney and liver samples reported noted no significant lesions or abnormalities in any of the samples (Table 5.11). These data suggest that there were no harmful effects to the internal organs in the growing animals that consumed a diet with algal meal.

DISCUSSION

The value of algal meal (oil extracted) increases considerably if it can be utilized in multiple applications and in large volumes. The composition of the algal meal makes it a logical livestock feed, as it is high in protein, fiber and minerals. Archibeque et al. (2009) compared the chemical composition on a dry matter basis of the algal meal from *Nannochloropsis oculata* to that of soybean meal (SBM), and steam flaked corn (SFC), two common grains fed to commercial livestock as protein sources. The total CP content of the algal meal was 35.28%, as compared to 51.55% in the SBM and 8.86% in the SFC, making this algal meal an adequate protein supplement for animal diets, in the same manner SBM and SFC is used today. Archibeque et al. (2009) also included a mineral analysis for the algal meal, and found it to be similar to both SBM and SFC. The research into feeding the oil-free meal from algaeoil production is minimal, and limited to a study in rats. Markovits et al. (1992) fed whole *Nannochloropsis* o. to adolescent rats, at both 5% and 10% inclusion levels. In their study, a mineral analysis of the algal meal was reported The mineral data presented by Markivits et al. (1992) is different than ours, and showed much decreased levels of Na (0.31% v. 1.07%), K (0.06% v. 1.55%), and Fe (102 ppm v. 238 ppm). This could be attributed to the growth medium of the algae as well as the extraction method utilized for our study.

An algal species from the same phyla (Heterokontophyta), *Schizochytrium* sp. has been evaluated for safety, and has been granted GRAS status by the FDA. Hammond et al. (2001, 2002) investigated the anti-toxicity of *Schizochytrium* in a series of four studies. This research

covered the feeding of Sprague-Dawley®™ rats for 13 weeks and evaluating them for any signs of toxicity at dosages up to 4000 mg/kg/day. The second study reviewed the feeding of gestational rats and rabbits, at levels of 180, 600 and 1800 mg/kg/day. The third study evaluated any reproductive toxicity and the final study focused on mutagenicity. All of the results showed no clinical signs of toxicity. These studies were isolated to the whole algae, with the fat included Dahm's et al. (2011) also evaluated the safety of the oil only from *Schizochytrium*, and in a 90 days study with rats, no adverse effects were noted. Although there is no data with *Nannochloropsis oculata*, our data is in agreement with Hammond et all (2001, 2002) and Dahms et al (2011), and does not suggest that the minerals contributed by the algal meal had any disadvantageous results in the animals.

In this current rabbit study, we also noticed the reduced fecal excretion of P (0.67 g/d vs. 0.92 g/d, $P<0.01$) in the algal fed animals. It is interesting to note that in a previous study with rats, we also noticed the reduced excretion of P in the feces of the animals fed the algal diet (89.25 g/d vs. 110.20 g/d, $P<0.01$). These algal fed rabbits also exhibited an increased apparent digestibility of P (44.53% vs. 29.35%, $P<0.01$). These data are very encouraging, as plants generally store P as phytic acid, rendering it unavailable to a non-ruminant animal (D'Mello et al. 2000). Our data would suggest that the P contributed by the algal meal is potentially more bioavailable to the animal that the P offered by traditional grains. The reduced excretion of the P would also offer livestock producers an advantage with less P being excreted into the environment.

As mentioned, there has not been much research published feeding *Nannochloropsis* o. meal to animals to compare to, our data does not suggest that the minerals contributed by the algal meal had any disadvantageous results in the animals. In fact, the digestibility of the macro minerals was either unaffected or enhanced in the Algal group. The same was seen with the trace

mineral digestibility data. These data support that algal meal as a useful and bioavailable source of minerals in an animal feed

The whole body composition data was generated in an effort to determine if there would be any toxic or harmful levels of minerals deposited in the carcass. Ours is the first study known to look at this, and we did not detect any levels of potentially toxic minerals in the bodies of the animals fed a diet with 10% algal meal. The effect of a high protein, mineral rich *Nannochloropsis* o. meal, on organ histology has only been evaluated in the previous study in rats that preceded this study. Our rabbit study also included histology for liver, kidney and spleen tissue. The lack of any difference in organ histology between the two groups further supports the safety of the algal meal as a potential feedstuff for growing animals.

It is interesting also note that in a previous study done with rats consuming a diet with 10% algal meal from *Nannochloropsis o.*, the liver weights were significantly *(P*<0.01) larger. Similar data had been reported by Markovits et al. (1992) in a study feeding rats 10% whole *Nannochloropsis o.*. In this study with rabbits, no weight differences were noted in any of the organs. These data that show no difference further support the safety and acceptability of this type of feedstuffs for young hind gut fermenters.

Based on this study, the algal meal from *Nannochloropsis oculata* is a safe, non-toxic potential feedstuffs for young growing rabbits. Further studies should be conducted to further understand the full usefulness of this type of biodiesel co-product in other animal species.

Table 5.1. Nutrient composition (DM basis) of Algal Meal use to formulate CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	CP, %	CF, %	ADF, %	NDF, %	Ash. %	TDN, %
Algal Meal	31.30	8.90	16.10	29.60	8.65	81

Table 5.2. Mineral composition (DM basis) of Algal Meal used to formulate CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	Al, ppm	As, ppm	B, ppm	Ba, ppm	Ca, %	Cd, ppm	Co, ppm	Cr, ppm	Cu, ppm	Fe, ppm	Hg, ppm	K, %	Mg, %	Mn, ppm	Mo, ppm	Na, %	P, %	Pb, ppm	S, %	Sb, ppm	Se, ppm	Tl, ppm	Zn, ppm
Algal Meal	60.0	<2.5	5.6	64.3	0.27	<0.3	0.61	<1.0	33.0	429.0	<10.0	1.34	0.34	34.9	<1.0	0.99	0.85	<2.5	0.50	<5.0	<10.0	<12.5	271.0

Table 5.3. Mineral composition (DM basis) of CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	Al, ppm	As, ppm	B, ppm	Ba, ppm	Ca, %	Cd, ppm	Co, ppm	Cr, ppm	Cu, ppm	Fe, ppm	Hg, ppm	K, %	Mg, %	Mn, ppm	Mo, Ppm	Na, %	P, %	Pb, ppm	S, %	Sb, ppm	Se, ppm	Tl, ppm	Zn, ppm
CON[1]	333.0	<2.5	28.4	28.1	1.00	<0.3	0.86	8.4	15.3	413.0	<10.0	1.83	0.40	104.0	1.5	0.25	0.67	<2.5	0.27	<5.0	<10.0	<12.5	75.9
ALG[2]	332.0	<2.5	34.2	28.1	1.03	<0.3	1.25	9.4	17.6	475.0	<10.0	1.83	0.39	99.3	1.5	0.36	0.67	<2.5	0.29	<5.0	<10.0	<12.5	99.6

1.Control: Harlan Lab's 2031 (alfalfa meal,soybean hulls,ground oats,wheat middlings, dehulled SBM,ground corn,dical P, cane molasses,salt, vitamins, minerals) + an additional wheat middlings,SBM, & soy oil to match increased CP & CF from algal meal.
2.Algal: Harlan Lab's 2031 + 10% algal meal

Table 5.4. Nutrient composition (DM basis) of CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	CP, %	CF, %	ADF, %	NDF, %	Ash. %	ME, kcal/g	DE, Mcal/kg
CON[1]	19.90	3.60	27.40	42.90	8.62	1.30	1.26
ALG[2]	19.70	4.20	23.70	42.60	8.76	1.34	0.68

1.CON: Harlan Lab's 2031 (alfalfa meal,soybean hulls,ground oats,wheat middlings, dehulled SBM,ground corn,dical P, cane molasses,salt, vitamins, minerals) + an additional wheat middlings,SBM, & soy oil to match increased CP & CF from algal meal.
2.ALG: Harlan Lab's 2031 + 10% algal meal

Table 5.5. Effect of feeding CON and ALG diets to young, growing New Zealand White Rabbits on body weight and ADG

		P VALUE	
RESPONSE	TREATMENT	DATE	TREATMENT*DATE
Weight, kg (all days)	0.82	<0.01	<0.01
Weight, kg d7*			0.68
Weight, kg d14**			<0.01
Weight, kg d21			0.67
Weight, kg d28			0.37
Weight, kg d35			0.42
Weight, kg d42			0.15
ADG, kg (all days)	0.50	<0.01	<0.01
ADG, kg d7*			0.92
ADG, kg d14**			<0.01
ADG, kg d21			<0.01
ADG, kg d28			0.56
ADG, kg d35			0.91
ADG, kg d42			0.44

*D7-13: Algal group fed 50/50 algal diet/control diet, Control group fed 100% control diet
**D14-42: Algal group fed 100% algal diet, Control group fed 100% control diet

Table 5.6. Macro Mineral Intake, Excretion, Retention and Digestibility Per Day for Young New Zealand White Rabbits Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
Ca				
Intake, g/d	1.95	1.86	0.08	0.39
Excreted Fecal, g/d	1.13	0.84	0.06	<0.01
Excreted Urine, g/d	0.23	0.22	0.03	0.94
Retained, g/d	0.73	0.78	0.03	0.21
Digestibility, %	42.20	55.37	1.72	<0.01
K				
Intake, g/d	3.57	3.30	0.14	0.18
Excreted Fecal, g/d	0.51	0.40	0.04	0.08
Excreted Urine, g/d	2.12	1.93	0.16	0.39
Retained, g/d	0.30	0.34	0.05	0.55
Digestibility, %	85.87	88.06	0.78	0.06
Mg				
Intake, g/d	0.78	0.72	0.03	0.14
Excreted Fecal, g/d	0.42	0.33	0.02	0.02
Excreted Urine, g/d	0.18	0.20	0.01	0.56
Retained, g/d	0.50	0.50	0.03	0.96
Digestibility, %	46.97	54.93	1.61	<0.01
Na				
Intake, g/d	0.49	0.64	0.02	<0.01
Excreted Fecal, g/d	0.24	0.32	0.02	0.01
Excreted Urine, g/d	0.18	0.22	0.02	0.15
Retained, g/d	0.27	0.34	0.05	0.29
Digestibility, %	50.37	50.13	2.74	0.95
S				
Intake, g/d	0.52	0.51	0.02	0.82
Excreted Fecal, g/d	0.19	0.20	0.01	0.78
Excreted Urine, g/d	0.21	0.19	0.02	0.31
Retained, g/d	0.35	0.41	0.04	0.35
Digestibility, %	63.23	61.98	0.87	0.32
P				
Intake, g/d	1.39	1.19	0.05	0.15
Excreted Fecal, g/d	0.92	0.67	0.05	<0.01
Excreted Urine, g/d	0.08	0.10	0.01	0.19
Retained, g/d	0.81	0.81	0.03	0.99
Digestibility, %	29.35	44.53	1.82	<0.01

Table 5.7. Trace Mineral Intake, Excretion, Retention and Digestibility Per Day for Young New Zealand White Rabbits Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
Al				
Intake, mg/d	59.87	59.76	0.00	0.99
Excreted Fecal, mg/d	65.30	49.83	0.00	0.01
Excreted Urine, mg/d	0.10	0.09	0.00	0.84
Retained, g/d	0.99	0.99	0.25	0.31
Digestibility, %	-0.75	12.61	2.08	<0.01
B				
Intake, mg/d	5.13	8.21	0.00	0.12
Excreted Fecal, mg/d	1.21	1.23	0.00	0.91
Excreted Urine, mg/d	2.82	2.84	0.00	0.95
Retained, g/d	0.56	0.42	0.16	0.53
Digestibility, %	78.38	18.60	0.96	<0.01
Ba				
Intake, mg/d	5.02	8.21	0.00	0.33
Excreted Fecal, mg/d	4.93	3.97	0.00	0.04
Excreted Urine, mg/d	0.03	0.03	0.00	0.35
Retained, g/d	0.89	0.95	0.06	0.51
Digestibility, %	10.35	18.60	1.60	<0.01
Co				
Intake, mg/d	0.17	0.22	0.00	<0.01
Excreted Fecal, mg/d	0.18	3.10	0.00	0.33
Excreted Urine, mg/d	0.01	0.01	0.00	0.56
Retained, g/d	0.80	0.73	0.97	0.34
Digestibility, %	-8.73	20.88	1.61	<0.01
Cr				
Intake, mg/d	1.32	1.43	0.00	0.33
Excreted Fecal, mg/d	1.46	1.81	0.00	0.42
Excreted Urine, mg/d	0.02	0.14	0.00	0.04
Retained, g/d	1.03	1.52	0.86	0.86
Digestibility, %	10.64	20.42	2.21	<0.01
Cu				
Intake, mg/d	2.98	3.34	0.00	0.14
Excreted Fecal, mg/d	2.99	2.91	0.00	0.67
Excreted Urine, mg/d	0.02	0.02	0.00	0.21
Retained, g/d	1.21	1.01	0.13	0.27
Digestibility, %	-0.28	8.53	1.20	<0.01

Table 5.8. Trace Mineral Intake, Excretion, Retention and Digestibility Per Day For Young New Zealand White Rabbits Fed CON and ALG Diets

Response	CON	ALG	SE	P Value
Fe				
Intake, mg/d	81.79	85.71	0.00	0.43
Excreted Fecal, mg/d	77.64	73.47	0.00	0.44
Excreted Urine, mg/d	0.77	1.62	0.00	0.01
Retained, g/d	0.76	0.84	0.09	0.50
Digestibility, %	3.79	14.60	1.67	<0.01
Mn				
Intake, mg/d	18.76	17.98	0.00	0.68
Excreted Fecal, mg/d	18.05	14.96	0.00	0.02
Excreted Urine, mg/d	0.06	0.09	0.00	0.07
Retained, g/d	0.98	0.97	0.00	0.24
Digestibility, %	11.15	16.89	1.55	0.02
Mo				
Intake, mg/d	0.29	0.27	0.00	0.14
Excreted Fecal, mg/d	ND	ND	ND	ND
Excreted Urine, mg/d	0.18	0.15	0.00	0.15
Retained, g/d	0.40	0.45	0.04	0.36
Digestibility, %	100.00	100.00	0.00	NA
Zn				
Intake, mg/d	15.24	17.98	0.00	0.01
Excreted Fecal, mg/d	15.94	21.10	0.00	0.16
Excreted Urine, mg/d	1.27	3.10	0.00	0.10
Retained, g/d	1.01	1.17	0.27	0.67
Digestibility, %	-6.76	-13.64	10.24	0.64

Table 5.9. Average Body Composition (DM basis) of young, growing New Zealand White Rabbits fed CON and ALG diets

Nutrient	CON	ALG	SE	P Value
Al, mg	11.42	5.24	0.00	0.01
B, mg	3.62	5.09	0.00	0.19
Ba, mg	5.66	5.95	0.00	0.89
Ca, g	31.72	27.30	3.92	0.44
Cr, mg	2.04	5.05	0.00	0.14
Cu, mg	13.25	18.13	0.00	0.46
Fe, mg	89.38	100.30	0.02	0.62
K, g	10.18	10.37	0.35	0.71
Mg, g	1.23	1.29	0.16	0.77
Mn, mg	1.43	1.44	0.00	0.94
Mo, mg	0.22	0.23	0.00	0.69
Na, g	3.81	3.72	0.17	0.72
P, g	19.65	17.55	1.85	0.43
S, g	10.31	10.90	0.46	0.38
Zn, mg	97.75	99.00	0.00	0.84

Table 5.10. Average Organ Weights of Young New Zealand White Rabbits Fed CON and ALG Diets

Organ	CON	ALG	SE	P Value
Blood, g	117.15	125.95	5.77	0.29
Blood, % Live BW	2.85	3.16	0.15	0.16
Digestive Tract[1],g	204.76	197.10	5.71	0.35
Digestive Tract, % Live BW	4.90	4.95	0.08	0.70
Lungs, g	13.64	12.82	0.43	0.18
Lungs, % Live BW	0.33	0.32	0.01	0.64
Heart, g	9.45	9.09	0.41	0.55
Heart, % Live BW	0.23	0.23	0.01	0.97
Kidneys, g	21.11	20.65	0.73	0.66
Kidneys, % Live BW	0.51	0.52	0.01	0.51
Liver[2], g	132.67	129.66	11.11	0.85
Liver, % Live BW	3.17	3.24	0.25	0.85
Spleen, g	1.53	1.70	0.09	0.19
Spleen, % Live BW	0.04	0.04	0.00	0.05
Brain[3], g	9.05	9.29	0.31	0.60
Brain, % Live BW	0.24	0.24	0.01	0.62

[1] Digestive Tract includes stomach, small intestine, large intestine, cecum
[2]Includes gall bladder
[3]Average brain weight is on rabbits 17,19,21,23 (C) and 10,20,22,24 (A)

Table 5.11.Organ histology of young, growing New Zealand White rabbits Fed CON and ALG Diets

ORGAN	HISTOLOGY	PRESENCE/ABSENCE	P VALUE
Spleen	Lymphoid Follicles	100%	NA
Liver	Lymphocytes	100%	NA
Kidney	Presence of Protein	50% C, 75% T	1.00

LITERATURE CITED

ALAT Training Manual. 2005. American Association for Lab Animal Sciences.

AOAC, 2005. Official Methods of Analysis. 18th ed Assoc. Off.Anal. Chem., Gaithersburg, MD.

Archibeque, S.L.,A. Ettinger, B.D. Willson. 2009. *Nannochloropsis* oculata as a Source for Animal Feed Acta. Agronomica Hungarica 57:245-248.

Dahms, I.F., P.A. Marone, E. Bailey-Hall, A.S. Ryan. 2011. Safety Evaluation of Algal Oil from Schizochytrium sp. Food and Chem. Tox. 49:70-77.

D'Mello, J.P.F., Officer, D.I.. Farm Animal Metabolism and Nutrition: Feed Enzymes. CABI Publishing, N.Y., N.Y. Pp. 405-426.

Hammond, B.G., D.A. Mayhew, M.W. Naylor, F.A. Ruecker, R.W.Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp.: I. Subchronic Rat Feeding Study. Reg. Tox. and Pharm. 33:192-204.

Hammond, B.G., D.A. Mayhew, J.F. Holson, M.D. Nemec, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp.: II. Developmental Toxicity Evaluation in Rats and Rabbits. Reg. Tox. and Pharm. 33:205-217.

Hammond, B.G., D.A. Mayhew, K. Robinson, R.W. Mast, W.J. Sander. 2001. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp. III. Single Generation Rat Reproduction study. Reg. Tox. and Pharm. 33:356-362.

Hammond, B.G., D.A. Mayhew, L.D. Kier, R.W. Mast, W.J. Sander. 2002. Safety Assessment of DHA Rich Microalgae from Schizochytrium sp. IV. Mutagentcity Studies. . Reg. Tox. and Pharm. 35:255-265.

Markovits, A., R. Conejeros, L. Lopez, M. Lutz. 1992. Evaluation of Marine Microalga *Nannochloropsis* sp. As a Potential Dietary Supplement. Chemical, Nutritional and Short Term Toxicological Evaluation in Rats. Nutrition Research. 12:1273-1284.

National Research Council. Mineral Tolerance of Animals. 2nd revised edition.

Chapter 6

Feeding *Nannochloropsis oculata* meal to adolescent New Zealand White Rabbits does 8not appear to adversely affect DE of the diet[5]

INTRODUCTION

The utilization of algae for biofuel has become more popular in recent years, as the demand for alternative fuel choices has increased Certain microalgae species produce large amounts (>30%) oil, and make viable oil sources for biofuel. The microalgae *Nannochloropsis oculata* is one of these high oil producing algae species, and is being researched extensively as an oil source. After the oil is extracted from the algae, a high protein meal remains, and its viability as a feedstuffs in being evaluated Past research in feeding the algal meal (oil extracted) to animals is scarce, as the main focus prior to 2010 has been on the potential health benefits from the PUFA in the algae. Markovits et al. (1992), Nitsan et al. (1999), Sukenik et al. (1993), Werman et al. (2003) and Villar et al. (1994) have all fed *Nannochloropsi sp.* to rats, and no adverse effects were noted When compared to common commercial livestock feeds, the algal meal from *Nannochloropsis* o., appears to be a potentially suitable protein supplement for animals and could be used in the same manner that SBM and SFC is used today (Archibeque et al.. 2009). Although animals have been fed whole *Nannochloropsis sp*, the DE of these diets have not been reported. Furthermore, there is no data reported on the consumption of diets prepared with algal meal (oil extracted). This is the first known study to gather diet, fecal and urinary data in order to calculate the DE of a diet formulated with the oil-free algal meal from

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biofuel manufacturing. Therefore, the objective of this study was to determine if feeding young rabbits a diet formulated with 10% algal meal (DM) had any effect on the DE of this diet.

MATERIALS AND METHODS

This experiment was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Algal Meal

The algal meal that was utilized in this study was supplied by SOLIX Biofuels Inc., Fort Collins, CO. A slurry was made from frozen algae, and the cells were lysed using high shear mixing. Hexane was added to the slurry, and the material was allowed to separate. The aqueous fraction was then re-extracted with hexane, and allowed to separate a second time. After this second separation, the aqueous portion was mixed with water and ethanol and evaporated at 100° C for ~24 hours. The resulting algal meal was then transferred to the Colorado State University Animal Sciences Department to be ground to a fine power using a Wiley Lab Mill model #4 and submitted for nutrient and toxic mineral analysis. The nutrient results for the algal meal are presented in Table 6.1. These results were shared with Harlan Laboratory and utilized to manufacture the Algal diet.

Animals and Treatments

A total of 24 6-8 week old New Zealand White male rabbits were obtained from Western Oregon Rabbit Company. The rabbits were received into the Colorado State University Laboratory Animal Resource center and immediately started on Harlan Teklad 2031 Global High Fiber Rabbit diet. The rabbits were held under standard laboratory conditions with a room temperature of 21°C, 37-45% humidity and a 12/12 light/dark cycle in IACUC approved solitary cage measuring 23.0” wide, 24.0” long and 15.0” high. After an eleven day acclimation period, the rabbits were randomly blocked according to body weight into two separate treatment groups,

Control (**CON**) and Algal (**ALG**), and the groups were then was stepped up onto the diets specifically formulated for this experiment. For 7 days, twelve rabbits were fed a 50/50 Control/2031 diet and twelve rabbits were fed a 50/50 Algal/2031 diet which incorporated 10% algal meal (sp *Nannochloropsis* oculata). On day 7, the rabbits were switched to 100% **CON** and 100% **ALG** diets. The CON diet and the ALG diets (Table 2) were formulated to be iso-nitrogenous, iso-caloric (Table 6.2) and to meet all of the nutritional needs for a young, growing rabbit (Nutrient Requirements of Rabbits, 2nd Revised Edition, 1977). The rabbits were weighed on d0 and then every 7 days after the study began. The CON group weighed an average of 3.51+/-0.75 kg initially (d 1 of 100% CON diet) and an average of 4.17+/-1.33 kg on d36, while the ALG group weighed an average of 3.45 +/-0.72 kg (d 1 of 100% ALG diet), and an average of 3.99+/-0.92 kg on d36. Overall, the CON group gained 0.66 kg throughout the study, an 18.80 % increase in total BW, and the ALG group gained 0.54 kg throughout the study, a 15.65% increase in BW. The ADG at the end of the study was 14 g/d (CON), and 10 g/d (ALG). Fresh water was made available at all times. Coprophagy was not prevented in order to encourage normal eating behavior.

Nutrient Balance Trial

From d 21 to 28 a balance trial was conducted and the feed intake, water intake, fecal and urinary output were measured for each rabbit every 24 hours. The rabbits were housed in their regular IACUC approved solitary cages, and there was no change to the environmental conditions. Remaining feed and water was weighed every 24 h, and recorded. Feed and water were both offered ad libitum. Urine from each rabbit was collected every 24 h into a plastic bowl containing 1mL of 6NHCl to maintain a pH of less than 3.0, and prevent volatilization of urinary N. Each day's urine was combined into a large resalable plastic bag *(P*er rabbit) and frozen at

-20° C. The feces from each rabbit was collected on a screen under the cage, captured and weighed every 24 h, stored in a plastic bag and frozen at -20° C.

Chemical Analysis

Samples of the algal meal, diet, feces and rabbit samples were analyzed for DM, crude fat and ash using AOAC 2005 methods. The lipid content of the rabbits was determined using acid hydrolysis (AHF) (AOAC 2005). Crude protein was determined using the Kjeldahl AOAC 2005 method, and calculated from total N values (N x 6.25). Soluble protein was determined using sodium borate-sodium phosphate buffer solution. NDF, ADF and lignin (Goering and Van Soest, 1970) were determined using a modified Van Soest using fiber bag technology (Ankom 200 and Daisy II Incubator, Ankom Technology Corp., Macedon, NY). Urine nitrogen was quantified using peroxysulfuric acid as described by Hach et al. (1985).

Bomb Calorimetry

Bomb calorimetry was conducted using a Parr 1231 bomb calorimeter, utilizing 2418.5915 MJ as the energy equivalent for the bomb and the sample container. The energy calculation was standardized using benzoic acid tablets (26.953 MJ/kg each), and for every ten samples a standard was run to ensure consistency. The wire used to ignite the sample was Parr No. 45C10, with a standard energy of 2.3 calories/cm, and ten cm were used per test. All diet, ort and fecal samples were run as they were in the bomb calorimeter. The urine energy was determined by initially obtaining DM values for each urine sample *(P*er animal), this value was recorded for each animal. Duplicate samples of pure cellulose (Nutricology) were then tested in the Parr bomb calorimeter for energy, which was determined to be 3.87 Mcal/kg. To prepare the samples of urine per animal, 1 g of cellulose was weighed, and 2mL of urine (as is) was added to the cellulose, these samples were then put in a 60° C oven for 24 hours, and the dried sample was then run in the Parr bomb calorimeter.

Statistical Analysis

Data for growth, diet energy, fecal and urinary energy as well as DE were analyzed using PROC MIXED procedure (SAS Institute Inc., Cary, NC) for repeated measures. The experimental unit was the individual rabbit, the fixed effect was treatment, and the random effect was date. Differences between treatments were considered statistically significant if $P \leq 0.05$ and trends if $P \leq 0.10$.

RESULTS

BW and ADG

Throughout the study the animals remained healthy. There was no significant effect from the ALG treatment on overall BW (P=0.82) or ADG (P=0.50), (Table 6.3).

Diet, Fecal and Urinary Energy Analysis

During the balance trial, orts, feces and urine were collected per animal every 24 hours, these data are presented in Table 6.4. The ALG animals consumed less feed (P=0.16), yet more water (P=0.20) than the CON animals. Subsequently, the ALG group produced more urine (P=0.03), and less feces (P=0.69). The orts from each rabbit were composited by animal to be analyzed The bomb calorimeter data (Table 6.5) on the ort samples themselves, revealed there was no difference between the CON and ALG diets (P=0.19), although the ALG diet had a slightly greater energy value. There was no difference noted in the fecal energy (P=0.16), however there was a difference noted in the amount of energy in the urine (P=0.01).

Digestible Energy (DE)

The DE was calculated from the energy in the diet consumed and the feces produced, these data are presented in Table 6.6. The actual GE was determined in the feed presented to the animals, and then the energy consumed was determined by the amount of feed eaten during the trial. These data show no difference between the two groups (P=0.21). The amount of energy

lost in the feces produced (based on the volume excreted) was also similar *(P*=0.93). These two factors were used to calculate the DE. There was no difference in DE between the two groups, although the DE of the ALG diet was decreased than the CON diet *(P*=0.19). The only difference noted was in the energy lost in the urine, *(P*=0.02). These data are reasonable, as the ALG animals consumed more water, resulting in a greater energy loss in their urine.

DISCUSSION

Digestible energy is a factor that is looked at by nutritionists as an estimate of the energy value of a feedstuffs or ration. Although it is only an estimate, it takes into account the amount of energy that is lost in the fecal material, and subtracts it from the Gross Energy (GE) of the ration consumed. In evaluating the oil-free algal meal from algaeoil production, it may be useful to compare the GE of algal meal with that of other biofuel co-products, i.e. DDGs. The GE of the algal meal used in this study was ~2.02 Mcal/kg, this is much decreased than the GE from standard DDGs, which is ~4.84 Mcal/kg (Ren et al., 2011). This is understandable, as the majority of the oil had been extracted from the algal meal, leaving a low-fat (8.9% DM basis), high protein (~30% DM basis) material. These data can be compared to an average fat content in DDGs 0f 10.76% (DM basis). *Nannochloropsis* o. (whole) has been fed to young rats with no adverse effects reported (Markovits et al. 1992), as well as pregnant and lactating rats (Mokady et al. 1995). In both of these studies the effect of the high EPA levels from the algae were evaluated. This was similar to the studies conducted by Sukenik et al. (1994) and Werman et al. (2003), where rats were fed the whole algae in order to determine the bioavailability of the EPA and the benefits on reducing the plasma and liver cholesterol levels. Nitsan et al. (1999) fed laying hens the whole *Nannochloropsis* sp. to determine if the eggs produced would be greater in EPA and DHA.

However, in all of these studies there was no mention of the effect on the DE of the diet with the algal material added. This is the first known study to take into account the differences in energy consumed and excreted with a diet that has 10% *Nannochloropsis* o. algal meal (oil extracted). Although no differences were noted in the final DE of the diets, it would be interesting to conduct further research and quantify the metabolizable energy (ME) of the diets with algal meal as well. This study supports previous work that there are no harmful effects on the health, growth or metabolism of the animal. The oil-free algal meal from *Nannnochloropsis oculata* should be considered as a potential feedstuffs for growing herbivore diets. It also suggests a need for further research into other species to determine the usefulness of the algal meal as a feed ingredient for other types of rations.

Table 6.1. Nutrient composition (DM basis) of Algal Meal used to formulate CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	CP, %	CF, %	ADF, %	NDF, %	Ash. %	ME, kcal/g	TDN, %
Algal Meal	31.30	8.90	16.10	29.60	8.65	2.01	81

Table 6.2. Nutrient composition (DM basis) of CON and ALG diets fed to young, growing New Zealand White Rabbits

Item	CP, %	CF, %	ADF, %	NDF, %	Ash. %	ME, kcal/g
CON[1]	19.90	3.60	27.40	42.90	8.62	1.30
ALG[2]	19.70	4.20	23.70	42.60	8.76	1.34

1.CON: Harlan Lab's 2031 (alfalfa meal,soybean hulls,ground oats,wheat middlings, dehulled SBM,ground corn,dical P, cane molasses,salt, vitamins, minerals) + an additional wheat middlings,SBM, & soy oil to match increased CP & CF from algal meal.
2.ALG: Harlan Lab's 2031 + 10% algal meal

Table 6.3. Effect of feeding CON and ALG diets to young, growing New Zealand White Rabbits on body weight and ADG

	P VALUE		
RESPONSE	TREATMENT	DATE	TREATMENT*DATE
Weight, kg (all days)	0.82	<0.01	<0.01
Weight, kg d7*			0.68
Weight, kg d14**			<0.01
Weight, kg d21			0.67
Weight, kg d28			0.37
Weight, kg d35			0.42
Weight, kg d42			0.15
ADG, kg (all days)	0.50	<0.01	<0.01
ADG, kg d7*			0.92
ADG, kg d14**			<0.01
ADG, kg d21			<0.01
ADG, kg d28			0.56
ADG, kg d35			0.91
ADG, kg d42			0.44

*D7-13: ALG group fed 50/50 algal diet/control diet, CON group fed 100% control diet
**D14-42: ALG group fed 100% algal diet, CON group fed 100% control diet

Table 6.4. Balance Trial Summary for young, growing New Zealand White Rabbits fed CON and ALG diets for 7 days

Response	CON	ALG	SE	P Value
Average Feed Intake, g/d	216.59	200.75	8.25	0.19
Average Water Intake, mL/d	405.44	446.70	22.11	0.20
Average Urine Produced, mL/d	144.02	188.71	13.38	0.03
Average Feces Produced, g/d	126.13	121.35	8.38	0.69

Table 6.5. Bomb Calorimeter Data on Samples of Orts, Feces and Urine collected During the 7 d Balance Trial with New Zealand White Rabbits

Response	CON	ALG	SE	P Value
Mcal/kg in Orts	4.33	4.37	0.10	0.19
Mcal/kg in Urine	0.32	0.26	0.06	0.01
Mcal/kg in Feces	4.47	4.30	0.34	0.16

Table 6.6. Bomb Calorimeter Data for Urine and Feces Produced, Diet Consumed and Digestible Energy (calculated) for Young, Growing New Zealand White Rabbits Fed CON and ALG Diets.

Response	CON	ALG	SE	P Value
Mcal/kg in Diet Consumed	7.32	6.80	1.17	0.21
Mcal/kg in Urine Produced	3.17	4.50	1.53	0.02
Mcal/kg in Feces Produced	6.06	6.13	2.16	0.93
DE, Mcal/kg	1.26	0.68	1.27	0.19

LITERATURE CITED

Abril, R., J. Garrett, S. Zeller, W. Sander, R.W. Mast. 2003. Safety Assessment of DHA-rich Microalgae from Schizochytrium sp. Part V: Target Animal Safety/Toxicity Study in Growing Swine. Reg. Toxic. Pharm.37:73-82.

ALAT Training Manual. 2005. American Association for Lab Animal Sciences.

AOAC, 2005. Official Methods of Analysis. 18th ed Assoc. Off.Anal. Chem., Gaithersburg, MD

Archibeque, S.L.,A. Ettinger, B.D. Willson. 2009. *Nannochloropsis* oculata as a Source for Animal Feed Acta. Agronomica Hungarica 57:245-248.

Boeckaert, C., N. Boon, I.Z. Abdulsudi, W. Verstraete, V. Fievez. 2006. Accumulation of Biohydrogenation Intermediates and Changes in the Rumen Protozoal Population After Micro Algae Feeding to Dairy Cattle. Comm. Appl. Biol. Sci. Ghent University. 71:83-86.

Boeckaert, C., B. Vlaeminck, J. Dijkstra, A. Issa-Zacharia, T. Van Nespen, W.Van Straalen, V. Fievez. 2008. Effect of Dietary Starch or Micro Algae Supplementation on Rumen Fermentation and Milk Fatty Acid Composition of Dairy Cows. J. Dairy Sci. 91:4714-4727.

Cornell Sodium Borate-Sodium Phosphate Buffer Procedure.1990. Incubated at ambient temperature. Cornell Nutrition Conference Proceedings. Pp. 85-86.

Franklin, S., K. Martin, R. Baer, D. Schingoethe, A. Hippen. 1999. Dietary Marine Algae (Schizochytrium sp.) Increases Concentrations of Conjugated Linoleic, Docosohexaenoic and Transvaccenic Acids In Milk of Dairy Cows. J. Nutr. 129:2048-2052.

Goering, H.K., P.J. Van Soest. 1970. Forage Fiber Analysis (Apparatus, Reagents, Procedures and Some Applications). Agric. Handbook No. 379. ARS-USDA, Washington, DC.

Hach, C.C., S.V. Brayton, A.L. Kopelove. 1985 Powerful Kjeldahl Nitrogen Method Using Peroxysulfuric Acd J. Agric. Food Chem. 33 (6) 1117-1122.

Herber-McNeill, S.M., M.E. Van Elswyki. 1998. Dietary Marine Algae Maintains Egg Consumer Acceptability While Enhancing Yolk Color. Poultry Science 77:493-496.

Hornicke, H., G. Bjonhag. 1980. Coprophage and Related Strategies for Digesta Utilization. Digestive Physiology and Metabolism in Ruminants. Pp. 707-730.

Hintz, H.F., H.F. Schryver, C.E. Stevens. 1978. Digestion and Absorption in the Hindgut of the Nonuminant Herbivores. J. An. Sci. 6:1803-1807.

Markovits, A., R. Conejeros, L. Lopez, M. Lutz. 1992. Evaluation of Marine Microalga *Nannochloropsis* sp. As A Potential Dietary Supplement Chemical, Nutritional and Short Term Toxicological Evaluation in Rats. Nutr. Res. 12:1273-1284.

Mathews, K., M. McConnell. 2009. Ethanol Co-Product Use in U.S. Cattle Feeding – Lessons and Considerations. www.ers.usda.gov/Publications/FDS/2009/04Apr/FDS09D01.

Nitsan, A., S. Mokady, A. Sukenik,. 1999. Enrichment of Poulry Products with Ω3 Fatty acids by Dietary Supplementation with the Algae *Nannochloropsis* and Mantur Oil. J. Agric. Food Chem. 47:5127-5132.

NRC for Domestic Animals, 2th Revised Edition.

Net Resources International. Hydrocarbons Technology.

Ren, P., Z. Zhu, B. Dong, J. Zang, L.Gong. 2011. Determination of energy and amino acid digestibility in growing pigs fed distillers' dried grains with solubles containing different lipid levels. Archives of Animal Nutrition. 65:303-319.

Sakaguchi, E., K. Kaizu, M. Nakamichi. 1992. Fiber Digestion and Digesta Retention from Different Physical Forms of the Feed in the Rabbit. Comp. Biochem. Physiol. 3: 559-563.

Sheehan, J., T. Dunahay, J. Benemann, P. Roessler. 1998. A Look Back at the U.S. Department of Energy's Aquatic Species Program-Bio-diesel from Algae, Closeout Report. NREL/TP-580-24190.

Slade, L.M., H.F. Hintz. 1969. Comparison of Digestion in Horses, Ponies, Rabbits and Guinea Pigs. J. Anim. Sci. 28:842-845.

Sukenik, A., H. Takahashi, S. Mokady. 1993. Dietary Lipids from Marine Unicellular Algae Enhance the Amount of Liver and Blood Fatty Acids in Rats. Ann. Nutr. Metab. 38:85-96.

Werman, M., A. Sukenik, S. Mokady. 2003. Effects of the Maine Unicellular Alga *Nannochloropsis* sp. to Reduce the Plasma and Liver Cholesterol Levels in Male Rats Fed on Diets with Cholesterol. Biosci. Biotechnol. Biochem. 67:2266-2268.

Villar, R., M.R. Laguna, I. Cadavid, J.M. Calleja. 1994. Effects of Aqueous Extracts of SixMarine Microalgae on Smooth Muscle Contraction in Rat Duodenum and Vas Deferens. Planta Med 60:521-526.

Chapter 7

Utilizing algae meal from *Nannochloropsis oculata* in livestock growth diets as a potential protein and energy source[6]

INTRODUCTION

The last 5 years have been financially challenging to the livestock industry, as commodity prices have hit historic highs. During this recession, the U.S. government renewed its interest in finding alternative energy sources. They began to look past corn ethanol, and in the direction of microalgae. One algae species in particular has drawn considerable interest due to its high oil content (>35% DM basis), *Nannochloropsis oculata*. As with corn ethanol, a valuable co-product is produced after the oil is extracted from the algae, an algal meal. This meal has a high level of crude protein (>30% DM basis) and compares well to other traditional crops as a livestock feedstuffs. Archibeque et al. (2009) reported that when compared to common commercial livestock feeds, the algal meal from *Nannochloropsis o*., appears to be a suitable protein supplement for animals and could be used in the same manner that SBM and SFC is used today. Research feeding algae to animals includes, Markovits et al. (1992), Nitsan et al. (1999), Sukenik et al. (1993), Werman et al. (2003) and Villar et al. (1994) who have fed whole *Nannochloropsis o.* to rats in order to determine the health benefits of the high EPA levels. Researchers Abrilet al. (2003), Boeckaert et al. (2006, 2008) and Franklin et al. (1999) have fed a similar alga (*Schizochytrium)* to swine, and dairy cattle to determine the benefits of the high DHA levels in production. The objective of this study was to compare the algal meal from

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Nannochloropsis o. to other corn ethanol co-products and determine the formulation feasibility of this new potential feedstuffs. Ours is the first known study to determine the price structure that algal meal needs to meet in order to be considered a practical and economic feedstuffs.

MATERIALS AND METHODS

This experiment was reviewed and approved by the Colorado State University Institutional Animal Care and Use Committee.

Algal Meal

The algal meal that was utilized in the rabbit study was supplied by SOLIX Biofuels Inc., Fort Collins, CO. A slurry was made from frozen algae, and the cells were lysed using high shear mixing. Hexane was added to the slurry, and the material was allowed to separate. The aqueous fraction was then re-extracted with hexane, and allowed to separate a second time. After this second separation, the aqueous portion was mixed with water and ethanol and evaporated at 100^0 C for ~24 hours. The resulting algal meal was then transferred to the Colorado State University Animal Sciences Department to be ground to a fine power using a Wiley Lab Mill model #4 and submitted for nutrient and toxic mineral analysis. The nutrient results for the algal meal utilized in the rabbit study are presented in Table 7.1. These results were shared with Harlan Laboratory and utilized to manufacture the Algal diet. The data collected from this algal meal was used to formulate theoretical rations using Dalex Livestock Solutions, LLC software.

Animals and Treatments

For the rat study, a total of 24 Sprague-Dawley®™ 8 week old male rats were obtained from Harlan Laboratories (Madison, WI). The rats were received into the Colorado State University Laboratory Animal Resource center and immediately started on Harlan Teklad 22/5 Rodent diet. The rats were held under standard laboratory conditions with a room temperature of 21^0C, 37-45% humidity and a 12/12 light/dark cycle in IACUC approved solitary cage measuring

10.5” wide, 19” long and 8” high. After a seven day acclimation period, the rats were randomly blocked according to body weight into two separate treatment groups, Control (**CONR**) and Algal (**ALGR**), and started on the diets specifically formulated for this experiment. The CONR diet and the ALGR diets were formulated to be iso-nitrogenous, iso-caloric (Table 7.2) and to meet all of the nutritional needs for young growing rats (NRC of Laboratory Animals, 4th Revised Edition, 1995). Twelve rats were fed the CONR diet and twelve rats were fed the ALGR diet which incorporated 10% algal meal. The CONR group weighed an average of 291+/-8 g initially and an average of 373+/-24g on d36, while the ALGR group weighed an average of 291+/-15g, and an average of 377+/-20g on d36. Overall, the CONR group gained 81.27 g throughout the study, a 27.86% increase in total BW, and the ALGR group gained 85.25 g throughout the study, a 29.18% increase in BW. The ADG at the end of the study was 2.32 g/d (CONR), and 2.44 g/d (ALGR).

For the rabbit study, a total of 24 6-8 week old New Zealand White male rabbits were obtained from Western Oregon Rabbit Company. The rabbits were received into the Colorado State University Laboratory Animal Resource center and immediately started on Harlan Teklad 2031 Global High Fiber Rabbit diet. The rabbits were held under standard laboratory conditions with a room temperature of 21^{0}C, 37-45% humidity and a 12/12 light/dark cycle in IACUC approved solitary cage measuring 23.0” wide, 24.0” long and 15.0” high. After an eleven day acclimation period, the rabbits were randomly blocked according to body weight into two separate treatment groups, Control (**CONB**) and Algal (**ALGB**), and the groups were then was stepped up onto the diets specifically formulated for this experiment. For 7 days, twelve rabbits were fed a 50/50 Control/2031 diet and twelve rabbits were fed a 50/50 Algal/2031 diet which incorporated 10% algal meal (sp *Nannochloropsis oculata*). On day 7, the rabbits were switched to 100% CONB and 100% ALGB diets. The CONB and ALGB diets were formulated to be iso-

caloric and iso-nitrogenous (Table 7.3) and to meet all the nutritional needs for young, growing rabbits (Nutrient Requirements of Rabbits, 2nd Revised Edition, 1977). The CONB group weighed an average of 3.51+/-0.75 kg initially (d 1 of 100% CONB diet) and an average of 4.17+/-1.33 kg on d36, while the ALGB group weighed an average of 3.45 +/-0.72 kg (d 1 of 100% ALGB diet), and an average of 3.99+/-0.92 kg on d36. Overall, the CONB group gained 0.66 kg throughout the study, an 18.80 % increase in total BW, and the ALGB group gained 0.54 kg throughout the study, a 15.65% increase in BW. The ADG at the end of the study was 14 g/d (CONB), and 10 g/d (ALGB).

In both studies, fresh water was made available at all times. Coprophagy was not prevented in order to encourage normal eating behavior.

Chemical Analysis

Samples of the algal meal, diet, feces samples from both studies were analyzed for DM, crude fat and ash using AOAC 2005 methods. The lipid content of the rabbits and rats was determined using acid hydrolysis (AHF) (AOAC 2005). Crude protein was determined using the Kjeldahl AOAC 2005 method, and calculated from total N values (N x 6.25). Soluble protein was determined using sodium borate-sodium phosphate buffer solution. NDF, ADF and lignin (Goering and Van Soest, 1970) were determined using a modified Van Soest using fiber bag technology (Ankom 200 and Daisy II Incubator, Ankom Technology Corp., Macedon, NY). Urine nitrogen was quantified using peroxysulfuric acid as described by Hach et al. (1985).

Mineral analysis was conducted by drying the feed and fecal samples at 75^0 C overnight, and digesting the samples at 95^0 C with nitric acid at a 1 ml nitric acid:100 mg sample ratio. After digestion 5 ppm Yttrium was used as an internal standard, and the samples were diluted with water to a final volume of 25 mL (50x dilution). One mL of the digested and diluted sample was further diluted to 1:10 with a 20% nitric acid and 5 ppm Yttrium solution (500x final dilution).

The final samples were then run on a Varian radial inductively coupled plasma (ICP) atomic emission spectrometer. The whole rat minerals were measured using the same method as for the feed and feces, however the final dilution was 50x, and 1 ppm of Yttrium was used as an internal standard and 500 ppm Cesium was used as and ionization quencher. Urine mineral content was quantified by digesting the urine in 1 mL nitric acid:1mL sample, and dried at 95 ^{0}C overnight. After digestion and drying, 1 ppm of Yttrium was used as an internal standard, and 500 ppm Cesium was used as an ionization quencher. The samples were diluted with water to a final volume of 25 mL (50x dilution) and run on a Varian radial (ICP) atomic emission spectrometer.

Bomb Calorimetry

Bomb calorimetry was conducted using a Parr 1231 bomb calorimeter, utilizing 2418.5915 MJ as the energy equivalent for the bomb and the sample container. The energy calculation was standardized using benzoic acid tablets (26.953 MJ/kg each), and for every ten samples a standard was run to ensure consistency. The wire used to ignite the sample was Parr No. 45C10, with a standard energy of 2.3 calories/cm, and ten cm were used per test. All diet, ort and fecal samples were run as they were in the bomb calorimeter. The urine energy was determined by initially obtaining DM values for each urine sample *(P*er animal), this value was recorded for each animal. Duplicate samples of pure cellulose (Nutricology) were then tested in the Parr bomb calorimeter for energy, which was determined to be 16.19 MJ/kg. To prepare the samples of urine per animal, 1 g of cellulose was weighed, and 2mL of urine (as is) was added to the cellulose, these samples were then put in a 60° C oven for 24 hours, and the dried sample was then run in the Parr bomb calorimeter.

Euthanasia

The rats and rabbits were humanely anesthetized on d 36 of the studies, via iso-flourene gas and exsanguination was via a fatal heart stick.

Formulations

Formulations were prepared on Dalex Livestock Solutions, LLC. software.

Calculations

Calculations for apparent digestibility and nutrient retention were made using the following formulas:

Apparent Digestibility = (Nutrient Intake – Nutrient in Feces)/Nutrient Intake X 100

Nutrient Retention = (Nutrient Intake (g) – Nutrient in Feces (g) – Nutrient in Urine(g))

Statistical Analysis

Data for growth, blood parameters, digestibility, intake, nutrient retention, organ weights and body composition were analyzed using PROC MIXED procedure (SAS Institute Inc., Cary, NC) for repeated measures. The experimental unit was the individual rat, the fixed effect was treatment, and the random effect was date. Differences between treatments were considered statistically significant if $P \leq 0.05$ and trends if $P \leq 0.10$.

RESULTS

BW and ADG

The rats stayed healthy throughout the 36 day study, and were weighed on d0 and every 7 days for the entirety of the study. There was no significant effect from the algal treatment on ADG ($P=0.57$), however there was a Treatment*Date effect ($P<0.01$) (Figure 7.1).

The rabbits also remained healthy for the duration of the study. They were weighed on d0 and then every 7 days after the study began. There was no significant effect from the algal treatment on overall BW ($P=0.82$) or ADG ($P=0.50$), (Table 7.4).

Nutrient Intake, Excretion, Retention and Digestibility: DM, Crude Fat, ADF, NDF and Ash

The data for the nutrient balance trial with the Sprague-Dawley©™ rats is presented in Table 7.5. There were differences noted in the intake of DM, ADF, NDF and ash ($P \leq 0.01$). The

difference in crude fat intake (*P*=0.05) indicates a possible trend towards a difference. There were also differences measured in the total excreted amounts of crude fat, ADF, NDF and ash (*P*<0.01). These data are reasonable, as the ALGR group consumed less feed during the balance trial (data not shown). The ALGR group showed less retention of DM, crude fat, and ADF (*P*≤0.01), while no difference was noted in NDF or ash retention (*P*>0.10). The apparent digestibility of DM, crude fat and ADF were different between the groups (*P*≤0.01), while no difference was seen in the apparent digestibility of NDF and ash (*P*>0.10).

Similar data for the New Zealand White Rabbits is shown in Table 7.6. However, as these were metabolically different animals, differences were noted from the rat study. In contrast to the rat study, no differences were noted in the intake of DM, crude fat, NDF or ash (*P*>0.10). There was a difference in ADF intake (*P*<0.01). The total excretion of DM and ADF showed no differences (*P*≥0.07), while the ALGB group excreted more crude fat than the CONB group (*P*<0.01), and more NDF and ash (*P*≤0.05). No differences were noted in the retention of DM, crude fat, NDF and ash (*P*>0.10), while ADF retention was decreased in the ALGB group (*P*<0.01). The apparent digestibility of DM, NDF and ash were improved in the ALGB group (*P*≤0.04), while the apparent digestibility of crude fat, and ADF were decreased (*P*≤0.03).

Nutrient Intake, Excretion, Retention and Digestibility: N and P

N and P metabolism data is presented in Tables 7.7 and 7.8. The rats indicated a difference in the intake of N and P (*P*<0.01), which is understandable as the animals consumed less feed during the trial (data not shown), the amount of each excreted in the urine was also different (*P*<0.05). N retention in the ALGR group was decreased than in the CONR group (*P*=0.02), while P retention was greater (*P*=0.92). The apparent digestibility of N was decreased in the ALGR group (*P*<0.01). In contrast to the rat data, there was no difference seen in the intake of N and between the two rabbit groups (*P*>0.10). No difference was noted in the urinary

excretion of N between the groups (*P*>0.10). N retention was greater in the ALGB group (*P*=0.03) while P retention was identical in both groups (*P*=0.99). There was no difference reported in the apparent digestibility of N (*P*>0.10), while P digestibility was improved in the ALGB group (*P*<0.01).

Ration Formulation: Ruminant and Monogastric

Compared to traditional ethanol co-products that are fed to livestock, algal meal (Table 7.9), has a nutrient profile that makes it a potential replacement for either DDGS or CDGS.

Ruminant Formulation

Theoretical formulations were developed to understand where algal meal could fit into a growth ration for beef cattle (Table 7.10). Our base formula consisted of corn silage (30% grain), corn (flaked), and DDGs, and the exercise was to determine the price that algal meal must reach in order to be utilized in the formula. We constructed the formula guidelines based on the NRC for Beef Cattle (Update 2000). We hypothesized at 589 kg steer, gaining 1.36 kg, with a minimum CP requirement of 13%, a minimum NE_g of 0.63 Mcal/lb, an NDF minimum of 15% and a maximum of 18% and a CF maximum of 5%. The rations showed that to meet these restrictions, algal meal could be the same price as DDGs ($290/MT) and it would be pulled preferentially into a least cost ration. DDGs would have to be ~$173/MT if algal meal was $209/MT in order for it to be used A third ration was then constructed to see at what price algal meal could increase to and still be used over DDGs at $209/MT. This exercise showed that up to $225/MT, algal would be used preferentially over DDGs at $209/MT. If algal meal cost $225/MT, then DDGs would need to drop to ~$204/MT to be pulled into a ration.

Monogastric Formulation

Theoretical swine formulas (Table 7.11) were also developed, to better understand the position algal meal would take in a monogastric diet. As swine diets are becoming increasingly

larger users of corn ethanol co-product (CDGS) this was an interesting exercise. We constructed the formula guidelines based on the NRC for Swine (1998). We hypothesized at 5-10 kg pig, consuming 500 g/d, with a minimum CP requirement of 23.7%, and a minimum ME of 1481 Kcal/kg. The base formulas for the swine diet contained traditional feeds such as corn grain, SBM (44%), and corn distiller's grain with solubles (CDGS). A similar approach was taken with the swine diets as with the beef cattle rations in determining where the algal meal price would need to be in order to be used preferentially over CDGS. In the scenario that algal meal is priced the same as CDGS, it will not be used. If CDGS is selling at $247/MT (2011), then algal meal would need to be $150/MT in order to be pulled into a least cost ration. However, if CDGS dropped by $2/MT, to $245/MT then algal meal would have to decrease $4/MT, or to $146/MT in order to be used preferentially over CDGS.

Value of Algal Meal as a Protein or Energy Source

When livestock producers are formulating rations for the animals in their care, the cost of the nutrients being offered to the animals is closely scrutinized. In general, protein and energy are the most costly nutrients to purchase. An analysis on the cost/kg of protein is presented in Table 12, and shows that the algal meal from *Nannochloropsis o*. could be approximately $0.66/kg, versus $0.69/kg for DDGS and $0.89/kg for CDGS. This narrow comparison is assuming algal meal at the same price as DDGS ($209/MT). Algal meal is still a more cost effective protein source ($0.72/kg) than CDGS when the price of algal meal increases to $225/MT. A similar analysis was made to determine the cost of energy from algal meal as compared to traditional grains, these data are shown in Table 7.13. If the price of algal meal fluctuates from $150/MT, to $209/MT and up to $225/MT, the price of energy (NEg) ranges from $0.11-0.16/Mcal. This compares to $0.18/Mcal for SBM (44%), $0.14/Mcal for DDGs and

$0.16/Mcal for cracked corn. Comparing ME cost/Mcal to CDGS ($0.09/Mcal), the cost of ME from algal meal ranges from $0.07/Mcal to $0.11/Mcal (using above prices).

DISCUSSION

In 2006 corn was trading at ~$3.10/bu, SBM (44%) was ~$209/MT and DDGs were ~$121/MT. By the middle of 2008, during a spectacular increase in energy pricing that skyrocketed crude oil prices to a historical high of over $100/barrel, feeds followed suit. The result was corn increasing to a high of $7.00/bu SBM reaching over $441/MT and the price for DDGs went up to ~$209/MT. These costs caused financial problems for anyone that used crude oil, or fed animals. As the markets began to stabilize in late 2008 and until recently in 2011, the prices for corn did come back down to around $3.70/bu yet DDGs remained consistent at ~$110-143/MT, however SBM never dropped to less than $330/MT. Therefore, it is necessary to note that in our ration formulation exercise, updated commodity prices were used, and if the price of SBM (44%) should decrease from its price of $330/MT, then it is possible these rations would change. It is also noteworthy that DDGs were trading at $209/MT in the Midwest during the winter of 2011-2012, and should this price decrease to more historical pricing, it is possible that algal meal would not be favored over ethanol co-products. However, taking the data as it was presented makes for an interesting case in further developing algae as a source of oil as well as algal meal. The co-product of algae oil production could be a valuable source of protein and energy for livestock diets. This of course is dependent on the price that algal meal can be made available to the agriculture market for. Comparing it to the corn ethanol co-product, DDGs, the cost of algal meal will need to at the same price or less in order to be considered as a source of protein and energy for beef cattle diets. If algal meal is compared to CDGS, the gap is wider, as the nutrient concentration in CDGS makes it a preferred source of nutrients over algal meal. The algal meal would have to be more than $100/MT less than CDGS before algal meal would be

formulated into a least cost swine ration (using 2011-2012 grain prices). As mentioned this is theoretical, as the commercial cost of algal meal (*Nannochloropsis o.*) is unknown at this time.

While ethanol co-products have been researched extensively (Stein et al. 2008, Klopfenstein et al. 2007), this is first known formulation analysis of *Nannochloropsis* o. algal meal. The comparisons drawn show that there is value in algal meal, however it will be cost dependent. The cost of protein and energy from algal meal on a per kg basis does show that it can be competitive to commonly fed feedstuffs. It is clear by the data generated in two studies (rats and rabbits) that algal meal can be included in a growth ration (up to 10% on a DM basis) and have no adverse health effects. The animals continued to grow normally and any metabolic differences can be attributed in a large part to their digestive tracts, and the challenge of digesting a highly fibrous material, like algal meal, without a rumen. Archibeque et al. (2009) compared the chemical composition (on a dry matter basis) of the algal meal from *Nannochloropsis oculata* to that of soybean meal (SBM), and steam flaked corn (SFC). The total CP content of the algal meal was 35.28%, as compared to 51.55% in the SBM and 8.86% in the SFC, making this algal meal an adequate protein supplement for animal diets, in the same manner SBM and SFC is used today. The soluble CP in the algal meal was comparable to the SBM (20.32% and 20.07% respectively). The B3 CP fraction was greater in the algal meal (63.52%), versus 1.82% in the SBM and 11.92% in the SFC. The ADF levels in the algal meal are comparable to other biofuel co-products, however without the benefit of a fermenting rumen, the bioavailability of nutrients will be compromised.

This analysis should encourage more vigorous research into the use of algal meal, in order to find ideal ration concentrations for all types of livestock. It should also provide algae researchers the encouragement to continue their work and while bringing a new fuel source, provide the agricultural community with a new, cost effective feed.

Table 7.1. Nutrient composition (DM basis) of Algal Meal used to formulate CONB and ALGB diets fed to young, growing New Zealand White Rabbits

Item	CP, %	CF, %	ADF, %	NDF, %	Ash. %	ME, kcal/g	TDN, %
Algal Meal	31.30	8.90	16.10	29.60	8.65	2.01	81

Table 7.2. Nutrient composition (DM basis) of CONR and ALGR diets fed to young, growing Sprague-Dawley™® rats

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	Ash. %	ME, kcal/g	P, ppm
CONR[1]	22.2	6.9	7.3	16.7	6.46	1.44	7915.0
ALGR[2]	21.6	7.9	6.4	18.0	6.79	1.51	7947.0

1.CONR: Harlan Lab's 2018 (ground wheat, ground corn,wheat middlings,dehulled SBM, calcium carbonate, brewers dried yeast, vitamins, minerals) + an additional 5% wheat middlings & SBM to match increased CP from algal meal.
2.ALGR: 2018 + 10% algal meal

Table 7.3. Nutrient composition (DM basis) of CONB and ALGB diets fed to young, growing New Zealand White Rabbits

Item	CP, %	Crude Fat, %	ADF, %	NDF, %	Ash. %	GE, Mcal/kg	DE, Mcal/kg
CONB[1]	19.90	3.60	27.40	42.90	8.62	4.33	1.26
ALGB[2]	19.70	4.20	23.70	42.60	8.76	4.37	0.68

1.CONB: Harlan Lab's 2031 (alfalfa meal,soybean hulls,ground oats,wheat middlings, dehulled SBM,ground corn,dical P, cane molasses,salt, vitamins, minerals) + an additional wheat middlings,SBM, & soy oil to match increased CP & CF from algal meal.
2.ALGB: Harlan Lab's 2031 + 10% algal meal

Table 7.4. Effect of feeding CONB and ALGB diets to young, growing New Zealand White Rabbits on body weight and ADG

RESPONSE	TREATMENT	P VALUE DATE	TREATMENT*DATE
Weight, kg (all days)	0.82	<0.01	<0.01
Weight, kg d7*			0.68
Weight, kg d14**			<0.01
Weight, kg d21			0.67
Weight, kg d28			0.37
Weight, kg d35			0.42
Weight, kg d42			0.15
ADG, kg (all days)	0.50	<0.01	<0.01
ADG, kg d7*			0.92
ADG, kg d14**			<0.01
ADG, kg d21			<0.01
ADG, kg d28			0.56
ADG, kg d35			0.91
ADG, kg d42			0.44

*D7-13: ALGB group fed 50/50 algal diet/control diet, CONB group fed 100% control diet
**D14-42: ALGB group fed 100% algal diet, CONB group fed 100% control diet

Table 7.5. Nutrient Intake, Excretion, Retention and Digestibility Per Day for Young Sprague-Dawley™® Rats Fed CONR and ALGR Diets

Response	CON	ALG	SE	P Value
OM				
Intake, g/d	20.28	15.63	0.75	<0.01
Excreted Feces, g/d	3.37	3.11	0.14	0.18
Retained, g/d	16.91	12.53	0.71	<0.01
Digestibility, %	83.12	79.91	0.84	0.01
DM				
Intake, g/d	21.68	16.77	0.80	<0.01
Excreted Feces, g/d	4.10	3.69	0.16	0.09
Retained, g/d	17.58	13.08	0.08	<0.01
Digestibility, %	80.83	77.73	0.95	0.03
Crude Fat				
Intake, g/d	1.50	1.32	0.06	0.05
Excreted Feces, g/d	0.17	0.23	0.01	<0.01
Retained, g/d	1.33	1.00	0.06	0.01
Digestibility, %	88.49	82.44	0.63	<0.01
ADF				
Intake, g/d	1.58	1.07	0.05	0.05
Excreted Feces, g/d	0.82	0.64	0.04	<0.01
Retained, g/d	0.76	0.43	0.06	0.01
Digestibility, %	47.45	40.36	3.16	<0.01
NDF				
Intake, g/d	3.59	3.02	0.15	0.01
Excreted Fecal, g/d	1.73	1.25	0.01	<0.01
Retained, g/d	1.86	1.77	0.14	0.67
Digestibility, %	51.60	58.28	2.63	0.09
Ash				
Intake, g/d	1.40	1.14	0.05	<0.01
Excreted Fecal, g/d	0.72	0.59	0.03	<0.01
Retained, g/d	0.68	0.55	0.05	0.09
Digestibility, %	47.64	47.91	2.48	0.94

Table 7.6. Nutrient Intake, Excretion, Retention and Digestibility Per Day for Young New Zealand White Rabbits Fed CONB and ALGB Diets

Response	CON	ALG	SE	P Value
OM				
Intake, g/d	178.12	164.48	6.77	0.17
Excreted Fecal, g/d	73.20	65.40	3.51	0.13
Retained, g/d	104.92	99.08	3.41	0.24
Digestibility, %	59.04	60.45	0.59	0.11
DM				
Intake, g/d	194.93	180.27	7.42	0.18
Excreted Fecal, g/d	81.98	72.61	3.93	0.11
Retained, g/d	112.94	107.66	3.65	0.32
Digestibility, %	58.08	59.95	0.60	0.04
Crude Fat				
Intake, g/d	7.02	7.57	0.29	0.19
Excreted Fecal, g/d	1.08	1.64	0.13	<0.01
Retained, g/d	5.94	5.93	0.18	0.97
Digestibility, %	84.93	78.74	1.00	<0.01
ADF				
Intake, g/d	53.41	42.72	1.89	<0.01
Excreted Fecal, g/d	35.22	30.58	1.70	0.07
Retained, g/d	18.19	12.14	0.91	<0.01
Digestibility, %	34.28	28.44	1.80	0.03
NDF				
Intake, g/d	83.62	76.80	3.17	0.14
Excreted Fecal, g/d	51.32	43.36	2.65	0.05
Retained, g/d	32.30	33.44	1.18	0.50
Digestibility, %	38.98	43.85	1.43	0.03
Ash				
Intake, g/d	16.80	15.79	0.65	0.28
Excreted Fecal, g/d	8.78	7.21	0.43	0.02
Retained, g/d	8.02	8.58	0.26	0.14
Digestibility, %	47.91	54.70	0.92	<0.01

Table 7.7. Nitrogen and Phosphorus Intake, Excretion, Retention and Digestibility Per Day for Young Sprague-Dawley™® Rats Fed CONR and ALGR Diets

Response	CON	ALG	SE	P Value
N				
Intake, g/d	0.77	0.58	0.03	<0.01
Excreted Fecal, g/d	0.14	0.16	0.01	0.07
Excreted Urine, g/d	0.29	0.37	0.02	0.01
Retained, g/d	-2.06	-3.41	0.02	0.02
Digestibility, %	81.62	72.94	0.71	<0.01
P				
Intake, mg/d	171.70	133.30	0.01	<0.01
Excreted Fecal, mg/d	110.20	89.25	0.01	<0.01
Excreted Urine, mg/d	18.08	31.00	0.00	<0.01
Retained, mg/d	196.80	248.10	0.36	0.92
Digestibility, %	34.86	32.44	2.89	0.56

Table 7.8. Nitrogen and Phosphorus Intake, Excretion, Retention and Digestibility Per Day for Young New Zealand White Rabbits Fed CONB and ALGB Diets

Response	CON	ALG	SE	P Value
N				
Intake, g/d	6.21	5.68	0.23	0.13
Excreted Fecal, g/d	1.98	1.84	0.11	0.40
Excreted Urine, g/d	2.43	2.11	0.19	0.25
Retained, g/d	0.03	0.17	0.42	0.03
Digestibility, %	68.35	67.99	0.74	0.74
P				
Intake, g/d	1.30	1.19	0.05	0.15
Excreted Fecal, g/d	0.92	0.67	0.05	<0.01
Excreted Urine, g/d	0.08	0.10	0.01	0.20
Retained, g/d	0.81	0.81	0.03	0.99
Digestibility, %	29.35	44.53	1.82	<0.01

Table 7.9. Nutrient Comparison of Traditional Grains and Algal Meal (DM basis) (*Nannochloropsis oculata*).

Response	Soybean Meal, 44%	Cracked Corn	Dried Distillers Grains	Corn Silage 30% Grain	Corn Distillers Grain Solubles	Algal Meal, Nanno. oc.
IFN	5-20-637	4-20-698	5-28-236	3-28-250	5-02-843	NA
DM, %	89.1	90	90.3	34.6	93.0	90.4
CP, %	49.90	9.8	30.4	8.65	27.70	31.3
NEg mcal/kg	1.40	1.55	1.5	1.08	NA	1.39
ME, kcal/kg	NA	NA	NA	NA	2820	2010
EE, %	1.60	4.06	10.7	3.09	8.40	8.90
ADF, %	10.0	3.30	21.3	26.6	16.3	16.1
NDF, %	14.9	10.8	46.0	46.0	34.6	29.6
Ash, %	7.20	1.46	4.6	3.59	NA	8.65
Ca, %	0.40	0.03	0.26	0.25	0.20	0.27
P, %	0.71	0.32	0.83	0.22	0.77	0.85
Mg, %	0.31	0.12	0.33	0.18	NA	0.34
K, %	0.22	0.44	1.08	1.14	NA	1.34
Na, %	0.04	0.01	0.3	0.01	NA	0.99
S, %	0.46	0.11	0.44	0.12	NA	0.50
Cu, ppm	22.40	2.51	10.6	4.18	NA	33
Fe, ppm	185	54.5	358	131	NA	429
Mn, ppm	35	7.89	27.6	23.5	NA	34.9
Zn, ppm	57	24.2	67.8	17.7	NA	271
Mo, ppm	0.12	0.60	1.80	0.53	NA	NA

Source: NRC Nutrient Requirements of Beef Cattle, Update 2000
NRC Nutrient Requirements of Swine, Tenth Revised Edition, 1998

Table 7.10. Theoretical Beef Cattle Formulations: 1300# steer, gaining 3#/d

Formulation	Standard Ration	Algal Meal @ Iso-Cost to DDGs	Algal Meal @ Greater Cost than DDGs
Corn Silage (30% Grain), %	10.00	10.00	10.00
Corn, Flaked, %	72.11	57.26	57.26
DDGs, %	17.89		
Tallow, %		0.09	0.09
Algal Meal (Nanno oc), %		32.65	32.65
Total Formula Cost (Ingredients) $/MT	$219.44	$214.59(1)	$219.99(2)

Cost Assumptions: Corn Silage: $72/MT, Corn, Flaked: $242/MT, DDGs: $209/MT
1. Algal meal at $209/MT
2. Algal meal at $225/MT while DDGs at $209/MT

Table 7.11. Theoretical Swine Formulations. 5-10 kg pig, consuming 500 g/d

Formulation	Standard Ration	Algal Meal
Corn Grain, %	51.70	55.11
SBM (44%), %	37.45	41.43
CDGS, %	10.85	
Algal Meal (Nanno oc), %		3.47
Total Formula Cost (Ingredients) $/MT	$300.45	$299.77(1)

Cost Assumptions: Corn Grain: $242/MT, SBM(44%): $330/MT, CDGS: $247/MT, Wheat (Hard Red Winter): $248/MT, Sorghum Grain: $251/MT
1. Algal meal at $150/MT and CDGS at $247/MT

Table 7.12. Cost of Protein - A Comparison of Traditional Feedstuffs and Algal Meal

FEEDSTUFF	$/MT	CP%	CP kg/MT	CP $/kg
SBM (44%)	$330	49.00	490.00	$0.67
DDGs	$209	30.4	304.00	$0.69
CDGS	$247	27.70	277.00	$0.89
Algal Meal @ Iso-Cost to DDGs	$209	31.30	313.00	$0.66
Algal Meal @ Greater Price than DDGs	$225	31.30	313.00	$0.72

Table 7.13. Cost of Energy: A Comparison of Traditional Feedstuffs and Algal Meal

FEEDSTUFF	$/MT	NEg Mcal/kg	ME Mcal/kg	NEg Mcal/MT	ME Mcal/MT	NEg $/Mcal	ME $/Mcal
SBM (44%)	$330	1.80		1800		$0.18	
DDGs	$209	1.50		1500		$0.14	
CDGS	$247		2.82		2820		$0.09
Corn Silage (30% Grain)	$72	1.08		1080		$0.07	
Corn, cracked	$242	1.55		1550		$0.16	
Algal Meal, *Nannochloropsis* o.	$150	1.39	2.01	1390	2010	$0.11	$0.07
Algal Meal, *Nannochloropsis* o.	$209	1.39	2.01	1390	2010	$0.15	$0.10
Algal Meal, *Nannochloropsis* o.	$225	1.39	2.01	1390	2010	$0.16	$0.11

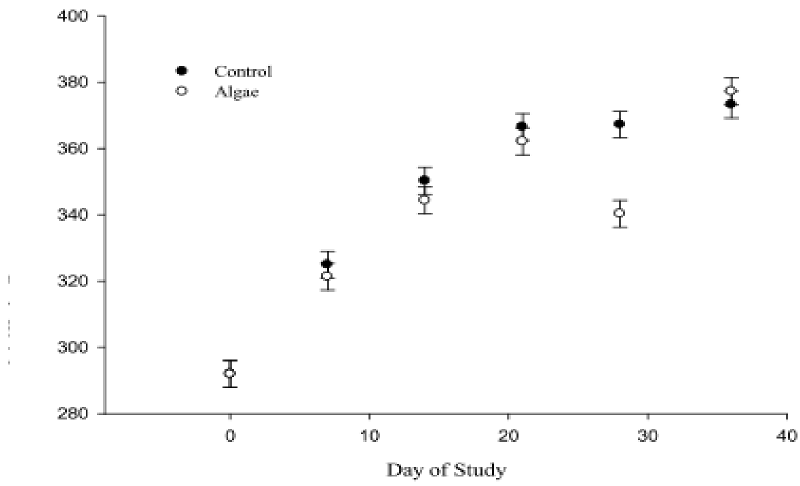

Figure 7.1. Body weight of growing Sprague-Dawley®™ rats fed CONR and ALGR diets

LITERATURE CITED

Abril, R., J. Garrett, S. Zeller, W. Sander, R.W. Mast. 2003. Safety Assessment of DHA-rich Microalgae from Schizochytrium sp. Part V: Target Animal Safety/Toxicity Study in Growing Swine. Reg. Toxic. Pharm.37:73-82.

ALAT Training Manual. 2005. American Association for Lab Animal Sciences.

AOAC, 2005. Official Methods of Analysis. 18th ed Assoc. Off.Anal. Chem., Gaithersburg, MD.

Archibeque, S.L., A. Ettinger, B.D. Willson. 2009. *Nannochloropsis oculata* as a Source for Animal Feed Acta. Agronomica Hungarica 57:245-248.

Boeckaert, C., N. Boon, I.Z. Abdulsudi, W. Verstraete, V. Fievez. 2006. Accumulation of Biohydrogenation Intermediates and Changes in the Rumen Protozoal Population After Micro Algae Feeding to Dairy Cattle. Comm. Appl. Biol. Sci. Ghent University. 71:83-86.

Boeckaert, C., B. Vlaeminck, J. Dijkstra, A. Issa-Zacharia, T. Van Nespen, W. Van Straalen, V. Fievez. 2008. Effect of Dietary Starch or Micro Algae Supplementation on Rumen Fermentation and Milk Fatty Acid Composition of Dairy Cows. J. Dairy Sci. 91:4714-4727.

Dalex Livestock Solutions, LLC. 5001 American Boulevard West, Bloomington, MN 55437.

Feedstuffs. 2012. Grain & Ingredient Cash Market Comparisons 2:84.

Franklin, S., K. Martin, R. Baer, D. Schingoethe, A. Hippen. 1999. Dietary Marine Algae (Schizochytrium sp.) Increases Concentrations of Conjugated Linoleic, Docosohexaenoic and Transvaccenic Acids In Milk of Dairy Cows. J. Nutr. 129:2048-2052.

Klopfenstein, T.J., G.E. Erickson, V.R. Bremer 2007. Board Invited Review: Use of Distillers By-Products in the Beef Cattle Feeding Industry. J. Anim. Sci. 86:1223-1231.

Markovits, A., R. Conejeros, L. Lopez, M. Lutz. 1992. Evaluation of Marine Microalga *Nannochloropsis* sp. As A Potential Dietary Supplement Chemical, Nutritional and Short Term Toxicological Evaluation in Rats. Nutr. Res. 12:1273-1284.

Nitsan, A., S. Mokady, A. Sukenik. 1999. Enrichment of Poulry Products with Ω3 Fatty acids by Dietary Supplementation with the Algae *Nannochloropsis* and Mantur Oil. J. Agric. Food Chem. 47:5127-5132.

Sukenik, A., H. Takahashi, S. Mokady. 1993. Dietary Lipids from Marine Unicellular Algae Enhance the Amount of Liver and Blood Fatty Acids in Rats. Ann. Nutr. Metab. 38:85-96.

Stein, H.H., G.C. Shurson. 2009. Board Invited Review: The Use and Application of Distillers Dried Grains with Solubles in Swine Diets. J. of Anim. Sci. 87:1292-1303.

Werman, M., A. Sukenik, S. Mokady. 2003. Effects of the Maine Unicellular Alga *Nannochloropsis* sp. to Reduce the Plasma and Liver Cholesterol Levels in Male Rats Fed on Diets with Cholesterol. Biosci. Biotechnol. Biochem. 67:2266-2268.

Villar, R., M.R. Laguna, I. Cadavid, J.M. Calleja. 1994. Effects of Aqueous Extracts of Six Marine Microalgae on Smooth Muscle Contraction in Rat Duodenum and Vas Deferens. Planta Med 60:521-526.

University of Missouri Extension. By-Product Feed Price Listing. 2011. http://agebb.missouri.edu/dairy/byprod/bplist.asp. 11/17.

USDA-Co Department of Agriculture Market News. 2012. Colorado Hay Report. 01/05.

USDA Livestock Market News. 2011. Texas Cash Grain Market. 10/04.

NRC. 1998. Nutrient Requirements of Swine. 10th Rev. Ed Natl. Acad. Press, Washington, DC.
NRC. 2000. Nutrient Requirements of Beef Cattle. 7th Rev. Ed Update. Natl. Acad Press, Washington, DC.