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Efficacy of Spirulina (Arthrospira platensis) Utilization in Broiler Diets

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Efficacy of Spirulina (Arthrospira platensis) Utilization in Broiler Diets

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by

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ABSTRACT

Spirulina (*Arthrospira platensis*) is a cyanobacteria microalgae that has potential as an animal feed ingredient. Five experiments were conducted to further assess its ability to serve as a protein source in broiler diets. Experiment 1 consisted of an amino acid digestibility and apparent metabolizable energy assay. The results indicated that the standardized ileal amino acid levels of spirulina are comparable with typical soybean meal.

Experiment 2 assessed the performance and processing parameters of broilers fed 10% spirulina in low crude protein diets, using published digestibility coefficients. Spirulina inclusion improved footpad quality and increased redness and yellowness of the breast, skin on thigh, and skinless thigh.

Experiment 3 utilized whole blood and liver samples from experiment 2. Results indicate that spirulina reduces systemic inflammation- and bacterial translocation-induced by low protein diet and could be a promising alternative protein source in poultry diets.

Experiment 4 examined the growth performance, carcass characteristics, woody breast myopathies, and breast fillet pigmentation during the grower and finisher phase. Spirulina was included up to 10% from 15 to 35 d and 5% from 34 to 49 d in standard corn/soybean meal diets. Results indicated no significant differences on any performance and carcass yields; validating digestibility values from experiment 1. Pigment deposition responded strongly to spirulina inclusion.

Experiment 5 assessed spirulina inclusion of 2.5% and dietary CP level in a 2x2 factorial. Performance parameters, water consumption, processing yields, litter quality, and litter flux measurements were recorded. Crude protein reductions inhibited performance parameters and

processing yields, while reducing water consumption and carbon dioxide flux. Spirulina inclusion reduced carcass and breast yield through decreased feed utilization.

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DEDICATION

I would like to dedicate my dissertation to my parents, Phyllis Selph, John Selph, Tonya Mullenix, and Jeff Mullenix. You all made this possible for me and I love you.

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Chapter III. Mullenix, G.J., C.J. Maynard, C.M. Owens, S.J. Rochell, W.G. Bottje, R.D. Brister, and M.T. Kidd. 2021. Spirulina platensis meal inclusion effects on broilers fed a reduced protein diet. J. of Appl. Poult. Res. https://doi.org/10.1016/j.japr.2021.100199.

Chapter IV. Mullenix, G.J., E.S. Greene, N.K. Emami, G. Tellez-Isaias, W.G. Bottje, G.F. Erf, M.T. Kidd, and S.Dridi. 2020. Spirulina Platensis inclusion reverses circulating pro-inflammatory (chemo)cytokine profiles in broilers fed low-protein diets. Front. Vet. Sci. in press. doi: 10.3389/fvets.2021.640968.

INTRODUCTION

The protein-gap classically refers to the idea that child malnutrition was the result of not receiving adequate protein quality. This concept, later claimed a fallacy, led to extensive research in alternative protein sources and an increased interest in microalgae (Semba, 2016). The term protein-gap could be suited to the difference between the anticipated increase in demand for soybean and its supply. Soybean meal (SBM) has been the gold-standard dietary protein source in poultry and swine diets since the 1940s. SBM rise to prominence was spurred by its potential as a dual-purpose crop, providing oil and protein. Its well-balanced amino acid (AA) profile, high AA digestibility, and adequate energy level complement most cereal-based diets (Ravindran, 2013). SBM increased usage has in many respects mirrored the rise in poultry production in the United States. SBM is currently the number one source of digestible amino acids (AA) in poultry and swine diets world-wide, yet classical poultry diets were formulated with multiple protein ingredients (Ruiz et al., 2020). Multiple protein rich ingredients were believed to provide broilers with an AA-balanced diet, due to their varying AA profiles. With the adoption of the ideal protein concept and precise AA feeding requirement there seems to be no benefit to the use of numerous protein ingredients in broiler formulation, outside of economic incentives (Leeson and Summers, 2001). Economic incentives can be enormous however, as diets account for approximately 70% of broiler production costs and constitutes a costly portion of broiler diets. Regionally affordable grains, oilseeds, animal meals, and ethanol byproducts are all currently used at a commercial level. Environmental concerns, costs, and consumer preferences could require nutritionists to look beyond the standard ingredients to meet future animal dietary protein needs.

I. LITERATURE REVIEW

Soybean Meal

Soybeans have been considered a staple for human consumption for centuries and it is believed to have been domesticated in China around 1700-1100 B.C (Hayward, 1970). The first recorded growth of soybeans in the United States was in Georgia in 1765 (Hymowitz, 1990). The use of soybean meal (SBM) in poultry diets wasn't utilized until the 1920-1930s following the demonstration by Osborne and Mendel (1917) that unheated SBM is inferior in nutrition quality to properly heated SBM. This predated the establishment of anti-nutritional factors in SBM, such as multiple trypsin inhibitor variants and lectins, by 30-40 years (Ruiz et al., 2020). Overprocessing of soybeans can also result in the decreased digestibility of lysine, arginine, and cysteine (Parsons, 2000). The soybean crushing industry in the United States has proven to be very efficient in oil extraction and soybean processing (figure.1), thus providing a SBM product that is consistent throughout the US (Sotak-Peper et al., 2017). World soybean production has increased over 1,200% since 1960; driven primarily by increased acreage planted and higher yields (FAO, 2021). Potentially limited yield increases, diseases, and environmental concerns over deforestation raise the question, can soybean production keep up with the increasing demand for animal feeds?

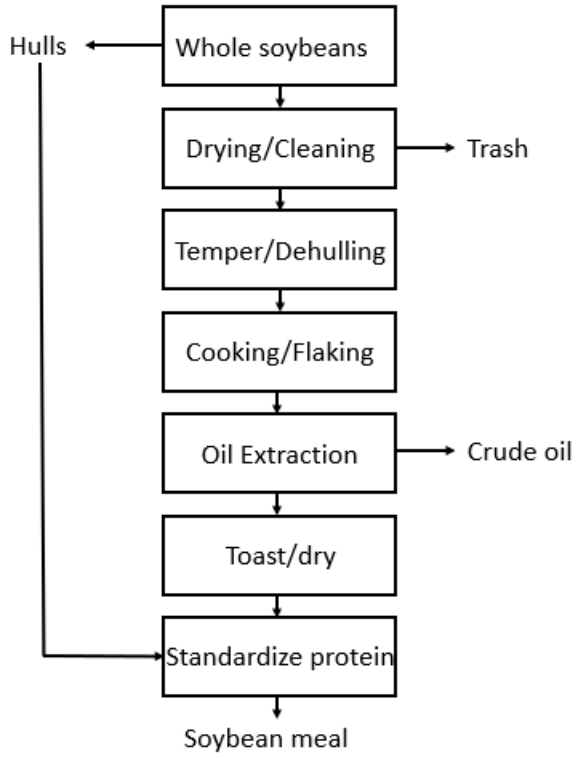

Figure 1. Flow chart of soybean oil extraction process (Adapted from FAO, 2002)

Single Cell Protein

Although this work is focused with microalgae replacement of SBM in broiler diets, a brief overview of emerging single cell proteins is needed. The term single cell protein (SCP) was coined in 1966 by Carol L. Wilson and refers to microorganisms like bacteria, microalgae, yeast, and fungi (Suman et al., 2015). The "promotion of the development of SCP for both animal feeding and direct utilization by man" was one of seven policy objectives put in place to avert the protein crisis (Semba, 2016). Many different medias and substrates have been used to produce SCP, with the goal of producing high protein food stock at an affordable price (Suman et al., 2015). Ultimately no SCP was able to fulfil the promise of a renewable cheap protein source, but interest in microalgae didn't wane because of its perceived nutritional value (Falquet, 2000).

ARTHROSPIRA PLATENSIS

Spirulina is commonly referred to as blue-green algae and grows in a variety of environments including soil, sand, marshes, brackish water, seawater, and fresh water (Cifferi, 1983). This ubiquitous organism (or collection of organisms) is actually a photosynthetic filamentous cyanobacteria, making the term blue-green algae a misnomer (Stanier and Van Neil, 1962). Cyanobacteria are considered among the oldest oxygen providing organisms on Earth and modern day *Arthrospira platensis* is morphologically similar to its ancient cousins (Koru, 2014). Spirulina (*Arthrospira platensis:* SP) is characterized as having multicellular cylindrical trichomes shaped as an open left-hand helix (Figure 2), yet the research has shown that helix geometry can be drastically impacted by physical and chemical conditions (Ali et al., 2012). *Arthrospira platensis* can often be found in naturally colonies or matts in tropical/subtropical water sources with high bicarbonate content, elevated pH, and salinity. Cohen (1997) displayed that SP has a multilayered cell wall composed mostly of peptidoglycan and lipopolysaccharides.

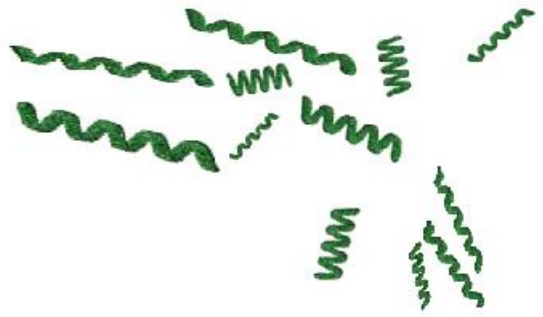

Figure 2. Microscopic view of microalgae Spirulina (Cyanobacteria), (Adapted from Koru 2012)

Taxonomy

Historically there has been much debate on the taxonomy of SP, which has led to confusion on how to best identify it. Spirulina was first isolated from a freshwater sample in 1827 by P.J. Turpin, and 14 years later Wittrock and Nordstedt named a microalgae sample

Spirulina jenneri f. platensis (Cifferi, 1983). The first taxonomic report for SP was written in 1852 by Stizenberger and was given the genus name *Arthrospira* due to its septa presence, helical form and multicellular structure (Stizenberger, 1852). Gomont (1892) grouped the microalgae by size and septa presence into two genera: *Arthrospira* and *Spirulina platensis*. Multiple research groups continued publishing conflicting articles on how to best classify SP considering size, septa presence, trichome length, transverse wall visibility, and other morphically differences (Gardner., 1917; Geitler., 1932; Welsh., 1961; Drouet., 1968). The currently accepted classification came in 1989 and determined these microalgae organisms were separated into two genera: *Spirulina* and *Arthrospira* (Tomaselli, 1997). There are 23 species of microalgae that are accepted within *Arthrospira* genus with *Arthrospira platensis* and *Arthrospira maxima* being the most prominent (Nowicka-Krawczyk et al., 2019). Most of the microalgae research conducted under the name Spirulina does in fact belong to the *Arthrospira* genus and has proven to be difficult to change. This confusion was extrapolated by the timing of the re-introduction of SP as human health food supplement in the 1970s and 1980s when the genus *Spirulina* was the common nomenclature. Whether it's due to consumer preference or researcher indifference, the term Spirulina in reference to the *Arthrospira* genus is acknowledged, accepted, and used throughout this manuscript.

General Safety

Spirulina is free of microcystins and poses no risk of toxicity when raised under normal conditions (Yang et al., 2011). Microcystins are the predominant toxins found in other cyanobacteria species that can cause necrosis of the liver (Woolbright et al., 2017). The environment that SP is raised in limits the possibility of other microorganisms entering the biomass, yet it's not impossible in open air production methods (Cifferi, 1983). Spirulina's

health-promoting properties and lack of toxicity has led to it being labeled “GRAS- General regarded as safe” and approved for human consumption in Argentina, Australia, Austria, Bahrain, Bahamas, Bangladesh, Belarus, Belgium, Brazil, Bulgaria, Canada, Chad, Chile, China, Colombia, Costa Rica, Croatia, Czech Republic, Denmark, Ecuador, Egypt, Ethiopia, Finland, France, Germany, Greece, Guam, Gulf States Haiti, Hungary, India, Iceland, Indonesia, Ireland, Israel, Italy, Jamaica, Japan, Kenya, Korea, Kuwait, Liechtenstein, Luxembourg, Macedonia, Malaysia, Mexico, Myanmar, Monaco, Netherlands, New Zealand, Nigeria, Norway, Peru, Philippines, Poland, Portugal, Romania, Russia, Saudi Arabia, Serbia, Singapore, Slovenia, South Africa, Spain, Sweden, Switzerland, Taiwan, Thailand, Togo, Turkey, Ukraine, United Kingdom, United States, Venezuela, Vietnam, Zaire, Zimbabwe (Koru, 2012).

History

The exact extent of which SP has been historically consumed by humans is largely unknown, but its certain that it was a regular food staple in the Aztec Empire (Farrar, 1966). Prior to the Spanish conquest into Mexico, SP was collected from local lakes, dried, and eaten by the local populace; referred to as Tecuitlatl. It is proposed that regional human consumption of SP stopped due to profound changes in social, political, and religious constructs brought on by the Spanish (Cifferi, 1983). The loss of environment for microalgae in leu of urban and agricultural development could also partially be to blame. Human consumption of SP was “rediscovered” in 1940 when a French phycologist described a sample obtained from a local market near Lake Chad in Africa (Dangeard, 1940). The dried hardened micro algae cake was referred to as Dihe by the local Kanembu people and used in sauces for accompanying millet meals. However, this publication received little regard until 1965 when a botanist participating in the Belgian Trans-Saharan expedition discovered a “curious substance green bluish, sold as dried

biscuits" in native markets in modern day Chad (Leonard, 1966). Leonard was the first to chemically analyze the microalgae that would come to be known as *Arthrospira platensis* and found a protein content close to 50%. Over 10,000 km away near Mexico City another species of SP was being examined by a French research group in the evaporation ponds of a sodium bicarbonate production facility. This research detailed the first growth requirements of *Spirulina maxima* that led to the development of Zarrouk's medium and the first large-scale production of SP (Sasson, 1997).

Microalgae production methods have a distinct advantage over terrestrial crops in that they're less impacted by unpredictable weather, diseases, pests, weeds, and variable soil quality (Hartman et al., 2011). The first large scale culture (Chlorella) was in Japan during the early 1960s, followed by the first large scale production of SP in the 1970s at Lake Texcoco in Mexico (FAO, 2008). Naturally occurring lakes or ponds can offset some of the costs associated with microalgae production, but are more susceptible to contamination. Production of microalgae has been commercialized and will be discussed in the subsequent section of this literature review.

COMPOSITION AND USES

Spirulina has been labeled as a superfood due to its high nutritional content of protein, amino acids, minerals, vitamins, pigments, and essential fatty acid content (Henrikson, 1994; Seyidoglu et al., 2017). Its protein level (55-70%) exceeds all other plant proteins, while still being inferior to meat and milk protein (Cifferi, 1983). Spirulina also has a favorable amino acid profile that can be included into modern broiler diets with current commercially available purified amino acid. Ross and Dominy (1990) stated that SP content is lower in total sulfur amino acids (particularly cystine) and lysine than SBM. The total lipid content is limited in SP (4-7%), and is primarily composed of palmitic acid (46%). It's also a good source of unsaturated

fatty acids, such as oleic acid, linoleic acid, and gamma-linolenic acid. Gamma-linolenic acid is limited in most feed ingredients, but can be maximized in SP under strict lighting conditions (Tanticharoen et al., 1994). Microalgae contains 13.6% carbohydrates such as glucose, mannose, galactose, and xylose (Seyidoglu et al., 2017). Salla et al. (2016) found that adding whey residue combined with harvesting at the correct time can produce SP with 58% carbohydrates. These carbohydrates are highly soluble and constitute a large portion of the total carbohydrate percentage (Seghiri., 2019).

Spirulina contains many essential minerals such as potassium, calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium, and zinc. The calcium and phosphorus levels are of particular interest as both minerals are essential for many biological functions including bone calcification, skeletal maintenance, metabolism, growth, blood clotting, enzyme activation, muscle contractions, intracellular signaling, cellular function and acid base balance (Veum, 2010). Spirulina cultures are very responsive to mineral availability; decreased NaCl levels increase lipid levels, while increased NaCl levels lower dry weight, chlorophyll, and certain xanthophylls (Ali et al., 2012). Spirulina is one of the richest sources of vitamins and bioactive pigments of any food source (Seyidoglu et al., 2017). Cyanobacteria are a rich source of chlorophyll, various carotenoids, and unique phycobiliprotein phototropic pigments. The two visible pigments responsible for SP being labeled “blue-green algae” are the green pigment provider chlorophyll and phycocyanin; a large blue-colored biliprotein that provides enormous benefits both financially and physiologically (Park et al., 2018). Phycocyanin stimulates the immune system with antioxidant, antimutative, antiviral and antitumor properties. Beta-carotene is considered a major carotenoid providing photosynthesis aid and anti-oxidant capabilities. Vitamin A is synthesized from beta-carotene, but the process can’t be reversed in poultry.

Vitamin A is necessary for the healthy development of bone, skin, and eyesight; but it's reported that corn provides adequate levels of beta-carotene at normal inclusion levels (Calislar, 2019).

Human Health

The health benefits associated with microalgae, and its value-added products, have been the principal component behind its increased utilization. Spirulina was reintroduced as a human health food substitute in the late 1970s and came with many claims of it being a cure-all for a multitude of ailments with no scientific support (Koru, 2012). It has since been proven to be beneficial in weight loss, diabetes, hypertension, viral protection, anticancer properties, cholesterol management, anemia, cardiovascular health, antioxidation, and anti-inflammation (Bishop and Zubeck, 2012). It's estimated that 70% of yearly SP production goes to the human health market. The full mechanistic extent of how SP impacts health aren't fully understood due to the dynamic nature of Spirulina's bioactive components and variability in the biomass (Seyidoglu et al., 2017). Microalgae has been used for various applications aside from human diet supplementation, such as animal nutrition, cosmetics, pharmaceuticals, CO_2 sequestration, bioenergy production, fertilizers, and waste water remediation (Kim, 2015).

Spirulina Utilization as an Animal Feed.

The notion of feeding SP to animals is not a new concept. Rich (1931) observed flamingos (*Phoenicoptera*) at numerous lakes in East Africa consuming primarily these "phytoplankton", which were later identified as cyanobacteria. *Arthrospira platensis* can serve as a replacement for numerous ingredients in broiler production, and has been included up to 10% (largely at the expense of SBM) without impacting performance parameters (Ross and Dominy., 1990; Toyomizu et al., 2001; Qureshi et al., 1996). Higher inclusion levels of 12 and 15% have been reported to be detrimental to broiler performance (Ross and Dominy., 1990; Pestana et al.,

2020). Evans et al. (2015) estimated the decrease in performance is a result of protein gelation, thus lack of enzymatic access to digesta.

Venkataraman et al. (1994) displayed that SP can be included into broiler rations at 14% and 17% when replacing fishmeal or groundnut cake, respectively. Performance parameters were maintained without a mineral and vitamin premix, which indicates SP could serve as a replacement for inorganic mineral substitution. Islam et al. (2020) used SP as a mineral replacement in broilers and concluded that SP can replace 50% of a vitamin and mineral premix on a 1:1 basis. Sugiharto et al. (2018) stated that SP could serve as a probiotic when supplemented at 1% into broilers diets at various stages of growth. The value-added aspect of SP inclusion into broiler meat or eggs could prove to be pivotal into its acceptance into poultry production. Strong pigmentation in broiler breast fillets are associated with SP levels higher than 1% (Toyomizu et al. 2001; Park et al. 2018). The consumer added value of the pigmentation is likely region specific. Spirulina inclusion can increase the polyunsaturated fatty acid profile of broiler meat and table eggs (Bonos et al., 2016; Pestana et al., 2020; Michalak et al., 2020). Marine fish species are a rich source of omega 3 fatty acids, of which they obtain from consuming microalgae or other algae-consuming fish (Lum et al., 2013). Spirulina utilization in marine species have been extensively studied and is likely the closest option to being economically feasible in animal production. Beal et al. (2018) described a method in which microalgae can be produced and used in commercial fish production that can be both economical and environmentally sustainable. Utilization of SP in marine animal diets also has a distinct advantage over terrestrial animal production because the costly step of drying the microalgae is forgone.

Production

All production methods need certain inputs like an inorganic carbon source, mixing, light source, and mixture of other nutrients (FAO, 2008). Zarrouk's medium (Zarrouk, 1966) was the first published and industry standard medium for many years. Nutrients needed for adequate biomass growth is one of the largest challenges facing small and large microalgae producers considering it accounts for 15-25% of total operation costs (FAO, 2008). Extensive research has been conducted to maximize SP growth, while minimizing nutrient supply and associative costs (Raoof et al., 2006; Madkour et al., 2012; Salla et al., 2016). Numerous carbon sources can be used to produce microalgae but carbon dioxide is the general preference for microalgae since it can be easily controlled and produces minor pH changes (Carvalho et al. 2006). Efficient utilization of the chosen carbon source is important because it makes up close to 50% of microalgae biomass and CO_2 is a major source of greenhouse gases (Becker, 1994). Dehydration of microalgae biomass can account for 20-30% of production costs (FAO, 2008). Drying in the sun was the classical way of dehydration, but it requires a large amount of space, time, and sunlight. This is not ideal for modern production methods, while techniques such as spray drying, drum drying, and freeze drying are not considered economically feasible (Liu et al., 2016).

There is not a standard production method for microalgae, but the term photobioreactor is used as an umbrella term for any mass cultivation system (Pulz, 2001). Photobioreactors are divided into open or closed air systems, with many variations. The most commonly used commercial photobioreactors are shallow raceway ponds mixed by a paddle wheel (Figure 3.), which provides benefits such as simpler construction, production, and reduced costs compared to closed systems (Liu et al., 2016). Open systems are however subject to water evaporation loss,

possible microbial pollution, uneven microalgae growth, and environmental factors. Closed systems have the benefit of producing denser biomass, due to their higher surface-to-volume ratio and increased light penetration (Carvalho et al. 2006).

Norsker et al. (2010) tested the production methods and economics of 3 different microalgae production systems operating at a small and large commercial scale. The research group concluded that tubular closed photobioreactors produce the most economically friendly biomass under Dutch climatic conditions. Zheng et al. (2013) stated that open raceway ponds are the most cost-efficient option for biomass production and over 90% of the world microalgae is produced in them. A standard production method is highly unlikely any time in the near future, so which photobioreactor is best depends on the situation and microalgae strain being utilized. There is sparce data available for yearly microalgae production, so it's difficult to have a true understanding of the industry's growth. It's estimated that 4,000 tons of SP were grown worldwide in 2004 (Shimamatsu, 2004) and 5,000 tons in 2019 (Sharma et al., 2019). These estimates must be taken with scrutiny due to the uncertainty of small-scale microalgae production. The limited production, costly mediums, and demand for SP byproducts equate to a product that is still well above consideration for use in most broiler diets. Chaiklahan et al. (2018) recently found peak financial return from SP production was when it was processed into phycocyanin. Current estimates and author correspondence of SP price is valued between $5,000 and $12,000/ton depending on biomass quality. It has been reported that microalgae residue (by-product of lipid extraction) can be utilized in animal diets when prices reach $1,000/ton (Borowitzka, 2013). If microalgae are expected to be used in place of oilseed, meat meals, or regional specific ingredients, then additional research is needed if SP is to be considered more

than a protein, energy, and amino acid source; i.e., as a probiotic, health promotor, or value-added product in poultry production.

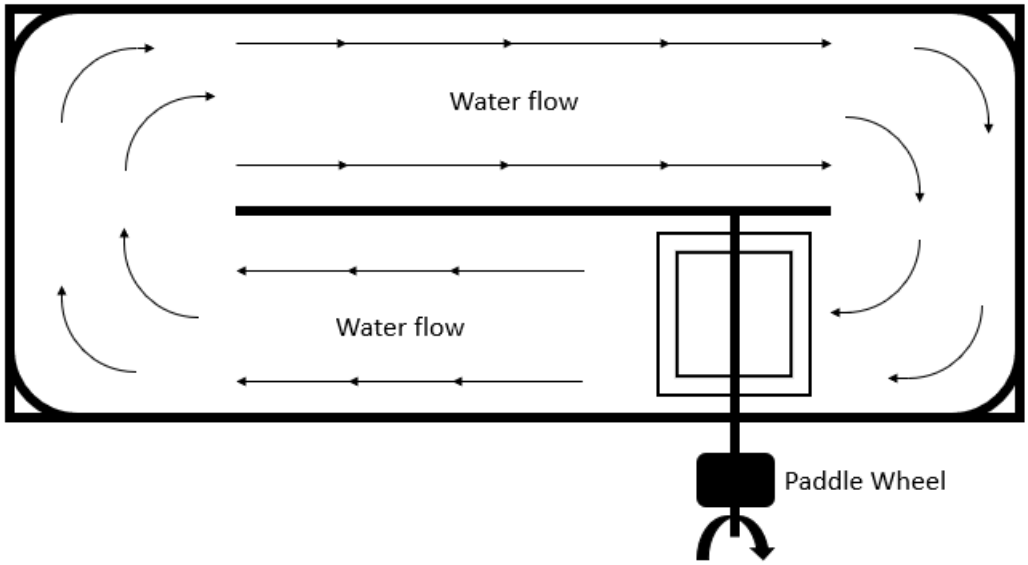

Figure 3. Open race-way pond design for Spirulina (Adapted from Shimamatsu, 2004)

FUTURE CONSIDERATIONS

The price difference between currently utilized protein sources and SP is too large to rationalize its use in animal diets (outside of specialty markets). Implementing microalgae into commercial poultry diets on a consistent basis will require advancements in the production, processing, and SP strains themselves. The growing interest for microalgae in CO_2 capturing, water mediation, and the biogas industry might be key to increasing production and driving down costs. Research is being conducted to maximize methodologies that would allow microalgae to serve multiple purposes including; water mediation and feed stock, CO_2 sequestration and biofuel, and biodiesel and biomethane (FAO, 2008). How to best process, store, and use recycled CO_2 is still being determined. Costs of microalgae production can be offset by using industrial and agricultural wastes, which could come with a higher risk of

contamination. Microalgae derived biodiesel is biodegradable, renewable, nontoxic and free of nitric oxides and sulfur oxides post combustion (Liu et al., 2016).

All modern commercial agricultural products have undergone genetic selection to bring out their full potential, but SP has proven to be difficult to manipulate (Kawata et al., 2004). While mutations of *Arthrospira platensis* have been found, it's believed that its full genetic potential has yet to be determined (Fujisawa et al., 2010). There are obvious opportunities for the increased usage of microalgae in animal diets, but many technological hurdles must be met. The modern commercial production of *Arthrospira platensis* (and all microalgae) is still very much in its infancy and extensive research could lead to a product that is economically and environmentally competitive.

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II. APPARENT METABOLIZABLE ENERGY AND DIGESTIBILE AMINO ACID VALUES OF SPIRULINA (ARTHROSPIRA PLATENSIS) MEAL FED TO 21D BROILERS

ABSTRACT

The use of Spirulina (*Arthrospira platensis*) meal as a novel ingredient in poultry feed is growing in interest. An anticipated growth of Spirulina utilization requires additional information on its nutrient utilization as broiler strains and Spirulina production methodology progress. Four batches of spirulina were analyzed for proximate analysis and AA content. This study consisted of two experiments that determined AME, AME_n, apparent (AID_{Coef}) and standardized (SID_{Coef}) ileal amino acid digestibility coefficients, ileal amino acid digestibility coefficient, and apparent and standardized ileal amino acid concentrations, and ileal amino acid level (SID) from one source of Spirulina. The average proximate analysis was 92.27% DM, 65.43% CP, 0.83% crude fat, and 1.20% crude fiber. The average AME_n was 2,820 (2,774-2,854) kcal/kg for 10, 20, and 30% Spirulina inclusion and 3,142 kcal/kg when regression analysis ($Y = 4.02X + 2,740$, $R^2 = 0.90$) was conducted and extrapolated to 100% inclusion. The average SID_{Coef} for Spirulina was 84% and digestible concentrations were; Met (1.36), Lys (2.55), Thr (2.70), Val (3.03), Ile (2.95), and Arg (3.52). These results indicate that Spirulina (*Arthrospira platensis*) meal is a high-quality protein substitute for use in broiler formulation.

INTRODUCTION

The poultry industry has relied heavily on soybean meal as a dietary protein source due to its high AA digestibility and nutrient profile. As populations and per capita income increases around the world there is an anticipated increase in poultry protein consumption (FAO, 2021). To meet the dietary protein needs of broilers, multiple sources will need to be utilized and microalgae, specifically Spirulina, is considered a promising alternative to soybean meal (Parisi et al., 2020).

Spirulina (*Arthrospira platensis*) is a blue-green cyanobacteria microalgae that has a high protein content (55-70%) with a balanced amino acid profile and rich source of vitamins, essential fatty acids, minerals, and phytochemicals (Liestianty et al., 2019). Spirulina is the term often used to describe the whole microalgae biomass as it's more than a sole protein source. The anti-oxidant and anti-inflammatory capabilities of Spirulina in poultry have also been displayed (Qureshi et al., 1996; Park et al., 2018; Mullenix et al., 2021a). Spirulina poses no risk of toxicity when raised in proper conditions and is free of microcystins; the predominant toxin in natural algal blooms (Yang et al., 2011).

Numerous experiments have been conducted to determine the performance results following Spirulina (*Arthrospira platensis*) inclusion into broiler diets with varying results (Ross and Dominy, 1990; Pestana et al., 2020; Mullenix et al., 2021b), yet less attention has been paid to its metabolizable energy level and digestible AA content. Metabolizable energy can be calculated by numerous procedures (each with their own benefits and short comings), yet AME_n is generally accepted for formulation (Lopez and Leeson, 2008). A more robust set of data is needed if Spirulina is to be utilized commercially within the ideal protein concept. The ideal protein concept was first described by Mitchell (1964) and is widely accepted by modern nutritionist to formulate to the AA needs of chickens. The high protein level of Spirulina (~60%) is a major incentive for its usage in animal diets, yet CP alone is insufficient in considering the quality of protein. Crude protein is calculated from total nitrogen, according to Kjeldahl method and Spirulina is comprised of 11.5% non-protein nitrogen including nucleic acids, amines, glucosamides, and cell wall material (Becker, 2007).

These factors emphasize the need for further AA analysis and determination of digestibility coefficients in Spirulina for its use in broiler formulation. The objective of this study

was to determine AME, AME_n, apparent (AID_{Coef}) and standardized (SID_{Coef}) ileal amino acid digestibility coefficients, ileal amino acid digestibility coefficient, and apparent and standardized ileal amino acid concentrations, and ileal amino acid level (SID) from one source of Spirulina (*Arthrospira Platensis*).

MATERIALS AND METHODS

All management practices and procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC) Protocol # 20031.

Bird husbandry and housing

Two experiments were conducted concurrently with Cobb 500 male by-products broilers raised to 20 and 21d, respectively. Three hundred and eighty-four, 1-day-old, chicks were obtained from a commercial hatchery (Cobb-Vantress hatchery, Fayetteville, AR) and reared in 48 metabolic cages (6 reps/treatment) within an environmentally controlled room. The metabolic cage dimensions were 61 cm x 61 cm, which allotted each bird 0.05 m^2 at day 14 when chick numbers were adjusted to 7 chicks per cage. Each cage was equipped with one trough drinker and one trough feeder. Supplemental paper feed trays and round jug waters were offered for the first 7 days to ensure access. Ambient room temperature was set at 32ºC at day 1 and gradually decreased to 27ºC at day 21.

Experimental design

All birds were fed a two-phase feeding program *ad libitum*, with a common starter mash diet from 1 to 14 d containing 21% crude protein and 3,248 kcal/kg. Ingredient composition of all experimental diets are shown in Table 1. All diets were mixed in a V-mixer at the University of Arkansas feed mill. Spirulina powder meal was sourced from a commercial manufacturer (Pond Tech., Markham, Canada), produced in alkaline fresh water, and underwent proximate

analysis for DM, CP, gross energy (GE), nitrogen (N), crude fat, crude fiber, and ash. Amino acid concentrations of the Spirulina were analyzed by ATC Scientific (Little Rock, AR), and NOVUS (St. Charles, MO) according to the Association of Official Analytical Chemists official methods (AOAC, 2005). All birds were weighed at the start of the experimental period (day 14) and at the conclusion of each respective trial to calculate final body weight (BW), body weight gain (BWG), and mortality corrected feed conversion ratio (FCR).

Experiment 1

The AME_n trial consisted of four dietary treatments with one basal diet and Spirulina replacing 10, 20, and 30% of all energy-providing ingredients. AME_n methodology was adapted from Lopez and Leeson (2008), which followed the classical total excreta collection assay. The Spirulina caloric level (at each inclusion level) was regressed against the level of Spirulina inclusion to generate a linear regression equation, which corresponds to the ME_n (kcal/kg) of Spirulina. Experimental diets were introduced on day 14 and allowed 4 days of adaption before 3 days of total excreta collection from 18-21d. Feed intake was monitored closely the last 3 days and excreta was collected/weighed daily each morning. All excreta were free from contamination (feed and feathers) and stored at -5ºC until post 3rd day collection by cage. Pooled excreta from each cage were homogenized and a 120 g sample was taken for analysis. All samples were stored at -20ºC until being lyophilized. Excreta was then ground with a coffee grinder and sent to the Central Laboratory at the University of Arkansas for DM, GE, and N analysis (Table. 2). AME was calculated for the basal and Spirulina diets by subtracting GE excreted from GE intake and dividing that value by feed intake. Gross energy was determined with a bomb calorimeter (Parr 6200 bomb calorimeter, Parr Instruments Co., Moline, IL.). To calculate AME_n nitrogen retention

was determined as the difference between N intake and N excreted, then multiplied by 8.22 kcal/g (Hill and Anderson, 1958).

$$AMEn=[(GE_{diet}\ kcal/kg\times FI\ kg)-((GE_{excreta}\ kcal/kg\times excreta\ output\ kg)+(N_{diet}\ g/g\times FI\ g-N_{EXRETA}\ g/g\times excreta\ g))]/(FI\ kg)$$

The following equation was used to calculate AME and AME_n for the ingredient Spirulina:

=AMEn Basal diet+[((AMEn test diet±AMEn Basal))/(% algae inclusion)]

Experiment 2

The AA digestibility assay determined AID and SID of Spirulina meal via the index method with two experimental treatments (8 reps/treatment). Spirulina was the sole protein source in one diet and a nitrogen free diet was used to calculate endogenous losses. An indigestible biomarker, titanium dioxide (TiO_2), was included at 0.5% and samples analyzed according to Short et al (1996). The experimental diets were offered from 14-20d and 16-20d for the Spirulina and NFD, respectively. On day 19 post-hatch, feeders were removed for 8 hours before allowing birds to eat 2 hours on day 20. All birds were humanely euthanized on day 20 by CO_2 inhalation, and the digesta content of the lower half of each ileum was pooled together by cage. The lower half of the ileum is defined as the terminal end of the smaller intestine between the Meckels diverticulum and ileo-cecal junction. The digesta samples were immediately frozen with liquid nitrogen and stored at -4ºC until being lyophilized. Excreta was then ground with an electric coffee grinder and analyzed (Table. 2) for DM, N, TiO_2 and AA concentration. Endogenous losses from the NFD were calculated my multiplying each AA concentration in the digesta by a ratio of TiO_2 in the diet to TiO_2 in the digesta. The following equations were used to calculate AID and SID; where (AA/Ti)diet = ratio of AA to titanium in the diet, (AA/Ti)ileal = ratio of AA to titanium in the ileal digesta, NFD EAA = endogenous amino acid flow, and Ingr. AA = ingredient amino acid.

$$\text{AID (\%)} = \frac{\left(\frac{AA}{Ti}\right)diet - \left(\frac{AA}{Ti}\right)ileal}{\left(\frac{AA}{Ti}\right)diet} \times 100$$

$$\text{SID(\%)} = \text{AID(\%)} + \frac{\text{NFD EAA}\left(\frac{\text{g}}{\text{kg}}\text{of DM}\right)}{\text{Ingr. AA}\left(\frac{\text{g}}{\text{kg}}\text{of DM}\right)} \times 100$$

Statistical Analysis

Data were analyzed using JMP Pro15 statistical software (version 15; SAS Institute, 2018) as a randomized, complete block design, with pen as the experimental unit. Nutrient analysis was shown by mean, SD, and CV (%). One-way ANOVA was used with $P<0.05$ indicating the probability of significant differences when appropriate. Significant differences among treatments were separated using a repeated t test. Linear regression for AME and AME_n were generated using fit model procedure of JMP.

RESULTS AND DISCUSSION

Considering corn and soybean meal are the primary two ingredients in broiler diets, due to their respective energy and protein level, any possible new ingredient should be of comparable chemical composition. The high protein level (~60% CP), gross energy level (5,144 kcal/kg), and balanced amino acid profile (Table. 3) emphasize the potential of Spirulina *Arthrospira platensis* as a feed ingredient in broiler production. Spirulina biomass as a partial soybean replacement requires further analysis as bird genetics and algae strains progress.

Metabolizable energy levels for Spirulina were calculated from multiple methodologies that produced varying results. In both calculations N correction had minimal impact on ME level with AME_n being 97% of AME. Wolynetz and Sibbald (1984) stated that birds fed *ad libitum* will have positive N retention, thus AME exceeds AME_n value. The mean AME and AME_n content was determined to be 2,913 (2,866-2,947) and 2,820 (2,774-2,854) kcal/kg for 10, 20,

and 30% Spirulina, respectively (Table. 4). These calculated values are in alignment with previous literature that stated Spirulina ME values for broiler were between 2,864 - 3,290 AME and 2,494 - 2,502 AME_n (Alvarenga et al., 2011; Blum et al., 1976; Tavernari et al., 2018).

Using regression analysis, ME values corresponding to varying Spirulina inclusion levels are $Y = 4.04X + 2,833$ ($R^2 = 0.90$) for AME and $Y = 4.02X + 2,740$ ($R^2 = 0.90$) for AME_n (Table. 4). Spirulina levels are extrapolated to the equivalency of 100% inclusion to 3,237 and 3,142 for AME and AME_n, respectively. Lopez and Leeson (2008) stated that ME values obtained from regression analysis (extrapolation to 100%) are a more reliable estimator than mean comparison, but should be interpreted with caution when lower levels of ingredient inclusion are used since larger extrapolation is needed.

Eighteen amino acid concentrations were determined in 4 batches of Spirulina on an as-is basis (Table. 3). Variability of AA concentrations between batches were minimal with the exception of alanine (CV=14.6), proline (CV=33.6), and glutamic acid (CV=50.3). The AA content of the algae used for the digestibility assay was the least of the 4 batches with the exception of proline and glutamic acid. Apparent and standardized ileal amino acid digestibility coefficients from experiment 2 are shown in Table 5. The average AID_{Coef} digestibility was 70.5%, with Met (76.9%) and Arg (75.9%) being the highest digested and Tyr (59%) and Cys (60%) being the lowest. To the authors knowledge, Tavernari et al. (2018) is the only other publication reporting Spirulina platensis AA digestibility coefficients for broilers, which is consistent with data from the current trial when similar AA are compared (71.1 vs 72.6%). There was a larger discrepancy when accounting for endogenous losses; comparing similar AA SID_{Coef} were 85% and 79.4% for the current trial and Tavernari et al. (2018), respectfully. The differences are likely a result of the aforementioned trial using previously determined ileal

endogenous losses from Brito et al (2009), which utilized Ross 308 broilers and a nitrogen free diet plus AA. Endogenous losses can vary between analysis methodology, choice of markers, age of birds, feeding regimes, laboratory and genetics (Parsons, 2020). The SID_{Coef} from Spirulina Platensis are less than those reported for soybean meal, yet exceeds canola meal and are comparable with other plant protein sources (Kim et al., 2012; Lemme et al., 2004).

The high AA concentration and digestibility coefficients equate to standardized amino acid contents (Table. 5) that are generally higher than 48% CP soybean meal (Rostagno et al., 2017). The average calculated SID values from all 4 batches that exceed soybean meal are Met (1.36), Thr (2.70), Val (3.03), Ile (2.95), Arg (3.52), Leu (4.58), Phe (2.41), Gly (2.55), Ser (2.66), Pro (2.16), Ala (3.79), Glu (5.73), Asp (5.14), and Tyr (1.87). Distinct differences from the current data and Tavernari et al. (2018) SID values emphasize the importance of further analysis of Spirulina Platensis for proper diet formulation.

CONCLUSIONS

The average AME_n for 10, 20, and 30% Spirulina diet was 2,820 (2,774-2,854) kcal/kg. Spirulina AME_n levels extrapolated from regression analysis to 100% inclusion was 3,142 kcal/kg. The average SID_{Coef} for Spirulina was 84% and calculated average SID values were; Met (1.36), Lys (2.55), Thr (2.70), Val (3.03), Ile (2.95), and Arg (3.52), Leu (4.58), Phe (2.41), Gly (2.55), Ser (2.66), Pro (2.16), Ala (3.79), Glu (5.73), Asp (5.14), and Tyr (1.87).

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TABLES

Table 1. Ingredient composition of experimental diets

	AME_n Trial[1]				AA dig. Trial	
Item	Basal, 0%	10%	20%	30%	Spirulina	NFD
Ingredients, g/kg						
Corn	582.12	521.72	461.31	400.91	–	–
Soybean meal	338.62	303.48	268.34	233.21	–	–
Soybean oil	37.96	34.02	30.08	26.14	35.00	35.00
Spirulina	–	100.00	200.00	300.00	300.00	–
Corn-Starch	–	–	–	–	–	–
Dextrose	–	–	–	–	567.70	843.20
Solkaflock	–	–	–	–	50.00	50.00
Limestone	9.02	9.02	9.02	9.02	9.02	7.02
Dicalcium phosphate	15.08	15.08	15.08	15.08	16.08	28.58
Titanium dioxide	5.00	5.00	5.00	5.00	5.00	5.00
Premix[12]	2.50	2.50	2.50	2.50	2.50	2.50
Sodium chloride	3.53	3.53	3.53	3.53	3.53	6.53
Potassium Sulfate	–	–	–	–	10.00	21.00
Choline Chloride	0.98	0.98	0.98	0.98	0.98	0.98
Santoquin	0.19	0.19	0.19	0.19	0.19	0.19
L-lysine HCl	1.40	1.25	1.11	0.96	–	–
DL-methionine	3.10	2.78	2.46	2.13	–	–
L-threonine	0.50	0.45	0.40	0.35	–	–
Calculated composition						
AMEn, kcal/kg	3137	3060	2984	2908	3122	3377
CP, %	20.40	24.34	28.28	32.23	18.18	–
dLys, %	1.188	1.288	1.389	1.489	0.671	–
dTSAA, %	0.899	0.972	1.045	1.118	0.499	–
dThr, %	0.764	0.930	1.096	1.262	0.736	–

[1]Spirulina replaced 10, 20, and 30% of all energy-providing ingredients evenly.
[2]Added per kg of finished feed: Vitamin A, 30,864 IU; vitamin D3, 22,046 IU; vitamin E, 220 IU; vitamin B12, 0.05 mg; menadione, 6.0 mg; riboflavin, 26.5 mg; D-pantothenie acid, 39.7; thiamine, 6.2 mg; niacin, 154.3; pyridoxine, 11.0 mg; folic acid, 3.5 mg; biotin, 0.33 mg; zinc, 100; iron, 15; manganese, 100; copper, 15; iodide, 1.20; selenium, 0.25; calcium, 69.

Table 2. Proximate analysis and amino acid Content of Spirulina Platensis

	Spirulina Source						
Proximate, %	1[1]	2	3	4	Avg.	SD	CV, %
CP	60.59	68.12	68.94	64.06	65.43	3.35	5.12
DM	91.61	94.00	93.38	90.09	92.27	1.53	1.66
Fat	0.61	0.42	0.42	1.88	0.83	0.61	73.24
Fiber	1.90	2.10	0.20	0.60	1.20	0.82	67.96
Ash	10.00	6.20	6.50	5.68	7.10	1.70	24.00
Amino Acid							
Met	1.49	1.60	1.59	1.60	1.57	0.05	3.0
Lys	2.75	3.11	3.21	2.77	2.96	0.20	6.9
Thr	2.94	3.32	3.35	3.08	3.17	0.17	5.4
Val	3.42	3.83	3.68	3.66	3.65	0.15	4.0
Ile	3.26	3.66	3.66	3.51	3.52	0.16	4.6
Arg	3.80	4.24	4.38	3.87	4.07	0.24	6.0
Leu	5.07	5.63	5.78	5.44	5.48	0.27	4.8
His	0.93	1.05	1.05	0.94	0.99	0.06	5.8
Phe	2.64	2.93	3.07	2.84	2.87	0.16	5.4
Trp	0.61	0.73	0.67	0.64	0.66	0.04	6.7
Cys	0.56	0.59	0.59	0.61	0.59	0.02	3.0
Gly	2.85	3.22	3.16	2.91	3.04	0.16	5.2
Ser	2.96	3.32	3.39	3.04	3.18	0.18	5.7
Pro	2.06	2.04	3.86	1.82	2.45	0.82	33.6
Ala	3.44	5.14	4.95	4.65	4.55	0.66	14.6
Glu	8.36	0.87	8.78	8.77	6.70	3.37	50.3
Asp	5.57	6.28	6.25	5.94	6.01	0.29	4.8
Tyr	2.13	2.37	2.52	2.61	2.41	0.18	7.5

[1]Spirulina utilized in metabolizable energy and amino acid digestibility trials

Table 3. Analysis methodology to determine chemical composition for Spirulina and/or digesta

Analysis	Method of analysis
Moisture	AOAC[1] official method 934.01, vacuum oven
Crude Protein	AOAC official method 984.13, Kjeldahl
Crude fiber	AOAC official method 978.10
Ash	AOAC official method 942.05
Total amino acids	AOAC official method 982.30, and AOAC 985.28, by HPLC
Gross energy	Bomb Calorimeter (Parr 6200, Parr Instruments, Co., Moline, IL)

[1]AOAC International (Official Method of analysis)

Table 4. AME and AME_n Regression equations of *Spirulina*-associated ME_n (kcal) to corresponding *Spirulina* inclusion (%) and mean determined using total collection method

Item	Intercept, kcal	Slope, %	R^2	At 100% inclusion	Mean, kcal
AME	2,833	4.04	0.90	3,237	2,913
SEM	6.28	0.29			2.23
P-value	<.0001	<.0001			<.0001
AMEn	2,740	4.02	0.90	3,142	2,820
SEM	6.20	0.29			2.31
P-value	<.0001	<.0001			<.0001

Table 5. Apparent and Standardized Ileal digestibility coefficients and amino acid digestibility values of Spirulina platensis

	Coefficients		Digestible concentrations			
Amino Acid	AID_{Coef}[1]	SID_{Coef}[1]	AID_{Trial}[1]	SID_{Trial}[1]	AID_{Avg}[2]	SID_{Avg}[2]
Met	76.9	86.8	1.15	1.29	1.21	1.36
Lys	70.4	86.1	1.94	2.37	2.08	2.55
Thr	71.3	85.2	2.10	2.50	2.26	2.70
Val	72.0	83.2	2.46	2.85	2.63	3.03
Ile	74.2	83.9	2.42	2.73	2.61	2.95
Arg	75.9	86.4	2.88	3.28	3.09	3.52
Leu	73.1	83.6	3.70	4.24	4.00	4.58
His	67.5	85.0	0.63	0.79	0.67	0.84
Phe	72.6	83.8	1.92	2.21	2.08	2.41
Trp	62.0	77.0	0.38	0.47	0.41	0.51
Cys	60.0	86.5	0.34	0.48	0.35	0.51
Gly	71.9	84.2	2.05	2.40	2.18	2.55
Ser	68.5	83.6	2.03	2.47	2.18	2.66
Pro	71.7	88.2	1.48	1.82	1.75	2.16
Ala	75.0	83.3	2.58	2.87	3.41	3.79
Glu	74.2	85.6	6.20	7.15	4.97	5.73
Asp	73.7	85.6	4.11	4.77	4.43	5.14
Tyr	59.0	77.5	1.26	0.47	1.42	1.87

[1]Data analyzed from batch 1 spirulina

[2]Values calculated from average AA content from 4 batches of spirulina and digestibility coefficients rom batch 1

III. SPIRULINA PLATENIS MEAL INCLUSION EFFECTS ON BROILERS FED A REDUCED PROTEIN

ABSTRACT

Apart from soybean meal, alternative sources of protein are a long-term concern facing commercial broiler producers. These alternative protein sources must be rich in protein, have a balanced amino acid profile, be highly digestible, be nutritionally safe for the bird, and ideally contain some other intrinsic value. *Spirulina platensis* is a microalgae that is growing in popularity due to its high protein level, health benefits, and environmental impact. Two experiments (in female and male Ross 708 broilers) were conducted to determine the effect of *Spirulina* inclusion in reduced crude protein diets on broiler growth, carcass yields, breast fillet color, breast myopathy, and footpad quality. The results showed that lowering crude protein impaired carcass yield in both experiments, while hindering growth performance more in male birds than female birds. *Spirulina* inclusion at 10% in a reduced protein diet improved footpad score in male broilers and increased meat and skin pigmentation in all birds. The inclusion of *Spirulina* into a commercial setting will always be driven by costs, but this study aids in expanding fundamental research to make that possible.

INTRODUCTION

With the global population anticipated to reach 9.7 billion (U. N., 2019) and poultry meat production expected to reach nearly 200 million tons by 2050 (Alexandratos and Bruinsma, 2012), there are obvious questions on how nutritionists will meet the protein needs of these broiler chickens. Soybean production would need to double by 2050 to meet monogastric animal feed production requirements, which are anticipated to fall short with the current crop yield gains of 0.9 to 1.6% per year (Ray et al., 2013). Multiple methods will need to be implemented to fill this “protein-gap” including lowering crude protein, increasing amino acid digestibility, and the use of alternative protein sources (Rutherfurd et al., 2002; Bryan et al. 2019; Pestana et al., 2020;

Selle et al., 2020). Algae has long been considered a potential source of protein for chickens (Grau and Klein, 1957) and multiple strains have been investigated to better understand their viability (Becker., 2007). Fewer than 10 of the 30,000 microalgae species identified are produced commercially (Gouveia et al., 2008). *Spirulina (Arthrospira) platensis*, a blue-green cyanobacteria microalgae, is particularly appealing as an alternative protein source considering its high protein content (55-70%) and amino acid profile, mimicking soybean meal (Table 1). *Spirulina* is also a rich source of other nutrients (i.e., vitamins, essential fatty acids, minerals, and pigments; Liestianty et al., 2019). These nutrients provide anti-oxidant and anti-inflammatory properties (Qureshi et al., 1996; Park et al., 2018; Ragab M. and Fathi M., 2018), while being free of the major algae toxins, microcystins, and pose no risks of toxicity (Yang et al., 2011; Al-Dhabi., 2013). In a companion study, Mullenix et al. (2021) reported that an inclusion of 10% *Spirulina* in low protein diets reduced inflammatory cytokines in circulation as well as bacterial translocation into the liver. The objective of this study was to determine the effect of *Spirulina* inclusion (replacing 50% of soybean meal) in reduced CP diets on broiler growth, carcass yields, pigment deposition, breast myopathy, and footpad quality.

MATERIALS AND METHODS

All management practices and procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC) Protocol # 21002.

Bird husbandry and housing

Three hundred and sixty (1-day-old Ross 708) broiler chicks were obtained from a commercial hatchery and feather sexed upon arrival to the University of Arkansas broiler research farm. Prior to arrival, chicks were vaccinated at the hatchery (*In ovo)* for Mareks and Newcastle diseases (½ dose Mareks (HVT)+ND *In ovo*, and ¼ dose SB1), and by spray for

infectious bronchitis (full dose Mass+Ark, and ½ dose Shor-Bron-D). After sexing, male and female chicks were divided and placed into two environmentally controlled rooms. Each room contained 15 floor pens (12 birds/pen; 0.09 m^2/bird) equipped with a tube feeder, nipple drinker line, and built-up top-dressed litter (15 mm). House temperatures were 32ºC at day one and gradually decreased to 20ºC at day 27. The birds received 23 hours of light until day 10 then light duration was decreased to 18 hours for the remainder of the experiment. Supplemental paper feed trays were placed for the first seven days and birds had free access to fresh water.

Experimental design

Two experiments were conducted concurrently with female birds raised to 35d (Experiment 1) and males to 37 days (Experiment 2). All birds were fed a two-phase feeding program ad libitum, with a common starter diet from 1 to 14 d containing 21% crude protein and 3,248 kcal/kg. Experimental grower diets (Table 2) were fed from 15 to 35 or 37 days of age in Experiment 1 and 2, respectively.

All birds were assigned one of three experimental diets: 1) standard corn/soybean meal positive control (Control); 2) reduced CP negative control (LCP); 3) reduced CP with *Spirulina* added to the formulation matrix (SP-LCP). The positive control diet represented an industry standard corn and soybean meal diet, which met or exceeded all nutrient recommendations from the commercial breeder (Aviagen, 2019) and contained 20% CP. The combination of reduced CP diets and rich whole protein sources necessitated formulating with higher levels of grain, thus increasing AME levels in two of the experimental diets. Diets 2 and 3 had a 15% reduction in CP and met all essential amino acid (EAA) needs (NRC, 1994) and included feed-grade Arg, Val, Ile, and Trp. Diet 3 had *Spirulina* meal inclusion of 10%, which represented half of the whole protein sources in the diet. Dried *Spirulina platensis* powder meal was sourced from a

commercial producer (Pond Tech., Markham, Canada) and analyzed for amino acid concentrations (ATC Scientific, Little Rock, AR; NOVUS, St. Charles, MO; University of Arkansas, Fayetteville, AR) prior to diet formulation. Digestible amino acid values were then obtained using published digestibility coefficients (Tavernari et al., 2018). Starter diets were fed as crumbles after pelleting whereas experimental diets were fed as pellets.

Pen weights and feed consumption were recorded at the start of the experimental period (day 15) and at the conclusion of the experiment prior to processing (Experiment 1, day 35; Experiment 2, day 37). Body weight gain (BWG), FCR, and mortality were then calculated for 15 to 35 or 37 days of age for Experiment 1 and 2, respectively. Feed was removed from all pens 10 hours prior to processing, while water access was maintained. On day of processing, all birds were processed at the University of Arkansas Pilot Processing Plant (Fayetteville Arkansas) via a commercial inline system. Birds were placed on shackles and electrically stunned (11 V, 11 mA for 11 s) before being exsanguinated, soft scalded (55 C for 2 min), de-feathered, and mechanically eviscerated. Footpad scores were recorded by one individual to limit observational error with a whole number increment scale: 0 - ideal foot pad with no redness or lesions; 1 – slight callus with minor redness smaller than a dime; 2 – lesions with significant calluses larger than a dime. Breast fillets were subjectively hand scored, by trained personal, for woody breast (WB) on a whole number increment scale with 0 being no signs of WB, 1 being mild to moderate WB, and score 2 being severe WB according to Tijare et al. (2016). Carcass and abdominal fat pad weights were recorded immediately post evisceration. Carcasses were chilled for 4 hours at 4^{o}C before breasts, tenders, wings, drums, and thighs were removed and weights recorded. Breasts and left thigh (skin-on) from each bird was collected and stored at 4°C for 24 hours for colorimetric analysis. Readings were taken with a colorimeter (CR-400; Konica

Minolta Sensing Inc., Sakai Osaka, Japan; size 102 (W) × 217 (H) × 63 (D) mm) using illuminant D65 and a 2-degree observer to determine the L* (Lightness), a* (redness), and b* (yellowness) values on the ventral side of the right breast fillet, and on the left thigh skin and left *ilotibialis* thigh muscle.

Statistical Analysis

Data were analyzed using JMP Pro15 (SAS Institute, 2018) as a randomized, complete block design, with pen as the experimental unit. One-way ANOVA was used with $P<0.05$ indicating the probability of significant differences. When appropriate, differences among treatments were separated using a repeated t test.

RESULTS AND DISCUSSION

Birds raised in both experiments exceeded breeder growth objectives (Aviagen, 2019), however, the lower protein treatments had higher than recommended FCR. The variability of the analyzed CP and AA from the formulated levels may be the result of undervaluing the protein level of soybean meal. No differences in 35 d BW were observed in Experiment 1 (females, Table 3). In Experiment 2 (male broilers), 37 d BW were significantly lower ($P=0.002$) in both LCP diets compared to the Control (Table 4). Decreasing CP to 17% has adverse effects on growth performance, even when diets are fortified with feed grade amino acids (Ferguson et al., 1998; Chrystal et al., 2019). However, a mild reduction in CP of 1 to 3% has been shown to have no adverse effects on live performance in trials conducted by Chrystal et al., 2019 and Harn et al., 2019. Reducing dietary CP to 17% in Experiment 1 had a minimal effect of lowering BWG by 5.8% (3.42 vs. 3.22 lb/bird), while the SP-LCP decreased BWG further ($P=0.002$) compared to the control diet (Table 3). Both LCP diets in Experiment 2 had a significant impact ($P=0.006$) on performance of birds by decreasing BWG by 9.4% compared to the Control (4.50 vs. 4.06

lb/bird) and there were no significant differences between the 2 LCP diets (4.03vs 4.08 lb/bird) (Table 4). Hernandez et al. (2012) showed that the BWG of male broilers raised to 48 d were more adversely affected by reducing CP than their female counterparts. This is in agreement with Pesti (2009), that concluded in a meta-analysis from numerous experiments that BWG has varying responses to dietary CP levels but does play a role in overall performance. *Spirulina* addition of 6 and 8% has been fed to broilers with no negative effects on performance (Ross et al., 1990; Toyomizu et al., 2001). Ross et al. (1990) displayed detrimental effects of higher inclusion rates of *Spirulina* on BW of broilers fed 12% *Spirulina* and BWG in cockerels fed 20%. BW was also impeded when Altmann et el. (2020) fed Ross 308 broilers, 22.1 and 12.5%, *Spirulina* in the grower and finisher phase.

Feed conversion ratio was compromised in both experiments with the reduction of CP by 9 ($P<0.001$) and 15 ($P=0.0013$) points, respectively (Table 3 and 4). *Spirulina* inclusion impacted the FCR differently in both experiments. The SP-LCP treatment had the highest FCR in Experiment 1 by 6 points, but improved FCR by 7 points in Experiment 2 when compared to the LCP diet. More modest amounts of *Spirulina*, 1 and 2.5%, have improved feed utilization by improving FCR by .48 and 4 points (Moujahed et al., 2011; Park et al., 2018). These increases in performance parameters are theorized to be from the chemical and physiological functions of *Spirulina*, which provide antimicrobial, antioxidant, and anti-inflammatory properties (Park et al., 2018). However, in the work herein it maybe that CP was decreased to a level where EAA or NEAA were limiting; thus, masking any *Spirulina* effect.

Processing differences were observed in female broilers in Experiment 1 (Table 3). The SP-LCP and LCP treatments showed a significant reduction ($P<0.05$) in % breast, % tender, and % drum yield compared to the Control diet. Processing weights of breast, tenders, wings, and

drums mirrored body weights and were significantly less in all parts except thigh in male broilers in Experiment 2 (Table 4). Thigh weight of the SP-LCP treatment was lower than the Control treatment (P=0.03), but not the other LCP treatment. The decreased yield responses, most notably breast yield are in alignment with Harn et al. (2019) in that reducing CP by 3% will impede processing yields. The reduction in breast yields were ameliorated with free Gly supplementation (Ospina-Rojas et al., 2014; Belloir et al., 2018). Fat deposition significantly increased in the LCP treatments in Experiment 2 similarly to male broilers fed reduced CP diets in a trial conducted by Corzo et al., 2007. Experiment 1 had no differences of fat deposition in either weight or yield in any treatment. No differences in wooden breast myopathy were observed between any dietary treatments in either experiment.

Average foot pad score was determined to be similar in Experiment 1 across all treatments (Table 3). In Experiment 2, the SP-LCP treatment had the least severity of footpad dermatitis in (Table 4), which was significantly less than the control diet. To the authors knowledge this has not been previously shown before in broilers fed *Spirulina*. Eichner et al. (2007) demonstrated improvement in footpad dermatitis in birds fed an all-vegetable diet by including corn gluten meal. Foot pad quality has been shown to be affected by numerous factors including genetics, environmental factors, bedding materials, and nutrition (Shepherd and Fairchild, 2010). The driving force on decreased severity of footpad dermatitis in the SP-LCP treatment is believed to be the combination of multiple factors; including decreased litter moisture, decreased N excretion, decreased carbohydrates from *Spirulina* replacement of soybean meal (6.46 vs. 35 %), and other nutrients in *Spirulina*.

Litter moisture is considered one of the most important factors in footpad dermatitis (Eichner et al. 2007). Reducing CP lowers water intake, thus water excretion in broilers

(Alleman and Leclerq, 1997). Hernández (2012) demonstrated that N and ammonia concentrations in litter decreased with dietary protein. Ammonia emissions were decreased as *Spirulina* supplementation increased linearly (P=0.005) from 0 to 1% in an experiment conducted by Park et al., 2018. Ammonia combined with water creates an acidic mixture impacting footpad dermatitis (Kamphues et al., 2011). Manure that adheres to the foot pad increases the incidences of footpad dermatitis and plant sources high in carbohydrates increase gut viscosity creating tacky caustic litter (Hess et al., 2004; Shepherd and Fairchild, 2010). Biotin in *Spirulina*, 550 ug/kg dm (Liestianty et al., 2019), could also play a role in footpad health in this study, even considering that additional biotin supplementation alone does not prevent or reduce footpad dermatitis (Mayne et al., 2007). Harms et al. (1977) showed that foot pad scores improved when biotin was given to broilers raised on dry litter, but not ones grown on wet litter.

Breast, thigh, and skin pigmentation increased significantly (P<0.005) in both experiments (Table 5 & 6), in both redness (a*) and yellowness (b*) when birds were fed 10% of *Spirulina*. Pigment contents can vary in *Spirulina* samples; zeaxanthin (0.1-0.7 mg/g), total carotenoids (0.28-2.23 mg/g), and C-Phycocyanin (1.1-9.1 mg/g) (Park et al., 2018). Zeaxanthin accumulation in breast fillets showed a direct correlation with breast yellowness indicating it is the main source of carotenoid pigmentation in *Spirulina* (Toyomizu et al., 2001). Skin and thigh also had higher pigmentation (P<0.05) in the *Spirulina* treatment, which is common with increased pigmentation. Toyomizu et al. (2001) reported breast redness was increased at 4% but not in the 8% SP fed diet, while breast and skin yellowness increased with additional *Spirulina*. An experiment by Raach-Moujahed et al. (2011) displayed that skin and breast yellowness increased with dietary inclusion of 2.5%, but not in the 5% treatment. No breast color differences

were observed when Park et al. (2018) fed broilers 1% *Spirulina*. The variation in color pigmentation data from the current study could be a result of different experimental designs, genetics, or *Spirulina* nutrient profile. Pigment deposition was not limited to breast, thigh and skin; although not recorded, there were visual color differences of shank, intestine, and blood plasma with *Spirulina* inclusion. Diet costs were not considered when formulating experimental diets, but post-hoc economic analysis shows the infeasibility of feeding *Spirulina* at this level, ~10% (Table 7) based on a current purchase value. More modest levels of algae and placing an economic value on pigmentation, as well as its probiotic potential, are necessary to further validate *Spirulina* as a cost effecting feed ingredient. Furthermore, the economic analyses presented (Table 7) indicates the cost of *Spirulina* will need to be similar to oilseeds and meat meals pending acceptance into practical broiler diets. Thus, algae production research and development that leads to increased commercialization will dictate the former.

CONCLUSIONS

Spirulina addition in low CP diets decreased incidences of footpad dermatitis and improved redness and yellowness pigmentation throughout the broiler, including the highly desired breast fillets. Reducing crude protein in the diet impaired FCR and processing yields in both trials, but impacted Experiment 2 male broilers performance parameters more than Experiment 1 female broilers.

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TABLES

Table 1. Proximate Composition and Amino Acid Analysis of *Spirulina Platensis* and Soybean meal

	Spirulina Platensis meal	Soybean meal[1]
Proximate Composition, %		
Dry Matter, %	91.61	93.32
Ash, %	10	6.58
Crude Fiber, %	1.9	3.2
Fat, %	0.61	1.76
Protein, %	60.59	49.69
AA Analysis, As Is		
Met., %	1.49	0.70
Lys., %	2.75	3.08
Thr., %	2.94	2.02
Val., %	3.42	2.31
Ile., %	3.26	2.36
Arg., %	3.80	3.56
Leu., %	5.07	3.89
His., %	0.93	1.25
Phe., %	2.64	2.61
Gly., %	2.85	2.09

Analyzed by NOVUS (AOAC, 2000)
[1]Analyzed Post-Hoc

Table 2. Composition of experimental diets, "as-fed" basis

	Crumble Common Feed (0-14 d)	Pellet Test Feed (15-35/37 d)[1]		
		Control	LCP	SP-LCP
Ingredient, %				
Corn	52.27	63.31	71.83	74.59
SBM (48%)	38.68	31.23	22.16	10.45
Spirulina Meal	–	–	–	10.00
Poultry fat	4.69	2.18	1.45	0.93
Dicalcium P	1.97	0.779	0.84	0.44
Limestone	1.08	1.08	1.11	1.39
NaCl	0.45	0.37	0.10	0.05
Na HCO3	–	0.14	0.58	0.58
Choline-Cl	–	0.08	0.11	0.18
Premixes[2]	0.20	0.14	0.14	0.14
Phytase	0.01	0.01	0.01	0.01
Coccidiostat	–	0.05	0.05	0.05
DL-Met	0.34	0.31	0.38	0.27
L-Lys HCl	0.20	0.21	0.49	0.51
L-Thr	0.10	0.11	0.23	0.12
L-Arg	–	–	0.22	0.23
L-Val	0.01	–	0.159	0.03
L-Ile	–	–	0.129	0.03
L-Trp	–	–	0.02	0.02
Estimated composition (% unless otherwise noted)				
Crude protein	21.4	20.1	17.0	17.0
AME (kcal/kg)	3,248	3,100	3,154	3,154
Calcium	0.96	0.84	0.84	0.84
Available phos.	0.48	0.42	0.42	0.42
TSAA	–	0.91	0.90	0.94
Lys	–	1.20	1.18	1.21
Thr	–	0.85	0.83	0.85
Ile	–	0.84	0.79	0.84
Val	–	0.93	0.91	0.96
Trp	–	0.24	0.20	0.21
Arg	–	1.30	1.23	1.30
TSAA, d	0.90	0.84	0.84	0.84
Lys, d	1.20	1.10	1.10	1.10
Thr, d	0.78	0.74	0.74	0.74
Ile, d	0.81	0.75	0.73	0.73
Val, d	0.90	0.83	0.83	0.83
Trp, d	0.24	0.21	0.18	0.18
Arg, d	1.32	1.21	1.16	1.16
Analyzed composition (%)				
Crude Protein	–	22.1	19.5	17.7
Met	–	0.5	0.5	0.4
Cys	–	0.4	0.3	0.3
Lys	–	1.3	1.2	1.1
Thr	–	0.9	0.8	0.8
Ile	–	0.8	0.7	1.0
Val	–	1.0	0.9	0.8
Trp	–	0.3	0.3	0.2
Arg	–	1.4	1.3	1.10

[1]Female birds were fed Pelleted test diets from 15 to 35 day. Male birds were fed Pelleted test diets from 15 to 37 day

[2]Added per kg of finished feed: vitamin A, 9,259 IU; vitamin D3, 6,614 ICU; vitamin E, 66 IU; niacin, 46 mg; d-pantothenic acid, 12 mg; riboflavin, 8 mg; pyridoxine, 3 mg; thiamine, 2 mg; menadione, 2 mg; folic acid, 1 mg; biotin, 0.1 mg; vitamin B12, 0.02 mg; manganese, 96 mg; zinc, 58 mg; copper, 2.7 mg; iodide, 1.9 mg; selenium, 0.16 mg.

Table 3. Performance and Processing Yield from female 35 day old broilers Experiment 1 fed diets differing in CP and CP source[1]

Parameter	Control	LCP	SP-LCP	SEM	*P-value*
Body Weight, lb	4.59	4.67	4.61	0.033	0.257
Day 15-35 Performance					
BW gain, lb/bird	3.42[a]	3.22[ab]	3.00[b]	0.064	0.002
Feed intake, lb/bird	5.67	5.62	5.60	0.101	0.863
FCR, lb/lb[2]	1.66[c]	1.75[b]	1.81[a]	0.013	0.001
Mortality, %	0.00	0.00	0.03	0.012	0.111
Day 35 processing					
Breast, lb[3]	1.00[a]	0.91[b]	0.85[b]	0.020	0.001
Tender, lb[4]	0.21[a]	0.19[ab]	0.18[b]	0.005	0.007
Wings, lb	0.36	0.35	0.34	0.007	0.086
Thigh, lb[5]	0.62	0.60	0.57	0.012	0.056
Drum, lb[5]	0.40[a]	0.37[b]	0.36[b]	0.004	0.001
Fat, lb	0.06	0.06	0.06	0.002	0.966
Carcass, lb	3.44	3.48	3.41	0.029	0.265
Yield, %	75.1	74.6	74.0	0.440	0.287
Breast yield, %[6]	21.7[a]	19.4[b]	18.4[b]	0.360	0.001
Tender yield, %	4.6[a]	4.1[b]	4.0[b]	0.091	0.001
Wing yield, %	7.9[a]	7.4[ab]	7.3[b]	0.127	0.016
Thigh yield, %	13.4[a]	12.8[ab]	12.4[b]	0.212	0.016
Drum yield, %	8.7[a]	7.9[b]	7.9[b]	0.074	0.001
Fat, %	1.37	1.33	1.34	0.043	0.841
Woody breast, avg[7]	0.48	0.52	0.54	0.042	0.608
Foot pad score, avg[7]	0.50	0.56	0.48	0.200	0.958

[1]Control diet contained 20.1% CP. CP was allowed to decrease to 17% in the Low CP and Low CP + algae diets resulting in L-Ile, L-Val, L-Arg, and L-Trp diet inclusions. The Low CP + algae diet allowed algae to replace 50% of soybean meal. Diets were formulated to identical minimum amino acid ratios to Lys.
[2]FCR=Mortality Corrected feed to gain ratio
[3]Breast=*Pectoralis major*
[4]Tender=*Pectoralis minor*
[5] Carcass parts are skin-on and bone-in
[6]Yields represent chilled carcass parts relative to live BW
[7] 0=none, 1=mild & 2=severe; means represent average score

Table 4. Performance and Processing Yield from male 37 day old broilers Experiment 2 fed diets differing in CP and CP source[1]

Parameter	Control	LCP	SP-LCP	SEM	*P-value*
Body Weight, lb	5.89[a]	5.34[b]	5.23[b]	0.108	0.002
Day 15-37 Performance					
BW gain, lb/bird	4.50[a]	4.03[b]	4.08[b]	0.093	0.006
Feed intake, lb/bird	7.36	7.34	6.95	0.220	0.352
FCR, lb/lb[2]	1.60[b]	1.75[a]	1.68[ab]	0.022	0.001
Mortality, %	0.05	0.07	0.02	0.028	0.462
Day 37 processing					
Breast, lb[3]	1.31[a]	1.10[b]	1.06[b]	0.026	0.001
Tender, lb[4]	0.25[a]	0.22[b]	0.20[b]	0.006	0.001
Wings, lb	0.45[a]	0.41[b]	0.39[b]	0.007	0.001
Thigh, lb[5]	0.75[a]	0.70[ab]	0.69[b]	0.014	0.030
Drum, lb[5]	0.54[a]	0.49[b]	0.48[b]	0.013	0.008
Fat, lb	0.06[b]	0.07[a]	0.07[a]	0.002	0.004
Carcass, lb	4.50[a]	3.99[b]	3.92[b]	0.076	0.001
Yield, %	76.4	74.9	75.0	0.429	0.042
Breast yield, %[6]	22.2[a]	20.7[b]	20.3[b]	0.228	0.001
Tender yield, %	4.2[a]	4.0[a]	3.9[b]	0.040	0.001
Wing yield, %	7.6[ab]	7.7[a]	7.5[b]	0.049	0.047
Thigh yield, %	12.7	13.18	13.2	0.208	0.195
Drum yield, %	9.2	9.1	9.1	0.111	0.805
Fat, %	1.08[b]	1.37[a]	1.38[a]	0.028	0.001
Woody breast, avg[7]	0.85	0.70	0.95	0.133	0.431
Foot pad score, avg[7]	0.54[a]	0.16[ab]	0.08[b]	0.099	0.017

[1]Control diet contained 20.1% CP. CP was allowed to decrease to 17% in the Low CP and Low CP + algae diets resulting in L-Ile, L-Val, L-Arg, and L-Trp diet inclusions. The Low CP + algae diet allowed algae to replace 50% of soybean meal. Diets were formulated to identical minimum amino acid ratios to Lys.
[2]FCR=Mortality Corrected feed to gain ratio
[3]Breast=*Pectoralis major*
[4]Tender=*Pectoralis minor*
[5] Carcass parts are skin-on and bone-in
[6]Yields represent chilled carcass parts relative to live BW
[7] 0=none, 1=mild & 2=severe; means represent average score

Table 5. Meat color from female 35 day old broilers fed diets differing in CP and CP source[1]

Parameter	Control	LCP	SP-LCP	SEM	*P-value*
Breast[2]					
L*	54.64	55.07	53.92	0.342	0.095
a*	2.41[b]	2.49[b]	5.77[a]	0.296	0.001
b*	9.56[b]	10.62[b]	23.71[a]	0.538	0.001
Skin[3]					
L*	73.59[ab]	74.58[a]	72.95[b]	0.405	0.044
a*	5.91[b]	5.26[b]	7.81[a]	0.320	0.001
b*	9.90[b]	10.65[b]	19.30[a]	0.497	0.001
Thigh[4]					
L*	56.46	56.49	55.77	0.413	0.412
a*	4.44[b]	4.25[b]	6.40[a]	0.323	0.001
b*	6.62[b]	7.70[b]	16.46[a]	0.404	0.001

[1]Control diet contained 20.1% CP. CP was allowed to decrease to 17% in the Low CP and Low CP + algae diets resulting in L-Ile, L-Val, L-Arg, and L-Trp diet inclusions. The Low CP + algae diet allowed algae to replace 50% of soybean meal. Diets were formulated to identical minimum amino acid ratios to Lys.
[2]Breast=Skinless *Pectoralis major*
[3]Skin=Left thigh skin
[4]Thigh=Skinless left thigh
[5]L*=lightness, a*=redness, b*=yellowness

Table 6. Meat color from male 37 day old broilers fed diets differing in CP and CP source[1]

Parameter	Control	LCP	SP-LCP	SEM	*P value*
Breast[2]					
L*	54.79	54.61	54.76	0.311	0.910
a*	2.71[b]	2.93[b]	6.13[a]	0.245	0.001
b*	9.91[b]	10.61[b]	23.89[a]	0.378	0.001
Skin[3]					
L*	74.67[ab]	75.26[a]	73.57[b]	0.402	0.033
a*	5.02[b]	5.19[b]	7.35[a]	0.273	0.001
b*	8.63[c]	10.47[b]	19.40[a]	0.258	0.001
Thigh[4]					
L*	58.12[ab]	58.88[a]	57.77[b]	0.262	0.031
a*	3.17[b]	3.44[b]	5.87[a]	0.220	0.001
b*	7.18[c]	8.64[b]	17.95[a]	0.237	0.001

[1]Control diet contained 20.1% CP. CP was allowed to decrease to 17% in the Low CP and Low CP + algae diets resulting in L-Ile, L-Val, L-Arg, and L-Trp diet inclusions. The Low CP + algae diet allowed algae to replace 50% of soybean meal. Diets were formulated to identical minimum amino acid ratios to Lys.
[2]Breast=Skinless *Pectoralis major*
[3]Skin=Left thigh skin
[4]Thigh=Skinless left thigh
[5]L*=lightness, a*=redness, b*=yellowness

Table 7. Economic Analysis of broilers fed diets differing in CP and CP source[1]

	Control	LCP	SP-LCP
Female 35d, Experiment 1			
Total feed cost/bird, $[2]	1.037	1.075	5.218
W.O.G./bird, $[3]	4.845	4.929	4.866
Gross bird profit, $[4]	3.808	3.854	-0.353
Male 37d, Experiment 2			
Total feed cost/bird, $[2]	1.299	1.355	6.440
W.O.G./bird, $[3]	6.217	5.636	5.520
Gross bird profit, $[4]	4.918	4.281	-0.920

[1]Control diet contained 20.1% CP. CP was allowed to decrease to 17% in the Low CP and Low CP + algae diets resulting in L-Ile, L-Val, L-Arg, and L-Trp diet inclusions. The Low CP + algae diet allowed algae to replace 50% of soybean meal. Diets were formulated to identical minimum amino acid ratios to Lys.
[2]Total feed cost/bird ($) = Cumulative feed intake (lbs) * Feed cost ($/lb.; ingredient prices were obtained from University of Arkansas sources and USDA – National weekly Feedstuff Wholesale Prices for May 25th, 2021[Corn= 200 $/ton, Soybean meal=354 $/ton, Spirulina meal= 15,422 $/ton])
[3]W.O.G. bird price ($) = Average bird weight (lb.) * WOGS ($; USDA Weekly National Whole Broiler/Fryer Report for May 28th, 2021 heavier than 3.51lbs.)
[4]Gross bird profit ($) = Whole bird price ($) – Total feed cost/bird ($)

IV. SPIRULINA PLATENSIS INCLUSION REVERSES CIRCULATING PRO-INFLAMMATORY (CHEMO)CYTOKINE PROFILES IN BROILERS FED LOW-PROTEIN DIETS

ABSTRACT

Proteins are considered the most expensive nutrients in commercial modern broiler production, and their dietary inclusion at low levels is pivotal to minimize feed costs and reduce nitrogen waste. The quest for an environmentally friendly source of proteins that favor the formulation of low protein diets without compromising broiler health, welfare, and growth performance has become a hotspot in nutrition research. Due to its high protein content, the naturally growing Spirulina microalgae is considered a promising nutrient source. The purpose of the present study was, therefore, to determine the effects of Spirulina supplementation on liver bacterial translocation, hematological profile, and circulating inflammatory and redox markers in broilers fed a low-protein diet. One-day-old Ross 708 male broilers (n = 180) were randomly assigned into one of three experimental treatments: standard diet as a control, low protein diet, and low protein diet supplemented with 100 g/kg of Spirulina. Target molecular markers were measured in the peripheral blood circulation using real-time quantitative PCR. Reducing dietary proteins increased bacterial translocation and systemic inflammation as indicated by proportions of basophils among blood leukocytes. The expression levels of circulating pro-inflammatory cytokines [interleukin (IL)-3, IL-6, IL-4, IL-18, and tumor necrosis factor-α], chemokines (CCL-20), and NOD-like receptor family pyrin domain containing 3 inflammasome were significantly upregulated in birds fed the low protein diet compared with the control. The inclusion of Spirulina reversed these effects, which indicates that Spirulina reduces systemic inflammation- and bacterial translocation-induced by a low protein diet and could be a promising alternative protein source in poultry diets.

INTRODUCTION

Global poultry meat production is projected to increase by 32% by 2030 and 59% by 2050 from production in 2012, with low- and middle-income countries making up most of this demand (FAO, 2018). The cost of feed, particularly proteins, and availability of feedstuffs are by far the most important factors in poultry production sustainability and competitiveness. Indeed, soybean meal (SBM) is the preferred source of protein in broiler diets, as it is the only economically feasible protein source currently used in many countries. However, the continuous increase in global demand for high-quality animal protein, which consequently needs an improved animal production and abundant animal feeds, may inevitably surpass the SBM supply. This may, in turn, affect the inflation and the market price of SBM, which is linearly rising. Furthermore, there has also been a precedent set to limit animal-derived protein sources for animal feed in the European Union via Regulation (EC) No. 999/2001 (EC, 2020). Thus, numerous innovative and effective approaches will need to be implemented to breach this looming protein gap to keep up with the worldwide demand for poultry meat. Reducing crude protein (CP) in broiler diets has been extensively studied, yet the underlying issues of increased fat deposition and decreased performance are still prevalent (Fraps et al, 1943; Belloir et al., 2017; Lemme et al., 2019). Implementing the ideal protein concept, computer least-cost formulation, and commercially produced crystalline amino acids allow for the lowest dietary CP diet thus far (Emmert et al., 1997; Kidd et al., 2013). The reduction of CP with supplemental crystalline amino acids alleviates the demand for SBM, yet alternative protein sources will also be important for meeting the anticipated increase in demand for protein in animal and poultry feed. Alternatives for SBM in broiler diets have been investigated, including traditional and novel options such as canola meal, blood meal, meat and bone meal, fish meal, peameal, sunflower meal, insect meal, and numerous algae species (Kocher et al., 2000;

Becker, 2007; Bingol et al., 2016; Bryan et al., 2019; Altmann et al., 2020). Many of the trials involving algae in broiler feed have focused on growth performance or meat quality with limited mechanistic explanations for their beneficial effects. Algae inclusion rates of up to 100 g/kg have maintained or increased performance parameters in chickens reared under conventional conditions (Toyomizu et al., 2001; Bonos et al., 2016; Fathi et al., 2018). Spirulina (Athrospira sp.) platensis is a filamentous spiral shaped blue-green cyanobacterium that grows naturally in warm and alkaline aquatic media and is particularly interesting as a possible animal feed protein source due to its high level of CP (43–70%) and balanced amino acid profile. In addition to essential fatty acids, vitamins, minerals, and pigments (Gouveia et al., 2008), Spirulina contains several compounds shown to have antioxidant, anti-inflammatory, immune-modulating, and probiotic properties (Qureshi et al., 1996; Uyisenga et al., 2010; Shanmugapriya et al., 2015: Park et al., 2018). Spirulina is also considered a nutritionally safe feed ingredient with no risk of mineral toxicity and free of the major algal toxin, microcystins (Yang et al, 2011; Falquet, 2017). Spirulina use as a feed ingredient for multiple species has been outlined (Holman et al., 2013), and one of the confounding issues put forth is the underlying mechanisms by which Spirulina impacts them. The purpose of this study was, therefore, to determine hematological parameters, inflammatory markers, and oxidative stress in broilers fed a low CP diet with and without Spirulina.

MATERIALS AND METHODS

Animals and Treatment.

A total of 180 1-day-old male (Ross 708) broiler chicks were obtained from a commercial hatchery and randomly allotted to one of the 15-floor pens (5 pens/diet; 12 birds/pen) in an environmentally controlled pilothouse. Chicks were reared in pens top-dressed with ∼4 cm of fresh wood shavings, and the temperature gradually decreased from 32◦C on day 1 to 20◦C on

day 27. The trial was conducted from February to March 2020. Birds received 23 h of light until day 10; then, light duration was decreased to 18 h for the remainder of the trial. Birds were given ad libitum access to feed (Table 1) and water throughout the trial. A standard corn–SBM basal diet (3,250 kcal/kg−1 , 21% CP) was fed to all birds until day 14, at which point experimental diets were introduced until 37 days of age. The experimental diets included an industry-standard level protein (∼20% CP) corn/SBM control (SCP), reduced (∼17%) CP corn/SBM diet (LCP), and LCP diet where Spirulina was included at the level of 100 g/kg (SP-LCP). All experimental diets were isocaloric and met all essential amino acid needs set forth by the primary breeder. Both low CP diets were formulated to be isonitrogenous.

Blood Sampling and Hematological Analysis

On day 37, blood (3 ml) was collected from non-fasted birds (10 birds/treatment; 2 birds/pen), via the brachial wing vein, in ethylenediaminetetraacetic acid-coated tubes and immediately placed on ice. The hematologic profile of 1 ml of whole blood samples was measured using the Cell-Dyn 3500 automated hematology analyzer calibrated for chicken blood (Abbott Diagnostics, Abbott Park, IL) within 3 h of sampling. Data collected included the percent of heterophils, lymphocytes, monocytes, eosinophils, and basophils and the calculated heterophil to lymphocyte (H/L) ratio. For gene expression analysis, 250 µl of blood was added to tubes containing 750 µl of TRIzol LS reagent according to manufacturer's recommendations (Life Technologies Corporation, CA, US). The birds' bodyweights were 2.61 ± 0.07, 2.60 ± 0.07, and 2.72 ± 0.08 kg for SCP, LCP, and SP-LCP, respectively

Liver Bacterial Translocation

As previously described by Tellez et al. (2015), a section of the right liver was aseptically removed from 30 chickens (10 birds/treatment), placed into sterile sampling containers, and

homogenized. Samples were then diluted 1:4 based on tissue weight with sterile 0.9% saline. Liver samples were then transferred to sterile 96-well Bacti flat-bottom culture plates and diluted 10-fold before being plated on tryptic soy agar to evaluate total counts of Enterobacteriaceae per gram of tissue. Samples were incubated under aerobic conditions at 37˚C for 24 h.

RNA Isolation, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was isolated from whole blood samples using Trizol LS reagent (ThermoFisher Scientific, Rockford, IL) according to manufacturer's recommendations. Take 3 Micro-Volume Plate using Synergy HT multimode microplate reader (BioTek, Winooski, VT) determined RNA concentrations and purity. Real-time quantitative PCR (Applied Biosystems 7500 Real-Time PCR System) was performed using 5 µl of complementary DNA, 1 µl of each forward and reverse specific primers, and 10 µl of SYBR Green Master Mix (ThermoFisher Scientific, Rockford, IL, United States) in a total 25-µl reaction. Oligonucleotide primers used for chicken cytokines, chemokines, inflammasomes, antioxidative, and 18S (housekeeping) genes are summarized in Table 2. The real-time quantitative PCR cycling conditions were 50◦C for 2 min, 95◦C for 10 min followed by 40 cycles of a two-step amplification program (95◦C for 15 s and 58◦C for 1 min). At the end of the amplification, melting curve analysis was applied using the dissociation protocol from the Sequence Detection system to exclude contamination with unspecific PCR products. 18S RNA was used to normalize the relative expression of targeted genes via the 2−11Ct method (Schmittgen et al., 2008). All values were compared relative with those in the SCP dietary control group.

Statistical Analysis

Statistical Analysis All data were analyzed as a complete randomized design with one-way ANOVA in JMP Pro v 15.0 (SAS Institute, Cary, NC, United States). Post-hoc analysis

assessment through multiple Dunnett comparisons was used when appropriate. Differences were considered significant at $P \leq 0.05$.

RESULTS AND DISSCUSSION

Hematological Analysis

Reducing CP by 3% in the diet (LCP) increased lymphocyte percent ($P < 0.01$) while decreasing that of heterophils ($P = 0.026$) and thereby resulted in a significantly lower H/L ratio ($P = 0.014$) (Table 3). The LCP group exhibited a significantly higher percent of basophil but no differences in monocyte or eosinophil percent compared with the SCP group. The inclusion of Spirulina reversed basophil percent to a similar level as the SCP (control) group. Spirulina inclusion had no impact on the H/L ratio or the percent of heterophils, lymphocytes, monocytes, or eosinophils (Table 3).

Liver Bacterial Translocation

The presence of bacteria in the liver reflects bacterial translocation from the gastrointestinal tract. Bacterial counts in the liver were elevated ($P < 0.05$) by the LCP diet compared with birds fed with the SCP control diet (3.34 ± 0.7 vs. 2.44 ± 0.4 log10 CFU/g). Bacterial translocation was ameliorated in birds fed the Spirulina LCP diet to levels (1.34 ± 0.4 log10 cfu/g) lower than in the liver of birds fed the SCP control diet

Circulating Inflammation-, Toll-like Receptor-, and Antioxidant-associated markers

The LCP diet upregulated ($P < 0.05$) the expression of pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-3 (IL-3), interleukin-18 (IL-18), and tumor necrosis factor-alpha (TNF-α), as indicated by expression of lipopolysaccharideinduced TNF-α factor (Figures 1A–D, 2A) and that of the regulatory cytokine interleukin-10 (IL-10) and interleukin-4 (IL-4) (Figure 2D). Similarly, the LCP diet upregulated the expression of circulating chemokine C-C motif

ligand 20CCL-20 (Figure 3A) but not that of C-C motif ligand 4 or C-X-C motif ligand 14 (Figures 3B–D). LCP supplementation increased the messenger RNA (mRNA) abundance of NOD like receptor family pyrin domain-containing 3 but not NOD-like receptor family CARD domain-containing 3, NOD-like receptor family CARD domain-containing 5, and nucleotide-binding oligomerization domain and leucine-rich repeat-containing X1 inflammasomes (Figures 4A–D). LCP diet upregulated the expression of myeloid differentiation primary response protein 88 (MyD88) but not that of toll-like receptors 3, 4, and 21 (TLR-3, TLR-4, TLR-21, and MyD88) (Figures 5A–D). Spirulina inclusion reversed the expression of all of the markers mentioned earlier to levels being expressed in birds fed with the SCP control diet (Figures 1–4). The LCP diet upregulated the blood expression of glutathione peroxidase 1 GPx-1 but not that of GPx-3 or superoxide dismutase 1 and 2 (SOD-1/2) compared with the control (SCP) group (Figures 6A–D). The inclusion of Spirulina reduced GPx-1 mRNA abundance to the control level; however, it non-significantly increased further the expression of GPx-3 and SOD-2 genes (Figures 6A, B, D)

Inflammation is a response to stress and can occur in multiple levels of severity, from immediate acute responses to long-term chronic conditions. There is an intertwining relationship between inflammatory response and oxidative stress, in which inflammation elevates free radicals to levels exceeding cell threshold, thus inducing oxidative stress (Surai et al., 2019). Stress-induced inflammation can be associated with a plethora of factors, including dietary nutrient composition, bacterial or viral infection, environmental factors, obesity, aging, and numerous diseases (Franceschi et al., 2000; Lu et al., 2014; Panahi et al., 2016; Ellulu et al., 2017; Perez, 2019; Dal Pont et al., 2020; Baxter et al., 2020). There is limited information on how low CP diets and Spirulina impact inflammatory biomarkers in broiler chickens. It is important to remember that

"low" or "high" CP level is relative regarding species, age, environment, and other factors. Excessive dietary protein intake can cause undigested protein or amino acids to enter the hindgut, increasing the amounts of opportunistic bacteria such as Clostridium perfringens in the ileum and ceca of broilers (Drew et al., 2004). Excess dietary nutrients/metabolites could cause metabolic (meta)-inflammation, which is different from pathological and physiological inflammation, and typically occurs in modern broilers production systems due to several factors such as ingredients used in the diet, nutrient excess in the diet, and high feed intake (Kogut et al., 2018). All forms of inflammation lead to an upregulation of certain inflammatory biomarkers (Khayyatzadeh et al., 2017). However, the mechanisms mentioned earlier could not be the case in our study, as, surprisingly, the LCP diet led to an increase in basophil concentration and inflammation-associated biomarkers. Basophil concentrations vary widely between avian species but are more highly concentrated in birds than their mammalian counterparts (Maxwell and Robertson, 1995). Basophils are highly granular leukocytes that are chemo-attracted to inflammation sites and undergo degranulation to release histamine and Th2 cytokines and increase expression of toll-like receptors (Yoshimoto, 2018). The significant increase in basophil counts in our study indicates a systemic inflammation that might be a result of increased bacterial translocation that was observed in the LCP-fed birds. This is supported by an increase in the expression of inflammatory cytokines and chemokines, including IL-3, IL-4, IL-6, IL-10, CCL-20, and TNF-α in response to the LCP diet. The increase in liver bacterial translocation might be the underlying cause of the observed inflammatory stress. Reducing dietary CP can increase the broiler's intestinal permeability and leakage, which in turn induces bacterial translocation (Fouts et al., 2012; Benton and West, 2012; Barekatain et al., 2019). Bacterial translocation increases pro-inflammatory cytokine production (Alexopoulou et al., 2017), which adversely impacts growth performance, as nutrients are directed

toward immune responses rather than growth (Emami et al., 2019). This is evidenced in our experimental conditions by lower bodyweight, bodyweight gain, and a 15-point increase in feed conversion ratio in LCP-fed birds compared with their SCP counterparts in unpublished data. It is worth noting that Kamely et al. (2020) reported no differences in inflammatory cytokine TNF-α and IL-1β expression in the abdominal cavity exudate of Ross 708 broilers. The discrepancy between the present study and Kamely et al. (2020) suggests that a low protein diet might affect the inflammatory system in a tissue-specific manner [systemic in our experimental conditions vs. local or abdominal cavity in Kamely et al. (2020)]. The difference in the experimental approaches (diet formulation, amino acid levels, etc.) might also contribute to the discrepancy mentioned earlier. Supplementation of Spirulina to LCP diets alleviated these negative effects and reduced inflammation as evidenced by amounts of IL-3, IL-4, IL6, IL-10, CCL-20, C3, and NLRP3 mRNA that were not different from those in the SCP control diet. This was in conjunction with a seven-point increase in feed conversion ratio in algae-LCP fed birds compared with LCP in unpublished data from this trial. Qureshi et al. (1996) noted that Spirulina enhances the immune response in chickens fed a standard CP diet, and numerous trials have shown that algae ameliorate the expression of inflammatory molecular signatures in other species (Gemma et al., 2002; Chei et al., 2020; Yang et al., 2020)

Inflammasome-forming NLRP3 and non-inflammasome forming NLRs (NLRC3, NLRC5, and NLRX1) help mediate inflammatory responses (Uchimura et al., 2018), such as the one witnessed in the LCP-fed birds. The NLRs are activated in response to pathogen associated molecular patterns and damage-associated molecular patterns; however, the exact underlying mechanisms for many NLRs in chicken have yet to be elucidated. In mammals, for instance, it is known that following microbial stimuli, NLR detects molecular patterns in the cytosol and

activates the NLRP3 inflammasome, which in turn activates caspase 1, resulting in the production of IL-18 and IL-1β (Franchi et al., 2009). Chen et al. (2020) suggested that NLRP3 inflammasome activation occurred via the TLR2- MyD88-nuclear factor-kappa B (NF-κB) signaling pathway when chickens were challenged with Mycoplasma gallisepticum. The upregulation of MyD88 expression in this study supports the involvement of the previously described pathway. MyD88 acts as a downstream signaling adaptor for all TLRs except TLR3 (Akira et al., 2006). MyD88 binds to TLRs and IL-1 receptor families to activate mitogen-activated protein kinases, activator protein 1, and NF-κB pathways (Deguine and Barton, 2014). NF-κB plays an important role in producing pro-inflammatory cytokines after stress induced by gram-negative bacteria infection, reactive oxygen species, oxidized low-density lipoprotein, and multiple other factors (Hewlings et al., 2017). Previous studies in mammals described several additional secondary signals, including plasma membrane disruption for bacterial toxins, lysosome destabilization, and liposome triggering mitochondrial reactive oxygen species (ROS), leading to inflammasome oligomerization and activation (Pelegrin et al., 2007; Cassel et al., 2009; Zhong et al., 2013). Although the mechanisms by which Spirulina reduces bacterial translocation and reverses the pro-inflammatory state induced by LCP are not known at this time point, it is conceivable that Spirulina might enhance gut integrity and merits further in-depth investigations. In addition to enhanced intestinal barrier integrity, Spirulina contains high levels (180 mg/g) of the biliprotein phycocyanin, which has been shown to have antioxidant, antibacterial, and anti-inflammation properties (Manconia et al., 2009; Abd El-Baky and El-Baroty, 2012; Liao et al., 2016; Sonani et al., 2017). Hao et al. (2019) recently described a regulatory mechanism by which phycocyanin inhibits NF-κB expression and inflammation in human lung cancer cells through downregulating toll/IL-1 receptor domain-containing adaptor protein. Park et al. (2018), on the other hand, showed

that Spirulina modulates the antioxidant system in broilers. In our experimental conditions, Spirulina was also shown to modulate the systemic antioxidant defense system by lowering GPx-1 and upregulating GPx-3 and SOD2, which is quite intriguing. GPx catalyzes the reduction of various hydroperoxides to H2O via oxidation of reduced GSH into its disulfide form (Li et al., 2000). SOD2, also known as mitochondrial manganese-dependent SOD, transforms toxic superoxide (a byproduct of the mitochondrial ETC) into hydrogen peroxide and diatomic oxygen (Li et al., 2000). The differential expression between GPx-1 and GPx-3 suggests a potential compensatory mechanism; in other words, GPx-3 expression was upregulated by Spirulina to compensate for the lack of GPx-1, which might be depleted due to elevations in ROS production. The upregulation of SOD2 could result from increased xanthophyll content or perhaps suggests enhanced cytoprotection and clearance of mitochondrial ROS by C-phycocyanin derived from Spirulina (Li et al., 2016), which might explain at least partly the reduction of inflammation and cytokine expression. Spirulina also has the xanthophyll lutein, which can increase SOD2 irrespective of ROS levels (Okamoto et al., 2019). In support of our hypothesis, phycocyanin is considered a principal component responsible for antioxidant activity in algae by scavenging hydroxyl radicals (Estrada et al., 2001). Furthermore, carotenoids such as β-carotene also protect cells from oxidative stress through quenching singlet oxygen damage (Tinkler et al., 1994).

CONCLUSIONS

This is the first report, to our knowledge, defining potential molecular targets by which Spirulina inclusion reduces systemic inflammatory activity, including cytokines, chemokines, bacterial translocation, and proportions of basophils in broilers fed a low protein diet.

Author Contributions

Conceptualization, G.M., M.K., W.B. and S.D.; Methodology, G.M., G.T., M.K. and S.D.; Software, G.M., N.E. and G.T.; Validation, G.M., E.G., N.E. and S.D.; Formal Analysis, G.M., E.G., G.T. and G.E.; Investigation, G.M., E.G., G.T., M.K. and S.D.; Resources, W.B, M.K., G.T., G.E. and S.D.; Data Curation, G.M. N.E. and S.D.; Writing – Original Draft Preparation, G.M.; Writing – Review & Editing, N.E. and S.D.; Visualization, SD; Supervision, G.M., M.K. and S.D.; Project Administration, W.B., M.K. and S.D.; Funding Acquisition, W.B., S.D., and M.K.

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Conflicts of Interest

The authors declare no conflict of interest.

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TABLES

TABLE 1 | Oligonucleotide real-time qPCR primers

Gene	Accession number[a]	Primer sequence (5′ → 3′)	Orientation	Product size (bp)
TNFα	NM_204267	CGTTTGGGAGTGGGCTTTAA	Forward	61
		GCTGATGGCAGAGGCAGAA	Reverse	
IL-18	GU119895	TGCAGCTCCAAGGCTTTTAAG	Forward	63
		CTCAAAGGCCAAGAACATTCCT	Reverse	
IL-3	NM_001007083.1	CAGCACCTCCTCCCTGTCA	Forward	64
		GGCTTCATTGCTGCCCTGTA	Reverse	
IL-4	NM_0010079.1	GCTCTCAGTGCCGCTGATG	Forward	60
		GAAACCTCTCCCTGGATGTCAT	Reverse	
IL-10	NM_001004414.2	CGCTGTCACCGCTTCTTCA	Forward	63
		CGTCTCCTTGATCTGCTTGATG	Reverse	
IL-6	NM_204628.1	GCTTCGACGAGGAGAAATGC	Forward	63
		GGTAGGTCTGAAAGGCGAACAG	Reverse	
C3	NM_205405.3	CCAGAGCCTGGTCACGATGT	Forward	62
		CGATACGGAAGGAAGGGATGA	Reverse	
CRP	NM_001039564	AAGCTCAGGACAACGAGATCCT	Forward	71
		TTTCCCCCCCCACGTAGAAG	Reverse	
NLRP3	XM_001233261	GTTGGGCAGTTTCACAGGAATAG	Forward	63
		GCCGCCTGGTCATACAGTGT	Reverse	
NLRC3	XM_015294675.2	CTCCAACGCCTCACAAACCT	Forward	93
		GCCTTTGGTCATTTCCATCTG	Reverse	
NLRC5	NM_001318435.1	CTCGAAGTAGCCCAGCACATT	Forward	80
		CATGTCCAGAGGTGTCAGTCTGA	Reverse	
NLRX1	XM_003642592.4	GGCTGAAACGTGGCACAAA	Forward	59
		GAGTCCAAGCCCAGAAGACAAG	Reverse	
GPx1	NM_001277853.2	TCCCCTGCAACCAATTCG	Forward	57
		AGCGCAGGATCTCCTCGTT	Reverse	
GPx3	NM_001163232.2	GGGCGCTGACCATCGAT	Forward	59
		CATCTTCCCCGCGTACTTTC	Reverse	
SOD1	NM_205064.1	TGGCTTCCATGTGCATGAAT	Forward	58
		AGCACCTGCGCTGGTACAC	Reverse	
SOD2	NM_204211.1	GCTGGAGCCCCACATCAGT	Forward	61
		GGTGGCGTGGTGTTTGCT	Reverse	
TLR-3	NM_001011691.3	GATTGCACCTGTGAAAGCATTG	Forward	67
		CGGGTATATATGCTTGAGTGTCGTT	Reverse	
TLR-4	NM_001030693.1	TCCTCCAGGCAGCTATCAAGAT	Forward	74
		GACAACCACAGAGCTCATGCA	Reverse	
TLR-21	NM_001030558.1	CTGCTGACCGACCTCTATCACA	Forward	61
		GGTTGAGGGTGCGCAGTCT	Reverse	
MyD88	NM_001030962.4	ACCTCAAAGATCATCCAGTCCAA	Forward	63
		TGGGACACAGTCAGTGTGCAT	Reverse	
CCLL-4	NM_001045831.1	CTTGCTGTCGGGTCCAATG	Forward	60
		CGAGGGAAGTGCTCTGTTTAAGA	Reverse	
CXCL-14	NM_204712.2	CCGGCTCGCCATGAAG	Forward	54
		ATCGCGATGACCAGCAGAA	Reverse	
CCL-4	NM_204720.1	CCTGCTGCACCACTTACATAACA	Forward	63
		TGCTGTAGTGCCTCTGGATGA	Reverse	
CCL-20	NM_204438.2	TGCTGCTTGGAGTGAAAATGC	Forward	62
		CAGCAGAGAAGCCAAAATCAAA	Reverse	
18s	AF173612	TCCCCTCCCGTTACTTGGAT	Forward	60
		GCGCTCGTCGGCATGTA	Reverse	

[a]Accession number refer to Genbank (NCBI). TNFα, tumor necrosis factor alpha; IL, interleukin; C3, complement component 3; CRP, C-reactive protein; NLR, NOD-like receptor; GPX, glutathione peroxidase; SOD, superoxide dismutase; TLR, toll like receptor; MyD88, myeloid differentiation primary response 88; CCLL4, chemokine-like ligand; CXCL, C-X-C motif chemokine ligand; CCL, C-C motif chemokine ligand.

Table 2 | Effects of a standard corn/soy (SCP), low crude protein (LCP) and *Spirulina* included low crude protein diet (SP-LCP) on blood lymphocyte profiles of broilers on d 37.

Parameter	SCP	LCP	SP-LCP	SEM	P value
Heterophils, %	55.21[a]	41.34[b]	41.80[b]	2.975	0.010
Lymphocytes, %	39.14[b]	48.39[a]	52.11[a]	2.982	0.026
Heterophil/lymphocyte	1.47[a]	0.86[b]	0.84[b]	0.143	0.014
Monocytes, %	2.16	3.00	2.62	0.467	0.472
Eosinophils, %	0.12	0.14	0.14	0.036	0.901
Basophils, %	3.37[b]	7.13[a]	3.33[b]	0.854	0.012

[a,b]Means in each row with different superscripts are significantly different ($P < 0.05$).

FIGURES

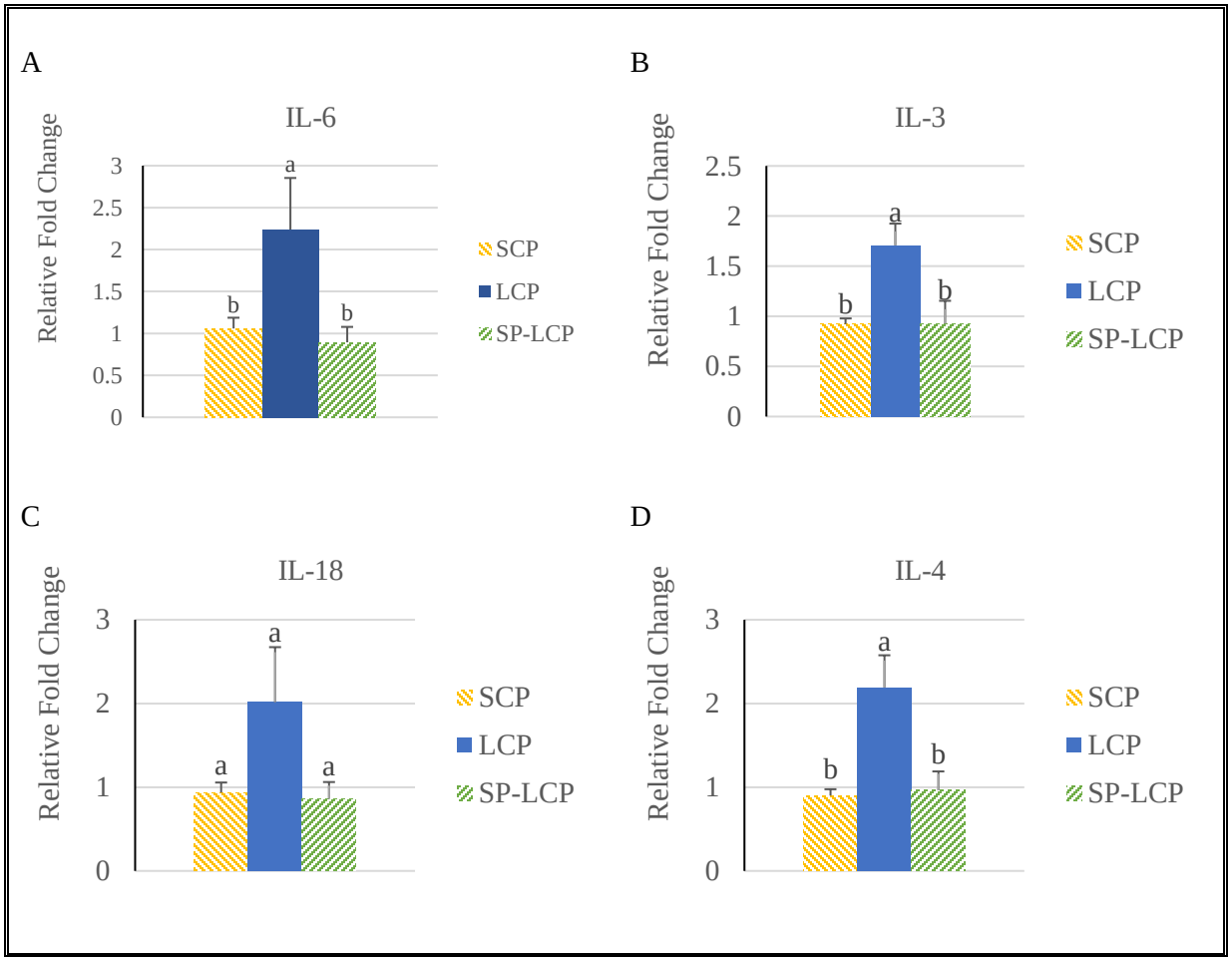

Figure 1. Effects of *Spirulina* on circulating cytokine expression profile. Relative mRNA abundance of circulating IL-6 (A), IL-3 (B), IL-18 (C) and IL-4 (D) in broilers on day (d) 37 were determined by real-time qPCR. Treatments include: standard corn/soy (SCP) diet as a control and low crude protein without (LCP) or with *Spirulina* (SP-LCP). Data are presented as mean ± SEM (n=8/ group). Means with different letters are significantly different ($P < 0.05$).

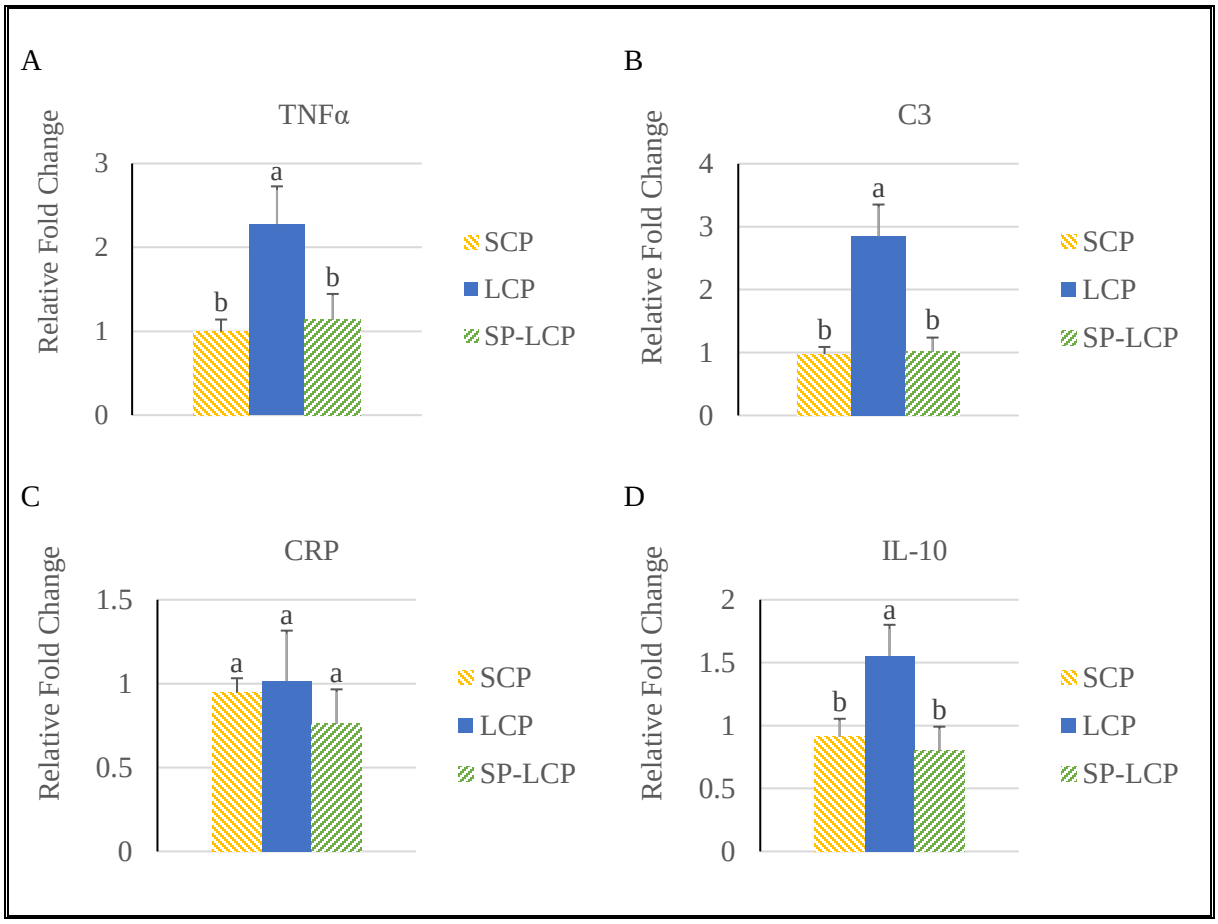

Figure 2. Effects of Spirulina on circulating TNFα, C3, CRP and IL-10 expression. Relative mRNA abundance of circulating TNF-α (A), C3 (B), CRP (C) and IL-10 expression in broilers on day (d) 37 were determined by real-time qPCR . Treatments include: standard corn/soy (SCP) diet as a control and low crude protein without (LCP) or with Spirulina (SP-LCP). Data are presented as mean ± SEM (n=8/ group). Means with different letters are significantly different ($P < 0.05$).

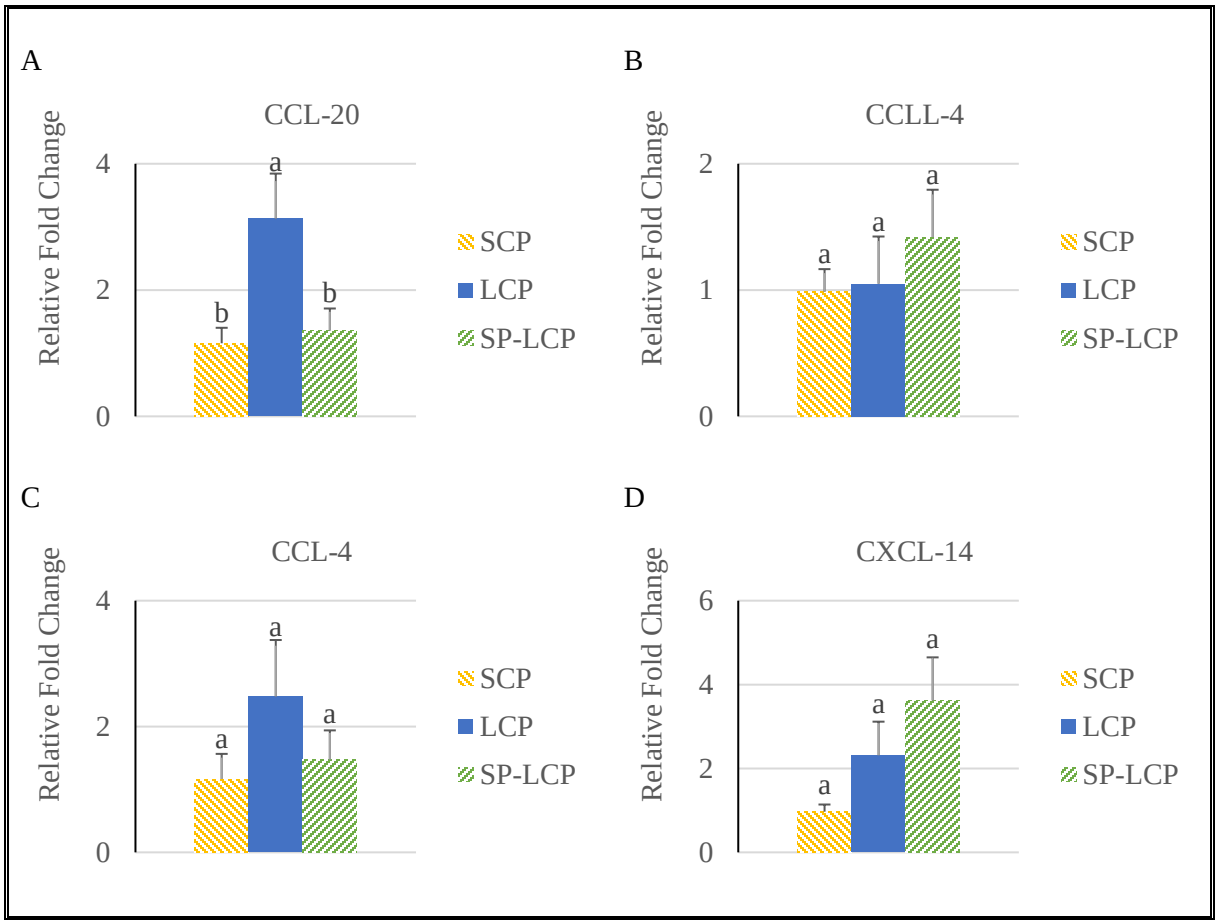

Figure 3. Effects of *Spirulina* on circulating chemokine expression profile. Relative mRNA abundance of circulating chemokine (C-C motif) ligand 20 (CCL-20) (A), chemokine-like ligand 4 (CCLL-4) (B), chemokine (C-C motif) ligand 4 (CCL-4) (C) and chemokine (C-X-C motif) ligand 14 (CXCL-14) (D) in broilers on day (d) 37 were determined by real-time qPCR. Treatments include: standard corn/soy (SCP) diet as a control and low crude protein without (LCP) or with Spirulina (SP-LCP). Data are presented as mean ± SEM (n=8/ group). Means with different letters are significantly different ($P < 0.05$).

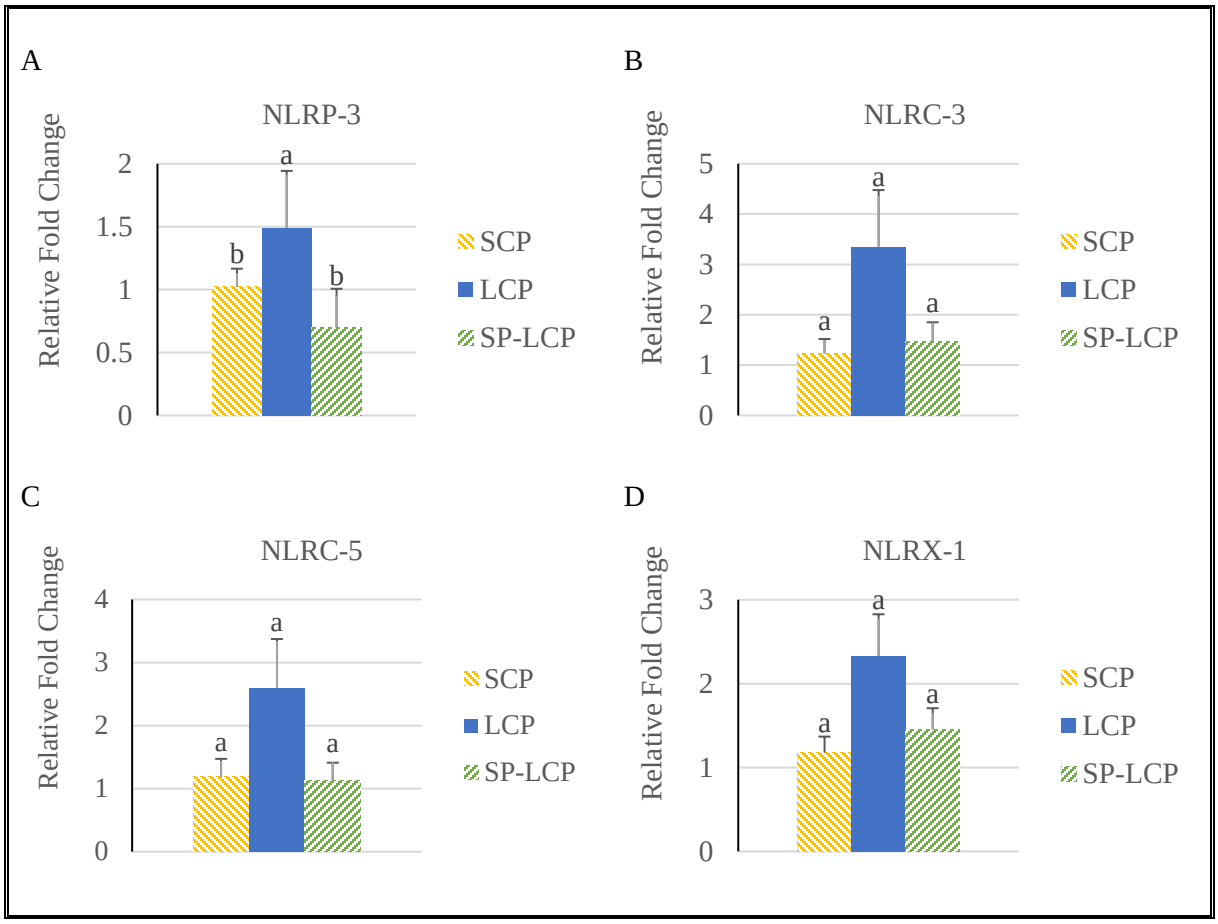

Figure 4. Effects of Spirulina on circulating Inflammasome Expression. Relative mRNA abundance of NOD-like receptor family pyrin domain containing 3 (NLRP-3) (A), NOD-like receptor family CARD domain containing 3 (NLRC-3) (B), NOD-like receptor family CARD domain containing 5 (NLRC-5) (C) and nucleotide-binding oligomerization domain, leucine rich repeat containing X1 (NLRX-1) (D) in broilers on day (d) 37 were determined by real-time qPCR. Treatments include: standard corn/soy (SCP) diet as a control and low crude protein without (LCP) or with Spirulina (SP-LCP). Data are presented as mean ± SEM (n=8/ group). Means with different letters are significantly different ($P < 0.05$).

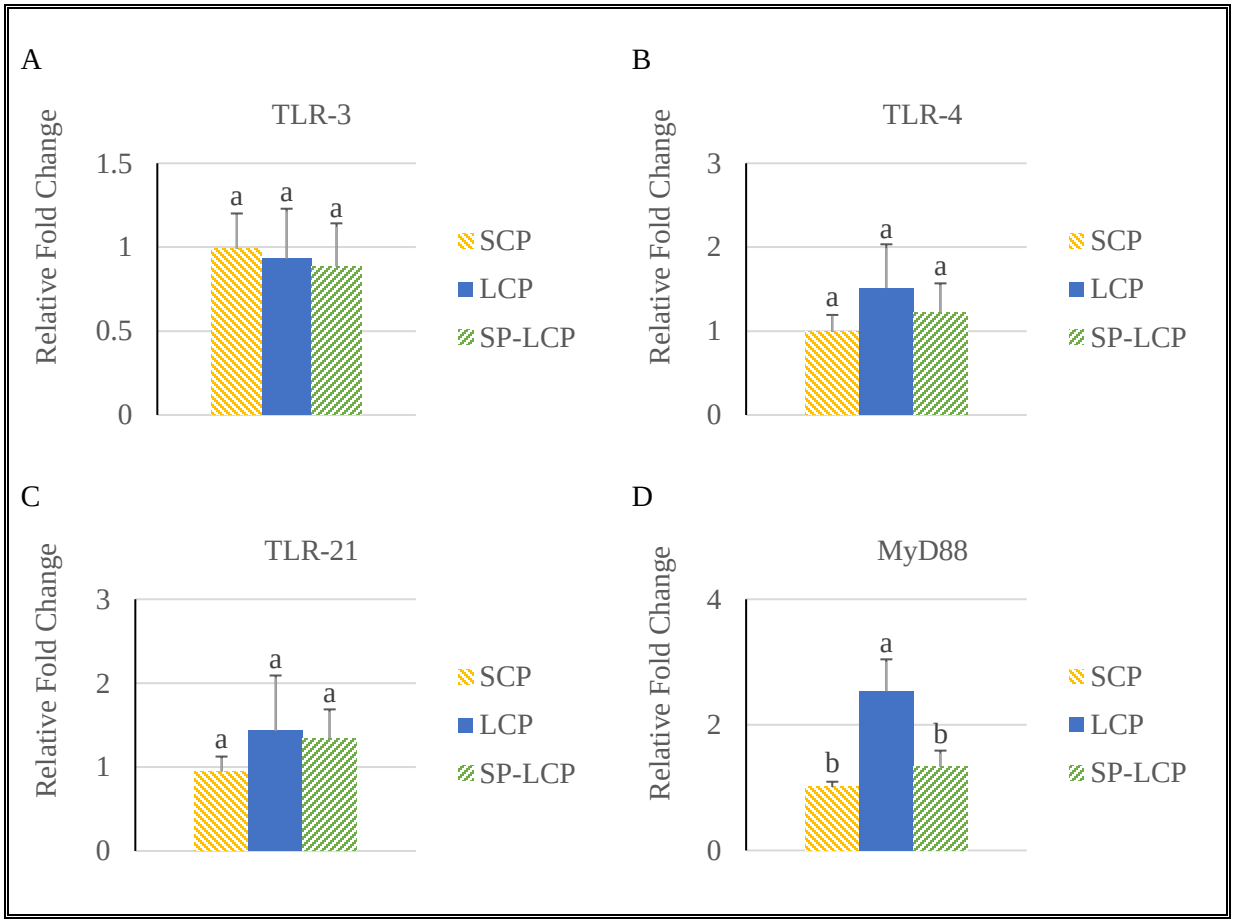

Figure 5. Effects of Spirulina on Circulating Toll-like Receptor Expression. Relative mRNA abundance of toll-like receptor 3 (TLR-3) (A), toll-like receptor 4 (TLR-4) (B), toll-like receptor 21 (TLR-21) (C) and myeloid differentiation primary response protein 88 (MyD88) (D) in the blood of broilers on day (d) 37 were determined by real-time qPCR. Treatments include: standard corn/soy (SCP) diet as a control and low crude protein without (LCP) or with Spirulina (SP-LCP). Data are presented as mean ± SEM (n=8/ group). Means with different letters are significantly different ($P < 0.05$).

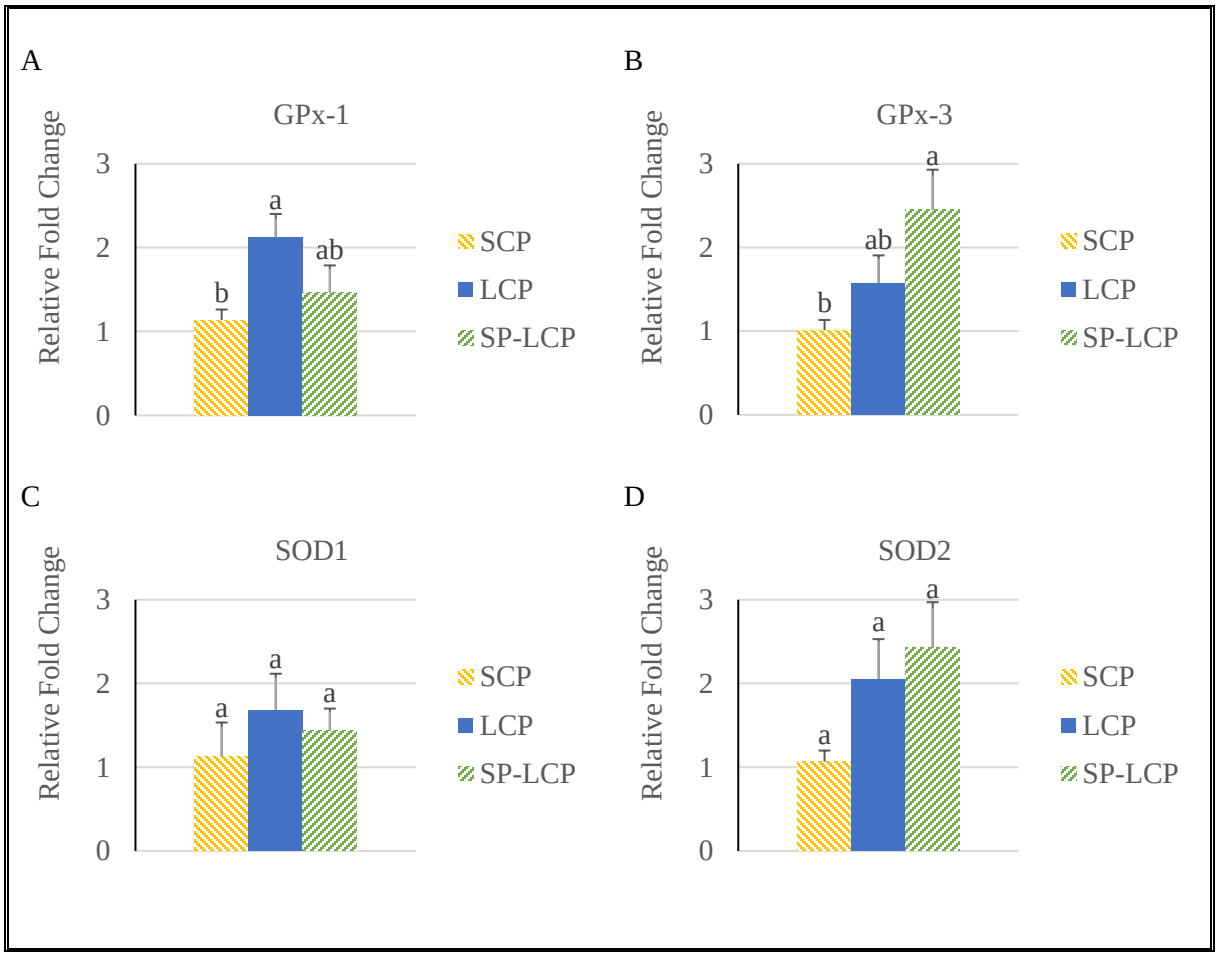

Figure 6. Effects of Spirulina on the expression of circulating antioxidant defense system. Relative mRNA abundance of glutathione peroxidase 1 (GPx-1) (A), glutathione peroxidase 3 (GPx-3) (B), superoxide dismutase 1 (SOD1) (C), and superoxide dismutase 2 (SOD2) (D) in the blood of broilers on day (d) 37 were determined by real-time qPCR. Treatments include: standard corn/soy (SCP) diet as a control and low crude protein without (LCP) or with Spirulina (SP-LCP). Data are presented as mean ± SEM (n=8/ group). Means with different letters are significantly different ($P < 0.05$).

V. SPIRULINA PLATENSIS MEAL TITRATION INTO MALE BROILER GROWER AND FINISHER

ABSTRACT

This study was conducted to examine the effect of feeding modern male commercial broilers Spirulina (*Arthrospira platensis*) on growth performance, carcass characteristics, woody breast myopathies, and breast fillet (*pectoralis major*) pigmentation during the grower and finisher phase. A total of 1,040 one day old Cobb 500 broiler chicks were allotted to one of two experiments and fed a common starter diet (1 to 14 d). Each experiment consisted of 6 treatments with experiment 1 being conducted from 15 to 35 d (grower phase) and contained 0 to 10% Spirulina. Experiment 2 was conducted from 34 to 49 d (finisher phase) and treatments contained 0 to 5% Spirulina. Body weight gain (BWG), mortality corrected feed conversion (FCR), and average feed intake (FI) were calculated for 15 to 34 and 34 to 48 d of age for Experiment 1 and 2, respectively. Five birds were randomly selected from each pen for processing on day 35 for Experiment 1 and day 49 for Experiment 2. Results showed no significant difference of means between Spirulina inclusion treatments on any performance parameters or carcass yields. Overall breast fillets showed low incidences of woody breasts and were not impacted by dietary Spirulina level. Breast fillet redness and yellowness increased linearly ($P<0.05$) through 5% Spirulina inclusion during the finisher phase. During the grower phase, max pigmentation seemed to reach max redness at 6% and yellowness at 8% dietary Spirulina level. In conclusion, the present findings indicate that Spirulina (*Arthrospira platensis*) can be fed at 10% from 15 to 34 and 5% from 34 to 48 without significantly impacting performance parameters, incidences of woody breast, or processing yields. Feeding increased levels of Spirulina heavily influences breast redness and yellowness.

INTRODUCTION

The search for alternative protein providing feedstuffs to soybean meal has been going on for the better part of a decade, yet it's nutrient profile and high digestibility make it a staple in most poultry diets around the world. The use of soybean meal will always be essential for poultry production, but if meat consumption increases as anticipated (FAO, 2021) there will be a need for accompanying protein sources (especially in countries with limited soybean production).

Microalgae is considered to be one possible alternative as it's viewed as environmentally friendly and has benefits beyond the scope of its protein level (Parisi et al., 2020). Spirulina (*Arthrospira platensis*) is one blue-green cyanobacteria microalgae that is particularly interesting since it has a high protein content (55-70%), balanced amino acid profile and is a rich source of vitamins, essential fatty acids, minerals, and phytochemicals (Seyidoglu et al., 2017). Spirulina is primarily used as a human health supplement and as a food colorant with an estimated 30% of current algal production going to animal feed (Becker, 2007). Cost continues to deter the further use of microalgae in animal feed; however, increased production of microalgae will likely drive down costs.

The limited production of Spirulina (5,000 tons/year) is a result of its costly production methods, but progress is being made on maximizing its growth potential (Beal et al., 2015; Tibbets, 2018). There are also incentives to increase microalgae production as it has a low aerial footprint, potential for waste water remediation and carbon scrubbing potential through closed system photobioreactors (Parisi et al., 2020). The closed system photobioreactors have a high initial capital investment, but are smaller, have a higher growth efficiency, use little to no water, and can mitigate some of the input costs associated with microalgae production in classical open

systems (Carvalho et al., 2006). These systems require continued development if microalgae strains are to be utilized on a commercial scale in animal production.

The sustainability aspect of microalgae, as well as its anti-oxidant and anti-inflammatory properties, could become more industry relevant as the poultry industry continues to move towards consumer preferences for value added products and antibiotic fee production (Qureshi et al., 1996; Lum et al., 2013; Park et al., 2018; Mullenix et al., 2021). There are numerous trials testing Spirulina's impact on broiler growth, with 10% being generally considered max inclusion level without hindering broiler performance. Thus, two titration studies were conducted to determine the impact of Spirulina (*Arthrospira platensis*) inclusion on growth performance and carcass characteristics of 35 and 49 d male broilers.

MATERIAL AND METHODS

All management practices and procedures were approved by the University of Arkansas Institutional Animal Care and Committee (IACUC) # 21055

Bird husbandry and housing

One thousand and forty (1-day-old Cobb 500) male broiler chicks were obtained from a local commercial hatchery (Springdale, AR) and transported to a solid walled broiler research facility at the University of Arkansas Research farm. All birds were reared in floor pens with reused built-up litter that was topped dressed with fresh pine shavings. Each pen was equipped with a section of continuous nipple drinker line (four nipples per pen), a hanging tube feeder, and supplemented with a cardboard chick tray for the first 10 days. The 1-day old chicks were randomly allocated into 120 pens (0.91 x 1.22 m) at 12 birds per pen, allowing 0.09m^2 per bird. House temperatures were set to 32°C at day 1 and gradually decreased to 18°C at the conclusion of the experiment. The lighting program was 24 hours of light from day 1 to 2, 23 hours of light

from day 2 to 11, and 18 hours of light from day 11 through the conclusion of the experiment. Feed and water were offered *ad libitum* for the duration of the trial. Birds were observed for overall health and mortality was recorded twice daily. A common crumble starter was fed to all birds from 1 to 14 days of age containing primary breeder recommendations of 21% CP, 1.2% dLys, and 3,000 kcal/kg AME_n.

Experimental Treatments

Experiment 1 utilized 720 broilers and had a two-phase feeding program with the experimental period from 15 to 35 day. A conventional corn/soybean meal grower "basal" diet and Spirulina based grower diet (10% Spirulina) were formulated within the ideal protein concept to have similar AME_n (3,085 kcal/kg), CP (22%), dLys (1.1%), dTSAA (0.84%), dThr (0.74%), dVal (0.85%), and dIle (0.73%) (Table. 1). Blending these two diets in ratios of 100:0, 80:20, 60:40, 40:60, 20:80 produced diets with 0, 2, 4, 6, 8, and 10% Spirulina with 10 replicates per treatment.

Experiment 2 utilized 720 broilers and had a three-phase feeding program with the experimental period from 34 to 49 day. The 0% Spirulina diet from experiment 1 was fed to all birds in experiment 2 from 15 to 34 d. A conventional corn/soybean meal finisher "basal" diet and Spirulina based finisher diet (5% Spirulina) were formulated within the ideal protein concept to have similar AME_n (3,150 kcal/kg), dLys (1.00%), dTSAA (0.76%), dThr (0.67%), dVal (0.77%), and dIle (0.66%) (Table. 1). Blending these two diets in ratios of 100:0, 80:20, 60:40, 40:60, 20:80 produced diets with 0, 1, 2, 3, 4, and 5% Spirulina with 10 replicates per treatment. All experimental diets were pelleted after mixing. Dried Spirulina platensis powder meal was sourced from a commercial producer (Pond Tech., Markham, Canada) and analyzed for amino

acid concentrations (NOVUS, St. Charles, MO) prior to diet formulation. Digestible amino acid coefficients and metabolizable energy of Spirulina Platensis from Mullenix et al. (2022).

Live performance and Carcass characteristics

Pen body weights and feed consumption were recorded at the start (Experiment 1, day 15; Experiment 2, day 34) and at the conclusion of each experimental period (Experiment 1, day 34; Experiment 2, day 48) prior to processing. Body weight gain (BWG), mortality corrected feed conversion (FCR), and average feed intake (FI) were calculated for 15 to 34 and 34 to 48 days of age for Experiment 1 and 2, respectively. Five birds were randomly selected from each pen for processing on day 35 for Experiment 1 and day 49 for Experiment 2. Birds were processed at the University of Arkansas Pilot Processing Plant (Fayetteville Arkansas) via a commercial inline system. Birds were placed on shackles and electrically stunned (11 V, 11 mA for 11 s) before being exsanguinated, soft scalded (55°C for 2 min), de-feathered, and mechanically eviscerated. Breast fillets were subjectively hand scored, by trained personal, for woody breast (WB) on a whole number increment scale with 0 being no signs of WB, 1 being mild to moderate WB, and score 2 being severe WB according to Tijare et al. (2016). Carcass and abdominal fat pad weights were recorded immediately post evisceration. Carcasses were chilled for 4 hours at 4°C before breasts, tenders, wings, and leg quarters were removed and weights recorded. Breast fillet from each bird was collected and stored at 4°C for 24 hours for colorimetric analysis. Readings were taken with a colorimeter (CR-400; Konica Minolta Sensing Inc., Sakai Osaka, Japan; size 102 (W) × 217 (H) × 63 (D) mm) using illuminant D65 and a 2-degree observer to determine the L* (Lightness), a* (redness), and b* (yellowness) values on the ventral side of the right breast fillet.

Statistical Analysis

Data were analyzed using JMP Pro15 (JMP, 2018) as a randomized, complete block design, with pen as the experimental unit. Treatments in both experiments were represented by ten replicate pens. Linear and quadratic analysis were analyzed using fit model procedure with standard least squares. Significance was indicated at $P<0.05$.

RESULTS AND DISCUSSION

Diet analysis and live performance parameters

The analyzed concentrations of CP and AA in experiment 1 were similar to the formulated values (Table. 1). The analyzed CP and AA content of the Spirulina diets in experiment 2 were less than calculated. In experiment 1, Spirulina inclusion level did not impact 34d BW, ADG, FI, FCR, or mortality (Table. 2). There were also no statistical differences on 48d BW, ADG, FI, FCR or mortality in experiment 2 (Table. 3). However, dietary Spirulina worsened FCR by 3% when increased from 0 to 5% of diet ($P=0.06$) in experiment 2.

Carcass characteristics and woody breast analysis

In experiment 1, carcass, breast, tender, wing, leg quarter and fat yield were not impacted by Spirulina inclusion into the diet (Table. 4). There was a quadratic response for carcass ($P=0.0342$) and breast yields ($P=0.0454$) in experiment 2, where yields were highest at 0 and 5% inclusion level (Table. 5). Tender, wing, leg quarter, and fat yields were not impacted by Spirulina level in experiment 2. As shown in Table 6 and 7, Spirulina inclusion level did not impact average WB score or distribution of WB scores in either experiment.

Breast colorimetric

A quadratic response ($P=0.0042$) to increasing dietary Spirulina was observed for breast yellowness in experiment 1 (Table. 8). Breast lightness ($P=0.0383$) and redness ($P=0.0018$)

showed a linear response to increasing dietary Spirulina level in experiment 1. In experiment 2, breast redness (P=0.0106) and yellowness (P<.0001) had a linear response to increasing dietary Spirulina inclusion (Table. 9). No significant differences were found for lightness (L*) in experiment 2.

Broiler final body weights exceeded the primary breeders target BW in both experiments (Cobb-Vantress, 2018). Both experiments averaged acceptable mortality (average mortality of 2.17 and 2.58% for experiment 1 and 2, respectively) and all broilers appeared healthy throughout the trials. The differences in analyzed and calculated values of the experimental diets are likely a result of over or under estimation of corn, SBM, or meat & bone meal AA content and CP level, as great caution was used during diet mixing and Spirulina analysis. Also, diets in experiment 1 contained meat & bone meal, whereas diets in experiment 2 were all vegetable based.

The performance results suggest that male broilers can be fed up to 10% Spirulina from 15 to 34 d and 5% from 34 to 48 d without significantly impacting BWG, FCR or FI. This is in alignment with previous findings that showed Spirulina platensis can be included into broiler diets at 6 (1 to 41 d), 8 (21 to 37 d) or 10% (1 to 35 d) without impacting performance parameters (Ross and Dominy., 1990; Toyomizu et al., 2001; Qureshi et al., 1996, respectively). Spirulina inclusion beyond 10% has mixed results with reports of 12 and 15% Spirulina diets having adverse effects on performance (Ross and Dominy., 1990; Pestana et al., 2020), while Venkartarman et al. (1994) substituted in 17% Spirulina into fishmeal and groundnut cake meal-based diets without hindering performance. The differences in performance results are likely a result of varying spirulina chemical composition, genetics, management practices, and experimental design. Evans et al. (2015) theorized that high Spirulina level diets (exceeding

16%) caused protein gelation, thus preventing thorough endogenous enzymes access to digesta. It's thought that protein gelation or the difference between calculated and analyzed diets may explain the (non-significant) 9-point increase between 2 and 3% Spirulina inclusion in experiment 2. Research is being conducted to address the viscosity concern of spirulina usage with exogenous enzymes (Pestana et al., 2020).

Literature regarding processing characteristic response to Spirulina inclusion is much more sparce considering skilled labor and costly equipment needed for processing an adequate number of replicates in an acceptable time frame. Toyomizu et al. (2001) and Altmann et al. (2018) found no differences in breast fillet yield, which aligns with the current trials that showed no significant difference of means between any yield values. Fat pad deposition was also unaffected by algae addition, yet Spirulina has been shown to influence meat quality by aiding in deposition of polyunsaturated fatty acids (Altmann et al. 2018; Bonos et al., 2016; Pestana et al., 2020).

Spirulina's prominent impact of muscle pigmentation is well established (Venkartarman et al., 1994), and Toyomizu et al. (2001) accredited zeaxanthin as the main source of carotenoid pigment from Spirulina for muscle tissue yellowness. Toyomizu et al. (2001) stated that breast yellowness increased in a linear fashion up to 8% inclusion, which relates with the current experiment 1 data. The 5% Spirulina inclusion diet showed the strongest yellowness in experiment 2 with a strong linear regression ($P<0.0001$). Raach-Moujahed at al. (2011) found an increase in breast yellowness at 2.5% Spirulina inclusion but not at 5% of the diet, while redness wasn't impacted. Toyomizu et al. (2001) found increased redness at 4% algae inclusion but not at 8%. Breast redness increased linearly ($P<0.05$) in both of the current experiments. Small usage

of microalgae (≤1%) has been reported in having no impact on breast muscle color (Raach-Moujahed at al. 2011; Park et al., 2018).

CONCLUSIONS

The present findings indicate that Spirulina (*Arthrospira platensis*) can be fed at 10% from 15 to 34 and 5% from 34 to 48 without significantly impacting performance parameters, incidences of woody breast, or processing yields. Feeding increased levels of spirulina heavily influences breast fillet (Pectoralis major) redness and yellowness.

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TABLES

Table 1. Composition of experimental diets, "as-fed" basis

	Experiment 1 (15-35 d)		Experiment 2 (34-45 d)	
	Basal[1]	Spirulina[1]	Basal[2]	Spirulina[2]
Ingredient, %				
Corn	58.63	61.57	67.62	73.37
SBM (48%)	33.47	21.99	27.72	18.44
Spirulina Meal	–	10.00	–	5.00
Meat and Bone Meal	3.00	3.00	–	–
Poultry fat	2.39	1.27	1.18	–
Dicalcium P	0.83	0.37	1.49	1.29
Limestone	0.60	0.92	0.87	1.04
NaCl	0.36	0.06	0.27	0.14
NaHCO[3]	–	–	0.22	–
Premixes[3]	0.18	0.18	0.18	0.18
DL-Met	0.22	0.22	0.16	0.16
L-Lys HCl	0.16	0.30	0.17	0.28
L-Thr	0.07	0.07	0.06	0.05
L-Val	0.09	0.06	0.07	0.05
Estimated composition				
Crude protein, %	21.8	21.9	19.8	19.3
AME, kcal/kg	3,085	3,085	3,150	3,150
Calcium, %	0.84	0.84	0.80	0.80
Available phos., %	0.41	0.41	0.40	0.40
TSAA, d	0.84	0.84	0.76	0.76
Lys, d	1.10	1.10	1.00	1.00
Thr, d	0.74	0.74	0.67	0.67
Ile, d	0.73	0.73	0.66	0.66
Val, d	0.85	0.85	0.77	0.77
Analyzed composition (%)				
Crude Protein	22.4	23.2	19.1	17.7
TSAA	0.95	1.02	0.80	0.82
Lys	1.32	1.36	1.12	1.09
Thr	0.95	0.97	0.80	0.77
Ile	0.92	0.99	0.78	0.71
Val	1.07	1.14	0.93	0.86

[1]Ratios of 100:0, 80:20, 60:40, 40:60, 20:80 were blended to produce diets with 0, 2, 4, 6, 8, and 10% Spirulina.
[2]Ratios of 100:0, 80:20, 60:40, 40:60, 20:80 were blended to produce diets with 0, 1, 2, 3, 4, and 5% Spirulina.
[3]Added per kg of finished feed: vitamin A, 9,259 IU; vitamin D3, 6,614 ICU; vitamin E, 66 IU; niacin, 46 mg; d-pantothenic acid, 12 mg; riboflavin, 8 mg; pyridoxine, 3 mg; thiamine, 2 mg; menadione, 2 mg; folic acid, 1 mg; biotin, 0.1 mg; vitamin B12, 0.02 mg; manganese, 96 mg; zinc, 58 mg; copper, 2.7 mg; iodide, 1.9 mg; selenium, 0.16 mg.

Table 2. Live performance of Cobb 500 males fed diets varying in Spirulina inclusion 14 to 34 d (Experiment 1)

Treatment (Spirulina, %)	34d BW, kg	ADG, g	FI, kg	FCR[1], g:g	Mortality, %
0	2.578	104.9	3.294	1.56	3.64
2	2.537	103.2	3.271	1.58	0.91
4	2.493	101.0	3.249	1.62	2.73
6	2.562	104.2	3.311	1.59	0.91
8	2.568	104.8	3.366	1.58	2.82
10	2.559	104.3	3.297	1.58	2.02
SEM	0.029	1.346	0.045	0.016	0.013
P-value					
Linear	0.8038	0.6943	0.3233	0.5461	0.6919
Quadratic	0.1798	0.1623	0.8240	0.1239	0.3839

[1]FCR=Mortality Corrected feed to gain ratio

Table 3. Live performance of Cobb 500 males fed diets varying in Spirulina inclusion 34 to 48 d (Experiment 2)

Treatment (Spirulina, %)	48d BW, kg	ADG, g	FI, kg	FCR[1], g:g	Mortality, %
0	4.086	108.0	3.430	2.04	1.82
1	4.108	110.0	3.450	2.01	1.82
2	4.079	110.3	3.508	2.04	3.64
3	4.030	105.9	3.478	2.13	2.73
4	4.081	105.8	3.500	2.12	3.64
5	4.103	106.5	3.476	2.11	1.82
SEM	0.0515	2.7815	0.0591	0.0474	1.449
P-value					
Linear	0.9164	0.2885	0.4688	0.0587	0.7034
Quadratic	0.4959	0.7543	0.4933	0.8664	0.3324

[1]FCR=mortality corrected feed to gain ratio

Table 4. Carcass Yields of 35 d Cobb 500 males fed diets varying in Spirulina inclusion

Treatment (Spirulina, %)	Yield[1], %					
	Carcass	Breast	Tender	Wing	Leg Quarter[2]	Fat
0	72.17	19.80	3.81	8.06	22.01	1.09
2	72.46	20.38	3.88	7.86	21.89	1.13
4	72.06	19.47	3.85	7.91	22.10	1.20
6	72.19	20.25	3.90	7.95	21.47	1.11
8	72.31	20.28	3.89	7.83	21.78	1.19
10	72.45	20.25	3.94	7.95	21.81	1.19
SEM	0.27	0.23	0.04	0.06	0.15	0.05
P-value						
Linear	0.6525	0.1965	0.0620	0.2350	0.1326	0.1959
Quadratic	0.5991	0.7331	0.8803	0.1437	0.4338	0.7228

[1]Yields represent chilled carcass parts relative to live body weight

[2]bone-in, skin-on thighs and drumsticks

Table 5. Carcass Yields of 49 d Cobb 500 males fed diets varying in Spirulina inclusion

Treatment Spirulina, %)	Yield[1], %					
	Carcass	Breast	Tender	Wing	Leg Quarter[2]	Fat
0	75.88	23.00	4.40	7.90	22.63	1.18
1	75.72	22.87	4.44	7.90	22.75	1.18
2	75.53	22.54	4.34	7.85	22.96	1.28
3	75.76	22.46	4.36	7.99	22.99	1.16
4	75.57	22.65	4.41	7.85	22.64	1.26
5	76.15	23.14	4.47	7.87	22.76	1.20
SEM	0.19	0.28	0.06	0.06	0.21	0.06
P-value						
Linear	0.4743	0.994	0.5742	0.7973	0.8469	0.6462
Quadratic	0.0342	0.0454	0.2000	0.6633	0.2543	0.5770

[1]Yields represent chilled carcass parts relative to live body weight

[2]bone-in, skin-on thighs and drumsticks

Table 6. Woody breast incidence of 35 d Cobb 500 males fed diets varying in Spirulina inclusion

		Distribution[1], %		
Treatment (Spirulina, %)	Average	0	1	2
0	0.40	66	28	6
2	0.44	66	24	10
4	0.26	84	6	10
6	0.28	78	16	6
8	0.36	74	16	10
10	0.36	73	18	9
SEM	0.08	5.68	5.37	3.88
P-value				
Linear	0.5035	0.2541	0.1589	0.7428
Quadratic	0.2760	0.1208	0.0519	0.9135

[1]Distibution based on the percent incidence of each score per pen observed in the four processed broilers

[2]Woody breast: 0=normal; 1=moderate myopathy; 2=severe myopathy

Table 7. Woody breast incidence of 49 d Cobb 500 males fed diets varying in Spirulina inclusion

		Distribution[1], %		
Treatment (Spirulina, %)	Average	0	1	2
0	0.64	52	32	16
2	0.66	50	34	16
4	0.58	52	40	8
6	0.52	58	32	10
8	0.52	58	32	10
10	0.64	50	34	16
SEM	0.09	6.64	7.07	4.57
P-value				
Linear	0.5257	0.7142	0.9448	0.6749
Quadratic	0.3068	0.7654	0.9290	0.3008

[1]Distibution based on the percent incidence of each score per pen observed in the five processed broilers

[2]Woody breast: 0=normal; 1=moderate myopathy; 2=severe myopathy

Table 8. Meat color of 35 d Cobb 500 males fed diets varying in Spirulina inclusion

	Yield, %		
Treatment (Spirulina, %)	L*	a*	b*
0	55.25	3.51	8.14
2	55.40	3.95	9.33
4	55.81	4.00	9.72
6	56.01	4.26	10.21
8	56.20	4.29	10.87
10	55.90	4.21	9.74
SEM	0.34	0.18	0.37
P-value			
Linear	0.0383	0.0018	0.0002
Quadratic	0.3185	0.1007	0.0042

a-c Means without a common superscript were determined to be significantly different ($P < 0.05$) by t test.
Breast=Skinless *Pectoralis major*
L*=lightness, a*=redness, b*=yellowness

Table 9. Meat color of 49 d Cobb 500 males fed diets varying in Spirulina inclusion

	Yield, %		
Treatment (Spirulina, %)	L*	a*	b*
0	56.99	2.94	8.89
1	57.30	3.31	9.82
2	56.50	3.46	9.82
3	56.53	3.42	9.90
4	56.37	3.52	10.22
5	56.50	3.63	10.62
SEM	0.493	0.188	0.275
P-value			
Linear	0.2034	0.0106	<.0001
Quadratic	0.7042	0.3754	0.5818

a-c Means without a common superscript were determined to be significantly different ($P < 0.05$) by t test
Breast=Skinless *Pectoralis major*
L*=lightness, a*=redness, b*=yellowness

VI. SPIRULINA (ARTHROSPIRA PLATENSIS) AND LOW PROTEIN IMPACT ON MALE BROILER GROWTH PERFORMANCE, WATER CONSUMPTION AND LITTER QUALITY

ABSTRACT

Six hundred and twenty-four male Cobb 500 broilers were used in this 2x2 factorial design. Two levels of dietary protein (17.5 and 20%) and spirulina (SP: 0 and 2.5%) were utilized to create the dietary treatments: Control, Control+SP, Low CP, and Low CP+SP. The experimental period was conducted from 14 to 35 day and feed intake, body weight gain, mortality, feed conversion ratio, water intake, water conversion ratio, and water intake/feed intake ratio were calculated. Litter samples and flux gases were collected from each pen before (13 d) and at the conclusion (34 d) of the experimental period. Ten birds from each pen were processed on day 35, and carcass yields calculated. Incidences of woody breast and footpad quality were recorded. Body weight gain and FCR were impaired in broilers fed the 17.75% CP diet. In addition, all carcass yields were also impaired and fat deposition increased in broilers fed the 17.75% CP diet. However, the low CP diet did benefit from less water consumption, better footpad quality, and less carbon dioxide litter flux. Spirulina inclusion of 2.5% did not impair body weight gain, but FCR was increased through higher feed intake. Spirulina also reduced carcass and breast yields, while increasing pigmentation in the breast fillets.

INTRODUCTION

Environmental impact of the agricultural industry is an important topic when discussing future sustainability as almost 30% of global greenhouse gas emissions come from current food production practices (Vermeulen et al., 2012). A U.S. broiler chicken industry sustainability report (NCC, 2020) stated that land use, carbon footprint, water consumption, fossil fuel usage, and particulate emissions have all decreased per kg of live weight production from 2010 to 2020. This was brought on by an industry wide effort to produce more efficient broilers for both financial and environmental reasons. Even with the poultry industry's ability to continue

producing more yield with less environmental impact there was an increase in total land use (5.4%), water consumption (5.4%), and particulate emissions (4.4%) in the previous decade (NCC, 2020). Although the poultry industry is considered the least environmentally taxing meat production, consistent and improved efficiency is needed (FAO, 2021).

Parisi et al. (2020) outlined the need for alternative environmentally friendly dietary protein sources going forward to meet this increased demand. Microalgae is one potential protein source that has been primarily produced for the human health market, but has enormous environmental incentives for its expanded use. Microalgae is renewable, has a high nutrient value, produced on less land that terrestrial crops, can serve as a waste water remediator, and can be used to sequester CO_2 from carbon polluters (FAO, 2008). It can also be produced within closed systems with essentially no water use. *Arthrospira platensis*, commonly referred to as spirulina (SP), (blue-green cyanobacteria) is the most commonly utilized microalgae due to its high protein content (60-70%), non-toxicity, balanced amino acid profile and is a rich source of vitamins, essential fatty acids, minerals, and bioactive phytochemicals (Bishop and Zubeck, 2012). Reductions in crude protein (CP) and supplementation of more non-bound amino acids is becoming more common place in the broiler diets (Liu et al., 2021). The objective of this study was to determine the effect of 2.5% SP inclusion and varying CP level of broiler diets on broiler growth, water intake, carcass yields, pigment deposition, breast myopathy, footpad quality, gas emissions, and litter quality.

MATERIALS AND METHODS

All management practices and procedures were approved by the University of Arkansas Institutional Animal Care and Committee (IACUC) # 21120

Bird husbandry and housing

Six hundred and twenty-four (1-day-old Cobb 500) male broiler chicks were obtained from a local commercial hatchery (Siloam Springs, AR) and transported to a solid walled broiler research facility at the University of Arkansas Research farm. All birds were reared in floor pens with reused built-up litter that was topped dressed with fresh pine shavings. Each pen was equipped with a section of continuous nipple drinker line (two nipples per pen), a hanging tube feeder, and supplemented with a cardboard chick tray for the first 10 days. The 1-day old chicks were randomly allocated into 24 pens (1.5 x 3.0 m) at 26 birds per pen, allowing 0.17m^2 per bird. House temperatures were set to 32°C at day 1 and gradually decreased to 18°C at the conclusion of the experiment. The lighting program was 24 hours of light from day 1 to 2, 23 hours of light from day 2 to 11, and 18 hours of light from day 11 through the conclusion of the experiment. Feed and water were offered ad libitum for the duration of the trial. Birds were observed for overall health and mortality was recorded twice daily.

Experimental Treatments

A two-phase feeding program was used, which consisted of a common starter phase and experimental period from 15 to 35 day. A common crumble starter was fed to all birds from 1 to 14 days of age containing primary breeder recommendations of 21% CP, 1.2% dLys, and 3,000 kcal/kg AME_n. A 2x2 factorial was utilized with two levels of CP (17.75 and 20%) and SP (0 and 2.5%); Control, Control+SP, Low CP, and Low CP+SP (Table. 1). The four experimental diets (6 replicates) were formulated within the ideal protein concept to have similar AME_n (3,085 kcal/kg), dLys (1.10%), dTSAA (0.84%), dThr (0.74%), dVal (0.85%), and dIle (0.73%), dArg (1.10%), and dTrp (0.18%). All experimental diets were offered as pellets. Dried spirulina (*Arthrospira platensis*) powder meal was sourced from a commercial producer (Pond Tech.,

Markham, Canada), and digestible amino acid coefficients and metabolizable energy values from Mullenix et al. (2021) were used for formulation. All protein containing ingredients were analyzed for amino acid concentrations (NOVUS, St. Charles, MO) prior to feed manufacturing.

Live performance and Water consumption

Group body weights and feed consumption were recorded at 14 and 34 d post-hatch. Feed intake, body weight gain, mortality, and mortality corrected feed conversion ratio (FCR) were calculated for the experimental period of 14 to 34 d. Water was offered ad-libitum and recorded throughout trial with self-contained water system (Hiltz et al., 2021). Water intake, mortality corrected water conversion ratio (WCR), and water intake/feed intake ratio were calculated for the experimental period of 14 to 34 d.

Carcass characteristics

Ten birds were randomly selected from each pen for processing on day 35. All birds were processed at the University of Arkansas Pilot Processing Plant (Fayetteville Arkansas) via a commercial inline system. Birds were placed on shackles and electrically stunned (11 V, 11 mA for 11 s) before being exsanguinated, soft scalded (55 C for 2 min), de-feathered, and mechanically eviscerated. Footpad scores were recorded by one individual to limit observational error with a whole number increment scale: 0 - ideal foot pad with no redness or lesions; 1 – slight callus with minor redness smaller than 1.8 cm; 2 – lesions with significant calluses larger than 1.8 cm. Skinless breast fillets were subjectively hand scored, by trained personal, for woody breast (WB) on a whole number increment scale with 0 being no signs of WB, 1 being mild to moderate WB, and score 2 being severe WB according to Tijare et al. (2016). Carcass and abdominal fat pad weights were recorded immediately post evisceration. Carcasses were chilled for 4 hours at 4^{o}C before breasts (boneless and skinless pectoralis major), tenders (boneless and

skinless pectoralis minor), wings, and leg quarters were removed and weights recorded. Parts yields were calculated as individual part divided by whole body weight. Breast from each bird were collected and stored at 4°C for 24 hours for colorimetric analysis. Readings were taken with a colorimeter (CR-400; Konica Minolta Sensing Inc., Sakai Osaka, Japan; size 102 (W) × 217 (H) × 63 (D) mm) using illuminant D65 and a 2-degree observer to determine the L* (Lightness), a* (redness), and b* (yellowness) values on the ventral side of the right breast fillet.

Litter quality and Gas flux measurements

Litter samples and gas emissions were collected from each pen before (13 d) and at the conclusion (34 d) of the experimental period. Protocols for sample collection followed Anderson et al. (2020). Briefly ammonia, nitrous oxide, methane, and carbon dioxide flux measurements were taken at 3 areas in each pen and analyzed by a Innova 1512 Photo-acoustic Multi-gas Analyzer (Innova Air Tech Instruments, Ballerup, Denmark). Litter samples were taken and analyzed for moisture, pH, electrical conductivity, ammonium-N, nitrate-N, total N, total C, C:N ratio, and soluble and total metals.

Statistical Analysis

Data were analyzed using JMP Pro15 (JMP, 2018) as a randomized, 2-way complete block design, with pen as the experimental unit. Treatments were represented by six replicate pens. Two-way ANOVA was used with $P<0.05$ indicating the probability of significant differences. When appropriate, differences among treatments were separated using a repeated Tukey's HSD test.

RESULTS AND DISSCUSSION

The results for feed intake, body weight gain, mortality, feed conversion ratio, water intake, water conversion ratio, and water intake/feed intake ratio as impacted by dietary CP level

and SP inclusion are included in Table 2. There were no interactive effects between CP level and SP inclusion on performance parameters. Mortality was not impacted by CP level or SP inclusion. Feed intake was not impacted by dietary protein level; which was to be expected since broilers consume feed to meet their AA and energy needs, assuming all AA requirements are met (Leeson and Summers, 2001).

Birds fed the lower CP diet had 112 less grams of body weight gain (P=0.0222) and 6 points higher FCR (P<.0001) than the standard CP fed broilers. Van Harn et al. (2017) found that male Ross 308 broilers were not impacted by mild reductions of CP, yet broilers have been proven to respond variably to minor dietary reductions in CP. Pesti (2009) found in a meta-analysis that overall performance is impacted by dietary CP levels. Male broilers are also more susceptible to adverse performance effects from reduction in CP (Hernandez et al., 2012). Dietary CP also impacted water intake (P=0.0011), and water to feed ratio (P=0.0237), which is in agreement with previous literature stating that reducing CP lowers water intake and water excretion (Alleman and Leclerq, 1997).

For the SP treatments it took 100 grams/bird more feed intake (P=0.0298) to reach the same body weight gain, which resulted in a 5-point elevation of FCR (P<.0001). This was perplexing considering Mullenix et al. (2021) fed SP up to 5% during the same grow out period without adverse performance impacts. One distinct difference was the former trial used primarily a corn/soybean meal diet, while the current trial used a substantial level of distiller's grains (Table. 1). Liu et al. (2021) observed that corn based diets maybe more suitable for reductions of dietary CP than wheat-based diets.

The decrease in performance in the SP treatments was also observed in the reduced carcass and breast yields (Table. 3). This is particularly interesting considering breast fillet size

has large economic consequences. Reducing CP negatively impacted all yield parameters ($P<0.05$) and increased adipose fat deposition. Fat deposition is undesirable to consumers and is a result of poor nutrient utilization (Leeson and Summers, 2001). Average score and incidences of WB is displayed in Table 4. There was a CP x SP interaction on average woody breast score ($P=0.0178$) and incidences of ideal "0" breast fillets ($P=0.0279$). Overall, there was little incidences of WB, but as CP was highest, SP was needed in the diet to maintain low myopathies.

The SP treatment breast fillets were redder (a*) ($P<.0001$), yellower (b*) ($P<.0001$), and had a darker hue (L*) ($P=0.0056$) (Table. 5). This is in alignment with previous research that showed SP inclusion above 1% significantly increases pigment in broiler breast fillets (Toyomizu et al., 2001; Park et al., 2018). The increased redness ($P=0.0002$) from the low CP treatments is possibly indicative of a higher myoglobin content or better oxidative stability of breast fillets (Pekel et al., 2020).

Average score and incidences of foot pad quality are displayed in Table 6. The low CP treatments displayed a better average ($P=0.0142$) and ideal "0" footpad ($P=0.0375$) than the standard protein treatments. Increased footpad quality is commonly associated with less litter moisture (Shepard and Fairchild, 2010). This contradicts the current research, which showed higher litter moisture ($P=0.0179$) in the low CP diets compared to the standard CP level diets; 40 vs. 36%, respectively. Litter analysis displayed no other differences for pH, electrical conductivity, ammonium-N, nitrate-N, total N, total C, C:N ratio, or soluble and total metals. Ammonia, nitrous oxide, and methane flux were not impacted by dietary treatment, while carbon dioxide flux ($P=0.0066$) was reduced in the lower CP diets (Table. 7). Classically litter nitrogen emissions (ammonia, nitrates, and nitrous oxide) are reduced through less nitrogen excretion in low protein fed broilers (Cappelaere et al., 2021). Park et al. (2018) displayed that SP

substitution into broiler diets lowered amounts excreta ammonia emission. The exact reasons for the discrepancies in emissions is not fully understood, but it must be noted that stocking density in the current trial was less than that in the industry to accommodate for the water intake system.

CONCLUSIONS

Reducing dietary CP to 17.75% impaired overall performance through decreased body weight gain and increased FCR. This decreased performance was reflected in decreased carcass yields and increased fat deposition. The low CP treatment birds consumed less water, had better footpad quality, and had litter with less carbon dioxide flux. Spirulina inclusion of 2.5% did not impair body weight gain, but FCR was increased through higher feed intake. Spirulina also reduced carcass and breast yields, while increasing pigmentation in the breast fillets

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TABLES

Table 1. Composition of Experimental diets

Ingredient, % as-is	Control	Control+SP	LowCP	LowCP+SP
Corn	58.75	60.95	67.24	66.93
Soybean meal	19.62	15.60	11.76	8.00
Spirulina meal		2.50		2.50
Distillers grains	15.00	15.00	15.00	17.32
Poultry fat	2.24	1.64	0.50	0.50
Meat & bone meal	1.50	1.50	1.50	0.41
Limestone	1.15	1.23	1.26	1.54
Dicalcium phosphate	0.37	0.26	0.44	0.52
Salt	0.23	0.09	0.23	0.10
Sodium bicarbonate	0.10	0.10	0.10	0.10
Choline chloride	0.05	0.05	0.05	0.05
L-Lysine HCl	0.40	0.45	0.63	0.70
DL-Methionine	0.21	0.21	0.28	0.28
L-Threonine	0.11	0.11	0.22	0.22
L-Arginine	0.10	0.13	0.31	0.35
L-Valine	0.03	0.03	0.17	0.17
L-Tryptophan	0.01	0.02	0.04	0.05
Vitamins[1]	0.05	0.05	0.05	0.05
Minerals[2]	0.08	0.08	0.08	0.08
Phytase	0.01	0.01	0.01	0.01
Calculated content, % unless noted otherwise				
CP	20.00	20.00	17.75	17.75
ME, kcal/kg	3,085	3,085	3,085	3,085
Ca	0.84	0.84	0.84	0.90
Na	0.17	0.17	0.17	0.17
DEB, mEq/kg	214.3	207.3	162.9	156.1
Available P	0.41	0.41	0.41	0.41
Digestible, Lys	1.10	1.10	1.10	1.10
Digestible, Met	0.55	0.56	0.59	0.59
Digestible, TSAA	0.84	0.84	0.84	0.84
Digestible, Thr	0.74	0.74	0.74	0.74
Digestible, Val	0.85	0.85	0.85	0.85
Digestible, Ile	0.73	0.73	0.73	0.73
Digestible, Leu	1.65	1.64	1.44	1.42
Digestible, Arg	1.10	1.10	1.10	1.10
Digestible, Trp	0.18	0.18	0.18	0.18

[1]The vitamin premix contributed (per kg of diet): vitamin A, 15,432 IU; vitamin D3, 11,023 ICU; vitamin E, 110 IU; niacin, 77 mg; d-pantothenic acid, 20 mg; riboflavin, 13 mg; pyridoxine, 6 mg; thiamine, 3 mg; menadione, 3 mg; folic acid, 2 mg; biotin, 0.2 mg; vitamin B12, 0.03 mg.

[2]The mineral premix contributed (per kg of diet): manganese, 100 mg; zinc, 100 mg; calcium, 69 mg; copper, 15 mg; iron, 15 mg; iodide, 1.2 mg; selenium, 0.25 mg.

Table 2. Performance parameters of male Cobb500 broilers fed 2 levels of crude protein (CP) with/without *Spirulina* (SP) inclusion from 14-35d.

Treatment		BWG[1]	F.I.[1]	W.I.[1]	FCR[1]	WCR[1]	W/F[1]	Mort
SP	CP	(kg)	(kg)	(L)	(g:g)	(mL:g)	(L/kg)	(%)
Interactive effects of Spirulina and Crude Protein (n=6)								
0	18	2.012	2.358	5.957	1.19	3.00	2.45	4.17
2.5	18	1.955	2.440	5.830	1.26	3.00	2.32	2.08
0	20	2.070	2.358	6.342	1.15	3.07	2.64	2.08
2.5	20	2.121	2.482	6.176	1.17	2.93	2.46	0.69
SEM		0.045	0.043	0.096	0.010	0.066	0.067	0.919
Main effect of Spirulina (n=12)								
0		2.041	2.361	6.149	1.17	3.03	2.55	3.13
2.5		2.038	2.461	6.003	1.22	2.97	2.39	1.39
SEM		0.032	0.030	0.068	0.007	0.047	0.048	0.650
Main effect of Crude Protein (n=12)								
	18	1.984	2.399	5.894	1.22	3.00	2.39	3.13
	20	2.096	2.423	6.259	1.16	3.00	2.55	1.39
SEM		0.032	0.030	0.068	0.007	0.048	0.040	0.650
P-value								
Spirulina		0.9390	0.0298	0.1429	<.0001	0.3266	0.0335	0.0734
CP		0.0222	0.5752	0.0011	<.0001	0.9980	0.0237	0.0734
SP × CP		0.2447	0.6803	0.8422	0.0577	0.2652	0.6950	0.7095

[1]BWG = Individual body weight gain; F.I. = individual feed intake; W.I. = individual water intake; FCR = mortality corrected feed conversion ratio; WCR = mortality corrected water conversion ratio; W/F = water intake/feed intake.

Table 3. Processing Yields[1] of male Cobb500 broilers fed 2 levels of crude protein (CP) with/without Spirulina (SP) inclusion from 14-35d.

Treatment		Carcass	Breast	Tender	LQ[2]	Wing	Fat
SP	CP[3]	%	%	%	%	%	%
Interactive effects of Spirulina and Crude Protein (n=6)							
0	18	73.12	19.42	3.50	23.10	7.57	1.57
2.5	18	72.65	18.95	3.48	23.03	7.58	1.73
0	20	74.02	20.77	3.68	22.57	7.83	1.42
2.5	20	73.02	19.93	3.57	22.57	7.58	1.53
SEM		0.276	0.236	0.052	0.212	0.063	0.076
Main effect of Spirulina (n=12)							
0		73.57	20.09	3.59	22.83	7.70	1.49
2.5		72.83	19.44	3.53	22.80	7.58	1.63
SEM		0.195	0.167	0.037	0.150	0.045	0.054
Main effect of Crude Protein (n=12)							
	18	72.88	19.18	3.49	23.07	7.58	1.65
	20	73.52	20.35	3.63	22.57	7.71	1.48
SEM		0.195	0.167	0.037	0.150	0.045	0.054
P-value							
Spirulina		0.0151	0.0122	0.2182	0.8765	0.0809	0.0761
CP		0.0327	<.0001	0.0194	0.0285	0.0485	0.0316
SP × CP		0.3455	0.4459	0.3517	0.8765	0.0485	0.7448

[1]Yields represent chilled carcass parts relative to live BW

[2]bone-in, skin-on thigh + drumstick

Table 4. Woody breast incidence of 35 d Cobb 500 males fed 2 levels of crude protein (CP) with/without Spirulina (SP) inclusion from 14-35d..

Treatment			Distribution[1], %		
SP	CP	Average	0	1	2
Interactive effects of Spirulina and Crude Protein (n=6)					
0	18	0.02[b]	98	2	0
2.5	18	0.02[b]	98	2	0
0	20	0.15[a]	88	8	4
2.5	20	0.02[b]	98	2	0
SEM		0.026	2.11	2.11	1.05
Main effect of Spirulina (n=12)					
0		0.08	93	5	2
2.5		0.02	98	2	0
SEM		0.018	1.49	1.49	0.75
Main effect of Crude Protein (n=12)					
	18	0.02	98	2	0
	20	0.08	93	5	2
SEM		0.018	0.07	1.49	0.75
P-value					
Spirulina		0.0178	0.0279	0.1295	0.1295
CP		0.0178	0.0279	0.1295	0.1295
SP × CP		0.0178	0.0279	0.1295	0.1295

[1]Distibution based on the percent incidence of each score per pen observed in the ten processed broilers

Table 5. Breast color of male Cobb500 broilers fed 2 levels of crude protein (CP) with/without Spirulina (SP) inclusion from 14-35d.

Treatment		L*[1]	a*[2]	b*[3]
SP	CP			
Interactive effects of Spirulina and Crude Protein (n=6)				
0	18	56.00	3.69	8.42
2.5	18	54.90	4.48	10.26
0	20	55.84	3.19	8.11
2.5	20	55.36	3.80	10.72
SEM		0.255		
Main effect of Spirulina (n=12)				
0		55.92	3.44	8.27
2.5		55.13	4.14	10.49
SEM		0.180	0.093	0.158
Main effect of Crude Protein (n=12)				
	18	55.45	4.08	9.34
	20	55.60	3.49	9.42
SEM		0.180	0.093	0.158
P-value				
Spirulina		0.0056	<.0001	<.0001
CP		0.5603	0.0002	0.7300
SP × CP		0.2258	0.5401	0.1025

[1]L*=Lightness
[2]a* = Redness
[3]b* = yellowness

Table 6. Footpad quality of 35 d Cobb 500 males fed 2 levels of crude protein (CP) with/without Spirulina (SP) inclusion from 14-35d.

Treatment			Distribution[1], %		
SP	CP	Average	0	1	2
Interactive effects of Spirulina and Crude Protein (n=6)					
0	18	0.32	70	28	2
2.5	18	0.53	53	40	7
0	20	0.72	40	48	12
2.5	20	0.67	47	39	14
SEM		0.099	8.08	7.53	3.49
Main effect of Spirulina (n=12)					
0		0.52	55	38	7
2.5		0.60	50	39	11
SEM		0.070	5.71	5.33	2.47
Main effect of Crude Protein (n=12)					
	18	0.43	62	34	4
	20	0.69	44	44	12
SEM		0.070	5.71	5.33	2.47
P-value					
Spirulina		0.4113	0.5698	0.8871	0.3282
CP		0.0142	0.0375	0.2256	0.0244
SP × CP		0.1944	0.1529	0.1753	0.6721

[1]Distibution based on the percent incidence of each score per pen observed in the ten processed broilers

Table 7. Litter flux measurements of male Cobb500 broilers fed 2 levels of crude protein (CP) with/without Spirulina (SP) inclusion from 14-35d.

Treatment1		NH_3	N_2O	CO_2	CH_4
SP	CP[3]	mg m^{-2} h^{-1}	mg m^{-2} h^{-1}	kg m^{-2} h^{-1}	mg m^{-2} h^{-1}
Interactive effects of Spirulina and Crude Protein (n=6)					
0	18	362.3	0.732	1.746	10.160
2.5	18	414.6	1.340	1.764	14.318
0	20	502.8	0.997	2.366	15.490
2.5	20	507.5	2.678	2.400	13.427
SEM		67.97	0.558	2.073	2.428
Main effect of Spirulina (n=12)					
0		432.5	0.864	2.056	12.825
2.5		461.0	2.009	2.082	13.873
SEM		48.07	0.395	1.466	1.717
Main effect of Crude Protein (n=12)					
	18	388.4	1.036	1.755	12.239
	20	505.1	1.838	2.383	14.458
SEM		48.07	0.395	1.466	1.717
P-value					
Spirulina		0.6798	0.0536	0.9014	0.6708
CP		0.1014	0.1664	0.0066	0.3716
SP × CP		0.7300	0.3478	0.9672	0.2148

[1] NH_3=ammonia; N_20=nitrous oxide; CO_2=carbon dioxide; CH_4=methane

OVERALL CONCLUSION

Chapter 2 suggests that Spirulina (*Arthrospira platensis*) has standardized digestible amino acids that are comparable with other prominent protein feedstuffs and a metabolizable energy level higher than soybean meal. Chapter 3 and 4 indicate that Spirulina substitution can benefit poultry production by increasing footpad quality and reducing systemic inflammation. Chapter 5 demonstrates that Spirulina can be added into corn and soybean meal-based grower (10%) and finisher (5%) diets without impacting performance parameters or processing yields. Chapter 6 demonstrated that Spirulina inclusion into corn, soybean, distillers' grain diets impeded feed utilization and breast meat yield. Data from chapter 6 also displayed that reductions of dietary crude protein improve footpad quality, reduces water consumption, and limits carbon dioxide gas flux from litter. Laboratory research needs to be conducted to characterize nutrient profile ranges of Spirulina. Subsequent broiler nutrition trials should be conducted to determine dietary inclusion ranges of spirulina.

APPENDIX A

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Michael Kidd
Fr: Billy Hargis - Ag-IACUC Chair
Date: October 3rd, 2019
Subject: IACUC Approval
Expiration Date: October 27th, 2021

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 20031 *Optimization of bioassays to determine and predict the metabolizable energy and digestible amino acid content of Spirulina Algea as a feed ingredient for broiler*.

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond October 27th, 2021 you can submit a modification to extend project up to 3 years from the orginal date approved, or submit a new protocol. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp

APPENDIX B

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Michael Kidd
Fr: Billy Hargis - Ag-IACUC Chair
Date: January 22nd, 2020
Subject: IACUC Approval
Expiration Date: December 31st, 1969

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21002 *Muiltiple assay approach measuring stress response of broilers fed low crude protein diets with Spirulina Aglea inclusion.*

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond December 31st, 1969 you can submit a modification to extend project up to 3 years from the orginal date approved, or submit a new protocol. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Garrett Mullenix, Xinge Xi, Wengian Wang, and Michael Kidd. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp

21002

APPENDIX C

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Michael Kidd
Fr: Billy Hargis - Ag-IACUC Chair
Date: August 25th, 2020
Subject: IACUC Approval
Expiration Date: August 21st, 2022

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21055 *Performance of Economic Implact of Spiruline Plantensis titration on modern commercial broilers.*

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond August 21st, 2022 you can submit a modification to extend project up to 3 years from the orginal date approved, or submit a new protocol. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Sam Rochell, Garrett Mullenix, Craig Maynard, Guillermo Tellez-Isaias, and Michael Kidd. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp

21055

APPENDIX D

DIVISION OF AGRICULTURE
RESEARCH & EXTENSION
University of Arkansas System

To: Michael Kidd
Fr: Billy Hargis - Ag-IACUC Chair
Date: June 21st, 2021
Subject: IACUC Approval
Expiration Date: June 18th, 2023

The Division of Agriculture Institutional Animal Care and Use Committee (Ag-IACUC) has APPROVED your protocol # 21120 *Reduced Protein with Spirulina Inclusion diets in broiler diets.*

In granting its approval, the Ag-IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the Ag-IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond June 18th, 2023 you can submit a modification to extend project up to 3 years from the orginal date approved, or submit a new protocol. By policy, the Ag-IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Michael Kidd, Garrett Mullenix, Craig Maynard, Savanna Crafton, Victoria Reid, Liz Greene, and David Reynolds. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The Ag-IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

BMH/tmp