Multilevel evaluation of prebiotic and probiotic supplementations, heat stress, and genetic strain effects on blood and intestinal epithelial cells of chicken

by

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Dedication

To Aryas

My beloved daughter, my life, my light
The sweetest sunshine ever.

TABLE OF CONTENTS

List of Tables

List of Figures

Abstract

There is a need to assess how dietary strategies might effectively benefit bird health, performance, and the ability to deal with environmental variability. Metabolomics is suggested as a useful approach for assessing chicken responses to management interventions and identifying key biomarkers and pathways associated with bird health and production. The purpose of this study was to examine the utility of metabolomics for assessing physiological responses of chickens of different genetic backgrounds to different pre- and probiotic supplements. Feeding *Chondrus crispus* seaweed as a source of prebiotic for 3 weeks had little effect on leukocyte counts and classic blood chemistry but significantly impacted feed intake, feed to egg ratio, and weight gain. In contrast, longer term supplementation with *Chondrus crispus* or *Ascophyllum nodusum* for 45 weeks affected plasma protein, enzyme profiles, and several blood chemistry values, with little effect on hen leukocyte counts and overall performance. Heat stress also affected the leukocyte count and some plasma chemistry parameters. Through the NMR approach, we provided quantitative data on 57 plasma and 59 intestinal epithelial cell metabolites to provide a resource to study physiological responses and biological functions in laying hens. We also detected differentially expressed metabolites and linked them to metabolic pathways using the KEGG database. In broilers, we assessed the impacts of *Bacillus subtilis* probiotic delivery routes on the plasma metabolome and identified nine, four, and three metabolic pathways associated with in-ovo, in-feed, and in-water probiotic treatments, respectively. Overall, our results showed that chicken plasma and epithelial cell metabolome were very sensitive to internal and external stimuli, including pre- and probiotic supplementation, heat stress, and genetic strain, often when production or more classical blood indicators of stress were not affected. These findings suggested that metabolomics could be successfully used to address the challenge of identifying pathways and mechanisms that explain the health effects of genetic and environmental factors.

List of Abbreviations Used

ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AN	*Ascophyllum nodosum*
ANOVA	Analysis of Variance
APRI	Atlantic poultry research institute
AST	Aspartate aminotransferase
BCFA	Branched chain fatty acids
CC	*Chondrus crispus*
CCAC	Canadian Council of Animal Care
CE	Capillary electrophoresis
DART	Direct analysis in real time ionization
EPC	Epithelial cell
H/L	Heterophil/lymphocyte
GC-MS	Gas chromatography mass spectrometry
GGT	Gamma-glutamyltransferase
GLDH	Glutamate dehydrogenase
GLM	General linear model
HPLC	High pressure liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
LC-MS	Liquid chromatography mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
PCA	Principal components analysis
PCR	Polymerase chain reaction
PLS-DA	Partial least-squares discriminant analysis
Q-TOF-MS	Quadruple time-of-flight mass spectrometry
SAS	Statistical analysis system
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
STC	Stroma cell
TCA	Tricarboxylic acid
TMAO	Trimethylamine N-oxide
TOF-MS	Time of flight mass spectrometry
UPLC	Ultrahigh pressure liquid chromatography
VIP	Variable importance in the projection
VLDL	Very-low-density lipoprotein

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CHAPTER 1. INTRODUCTION

1.1. Introduction

Metabolomics is used to characterize the chemical composition of animal biofluids and tissue samples. Various analytical assays, along with pattern recognition and statistical data analysis methods, can be utilized to effectively place the experimental observations into a relevant biological context (Chen et al., 2015; Karnovsky et al., 2020). Metabolomics has been applied in poultry studies to illustrate bird responses to some production and health traits (Huber et al., 2010; Ji et al., 2012; Cajka et al., 2013; Shen et al., 2014). In poultry science, it is important to detect key biomarkers and metabolic pathways to better understand chicken physiological responses to environmental and management conditions. The development of natural prebiotics and probiotics for poultry production is important to reduce conventional antibiotic use and promote better animal health and welfare. Chicken studies have highlighted the utilization of seaweed supplements to enhance production outcomes and promote overall gut health. (Carrillo et al., 1990; Carrillo et al., 2008; Holt et al., 2011; Souza et al., 2012). It is also important to consider the route of probiotic delivery as it can influence the survival and interaction of probiotic microorganisms within the host's digestive system (Shori, 2017). It is expected that the administration of these natural prebiotics and probiotics in diets will continue to increase, though more work is needed to understand how they can impact blood function and intestinal health more generally in the long term.

Chickens respond to heat stress by generating a combination of behavioral, biochemical, and physiological changes. Heat stress as a critical environmental stressor, can cause

significant decreases in chicken performance and productivity by reducing feed intake, growth rate, egg production, egg quality, and feed efficiency (Sahin et al., 2009). However, many causes of these changes have remained unknown, and more research is needed to examine the inhibitory effects of heat stress on blood factors. Examining blood parameters may provide useful clinical indicators of how birds respond to different dietary interventions or environmental stressors (Etim et al., 2014).

The existing knowledge gap lies in the assessment of the applicability and utility of metabolomics in understanding chicken biological and physiological responses. Therefore, we aimed to explore the potential of utilizing the plasma and epithelial cell metabolome in order to gain insights into how chickens with different genetic backgrounds respond to environmental factors. Blood chemistry and hematology were also used as indicators of the metabolic response to external change. Thus, this study was planned to use prebiotic supplementation, thermal heat stress, and bird strain to investigate the utility of plasma and intestinal epithelial cell metabolome, as well as the plasma biochemical and haematological parameters in relation to physiological responses. Furthermore, we conducted a separate study to investigate the impact of probiotic delivery routes on the plasma metabolome in broilers, aiming to assess the response to probiotic intervention.

1.2. Metabolomics: An Emerging Field of Omics Science

Metabolomics, a newly emerging field of "omics" research, uses advanced analytical chemistry techniques to comprehensively characterize small molecule metabolites in biological matrices such as cells, tissues, and biofluids (Wishart, 2008). Metabolomics, as an increasingly popular approach, differs from other "omics" fields by focusing on smaller,

often neglected molecules with molecular weights less than 1500 daltons, which are smaller than most proteins, DNA, and other cellular macromolecules (Zhang et al., 2010). The collection of these small molecules found in a given biological sample is called the metabolome. No single instrument or separation method can detect all metabolites present in a metabolome; instead, researchers harness the power of complementary techniques such as nuclear magnetic resonance spectroscopy (NMR), gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), inductively coupled plasma mass spectrometry (ICP-MS), emerging capillary electrophoresis (CE), and several targeted HPLC-based assays (Blow, 2008). The development of these modern metabolomics techniques could enable the separation, identification, and absolute quantification of compounds for a wider range of research disciplines.

More recently, advances in this field, have made it possible to characterize thousands of metabolites in biological matrices, promising richer and more complete analysis capabilities. Just like genomics, transcriptomics, and proteomics, the field of metabolomics has experienced tremendous advancements in analytical technologies for absolute metabolite quantification. Technological improvements in mass spectrometry (improved sensitivity and mass resolution), NMR spectroscopy (improved sensitivity with higher field magnets and cryoprobes), and chromatography (faster separations with HPLC and ultra-high-pressure liquid chromatography, or UPLC) have allowed metabolomics to grow tremendously. Likewise, significant developments in the software and databases have allowed scientists to gain a more complete picture of system-wide metabolism and biology. As a result, metabolomics has the potential to enable more complete chemical analysis of many complex biological matrices in different jurisdictions.

1.3. Applications of Metabolomics in Animal Science

In recent years, metabolomics has been ideally positioned to be used in many areas of clinical studies (biomarker identification for disease diagnosis, prognosis, or monitoring), nutritional analysis, and crop research (Wishart, 2008; Kim et al., 2016). It is also being increasingly used in animal science, livestock research, and livestock monitoring (Fontanesi, 2016). In fact, metabolomics can be an excellent tool for determining the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli and measurement of the body's biochemical reactions products (Nicholson et al., 1999; Oresic et al., 2014).

The complete metabolic profile could also open the door for the development of biomarkers for specific physiological responses of animals to different stressors (Oresic et al., 2014). It is becoming increasingly clear that, of all the omics techniques, metabolomics has the greatest potential for biomarker discovery to address the challenge of finding pathways and mechanisms that consistently explain the health effects of environmental factors.

Likewise, metabolomics is allowing researchers to focus on quantitative measurement of the end products of genetic, epigenetic, and environmental interactions as well as phenotype characterization (Nicholson et al., 1999; Oresic et al., 2014). Changes in the concentration of metabolites could reflect the interactions between genes and the environment. Furthermore, the combination of transcriptomics, genomics, proteomics, and metabolomics can help to gain a better understanding of host-microbiome interactions to bridge the gap between gene expression, metabolic, and physiological responses (Le Roy et al., 2016).

As such, metabolomics can be increasingly applied in the poultry industry, affecting chicken health, welfare, and productivity. The metabolomics approach in poultry science has the potential to investigate quantitative and qualitative metabolic variations caused by environmental conditions or genetic mutations. This tool can also be used to investigate the impact of diet, drugs, and infections on chicken systemic metabolism. Metabolic studies can evaluate the impact of nutrition and food on a chicken's metabolism and provide a useful framework for nutritional research. More specifically, it could have a great impact on the evaluation of chicken meat quality, egg quality, and productivity in poultry farming. However, despite its promise and the potential advantages of metabolomics in addressing questions in livestock and animal science, the application of metabolomics in livestock species (especially about poultry science) is not particularly widespread. A review of PubMed and Google Scholar indicates that fewer than three dozen papers on chicken metabolome characterization have been published with the word "metabolomics" in the title. Therefore, metabolomics has the potential to be a route to delivering significant improvements in poultry production systems.

1.3.1. Literature Survey on Poultry Metabolomics

Based on the data collected from our literature survey, the majority of metabolomic studies among all avian species were performed on chicken, as it is one of the most widely consumed meats in the world. However, there have been a few metabolomics studies on ducks, small birds, and nestling birds (Fathi et al., 2017; Bonnefont et al., 2014; Liu et al., 2013; Fischer et al., 2013). Poultry metabolomics studies can be categorized into different areas, including chicken physiology, chicken nutrition, chicken health, chicken production,

chicken reproduction, chicken products, and human health (Goldansaz et al., 2017). The majority of poultry metabolomic studies were in the areas of poultry physiology (mainly analysis of different tissues, body organs, biofluids, etc.) and poultry products (egg and meat products).

To date, there have only been a few NMR studies on chicken biofluids or tissue samples that resulted in both the quantitation and identification of specific chemical compounds (Sundekilde et al., 2017; Fotakis et al., 2017; Beauclercq et al., 2016). There are quite a few NMR studies in this field reporting no absolute concentrations but rather reported detection of chemical compounds in chicken (Niemuth and Stoskopf, 2017; Le Roy et al., 2016). One of the most complete NMR studies was a report by Fotakis et al., (2017) that investigated the effect of flavonoids and vitamin E as dietary supplements on the metabolic fingerprint of chicken plasma. They identified 37 metabolites, including the total lipid content and amino acid content, by NMR based metabolomics (Fotakis et al., 2017). Another notable NMR study detected a total of 19 different metabolites in heparinized whole chicken blood (Niemuth and Stoskopf, 2017). Beauclercq et al., (2016) also used NMR to show the linkage between the metabolome and poultry meat quality and detected 46 chicken muscle metabolites.

1.3.2. Trends in Biomarker Discovery in Poultry Metabolomics Research

Metabolomics has been successfully applied to identify multiple potential candidate biomarkers, which has an enormous impact on poultry science. Based on our survey on poultry metabolomics, the quality of biomarker discovery studies is not particularly good. The majority of metabolomic biomarker papers have covered biomarker discovery

methods and presented a standard operating procedure for sample collection, preparation, data analysis, and modeling (Hermo et al., 2016; Morales-Gutierre et al., 2015; Huber et al., 2010). There are limited publications focusing on poultry biomarker compounds related to environmental exposures, nutrition, health, production, and contributors to chicken egg and meat products (Fotakis et al., 2017; Tomonaga et al., 2018; Zampiga et al., 2018; Hofmann et al., 2019; Brugaletta et al., 2020; Chen et al., 2021). Some of the most compelling published studies regarding the reported biomarker studies in chicken include those by Huber (2010), who identified 20 biomarkers, and a study by Shen (2014), who identified 14 biomarkers detected by ultra-performance liquid chromatography/quadruple time-of-flight mass spectrometry (UPLC/Q-TOF/MS).

Huber et al., (2010) focused on biomarker detection in chicken and showed the remarkable differences between organic and conventional feeding on the immune response of the chicken. They observed modulation in the levels of glyceric acid, monomethyl phosphate, sugars (ribose, ribulose, and fructose), amino acids (alanine and methionine), vitamin E, and a-ketoglutaric acid in chicken liver. These biomarkers can potentially enable future studies in human subjects regarding the modulation of the immune response by organic foods (Huber et al., 2010). Shen et al., (2014) detected 14 potential biomarkers of ascites as one of the three major metabolic diseases in poultry production using UPLC/Q-TOF/MS. Another reported biomarker study was conducted by Beauclercq et al., (2016) to characterize the metabolomics signature of broiler chicken tissues that were subjected to a pharmacological treatment (enrofloxacin). The Beauclercq study identified 22 biomarkers as the most important features contributing to the separation between non-medicated, medicated, and post-treatment samples. They also detected 31 metabolites as residues of

enrofloxacin in the liver, kidney, and muscle tissues of broiler chickens using the MS/MS spectrum (Beauclercq et al., 2016).

Metabolomics can also be used as a tool to detect the fraudulent adulteration of products used as chicken feed, including dietary supplements and chicken food. Cajka et al., (2013) revealed the differential metabolites in response to the presence or absence of chicken bone meal during the feeding of chickens (Cajka et al., 2013). In this study, a broad range of polar and non-polar metabolites, including amino acids, organic acids, sugars, peptides, triacylglycerides, and diacylglycerides, were isolated using a direct analysis in real time ionization-mass spectrometry (DART-MS) assay. Another notable LC-MS study reported changes in the chemical composition of different tissues from medicated broiler chickens to produce bioinformation to discriminate whether there is pharmacological adulteration in food analysis (Hermo et al., 2016). The LC-ESI-LTQ-Orbitrap mass spectrometer method was carried out using a HPLC system for the quantitative determination of amoxicillin and the qualitative analysis of metabolomic profiles in different biological tissues, including chicken muscle, liver, and kidney. The applied methodology proved excellent analytical methods for the detection of food fraud and adulteration and to assess possible biomarkers corresponding to endogenous metabolites.

Recently, there have been a few poultry metabolomics studies focusing on changes in metabolite levels associated with the phenotype. The link between food, drugs, dietary supplements, and chicken metabolites has recently been revealed and has generated compelling results not only for chicken farmers but also for industry experts and researchers. These include papers describing the application of metabolomics towards predicting feed efficiency in chickens (Metzler-Zebeli et al., 2017). In this study, some

phenotypic measurements were assessed to show the environment's effects on feed efficiency, growth performance, and serum metabolites. The other interesting study on broiler chicken tissue metabolomics was a report by Ji et al., (2012), who reported the response of chicken adipose tissue to two energy manipulations: fasting and insulin deprivation. Tissue metabolomic analysis was done using LC-MS/MS to characterize the chemical composition of chicken abdominal adipose tissue in chickens (Ji et al., 2012). This study reported a total of 92 detected metabolites, of which 12 were significantly different between insulin-neutralized and fasted. Beauclercq et al., (2016) also showed the association between metabolites and poultry meat quality. Muscle glycogen storage and meat ultimate pH were taken as the major factors that could influence the processing ability and sensorial quality of poultry meat. They identified and quantified 46 chemically distinct metabolites as well as glycogen and meat pH biomarkers in the serum and muscle of two chicken lines (Beauclercq et al., 2016). The detection of these predictive biomarkers can open new perspectives for poultry selection and production and affect the competitiveness of the poultry industry.

Recently, there have been several poultry metabolomics studies that investigated the association between metabolite profiles and chicken disease, which can help the poultry industry, farmers, and researchers (Sundekilde et al., 2017; Shen et al., 2014). A previous study demonstrates the strengths of metabolomics in dealing with chicken muscle dystrophy (Sundekilde et al., 2017). This study was designed to reveal that pectoralis muscle dystrophy in chickens was associated with the chemical composition of pectoralis muscle samples by quantitating the content of muscle samples using a high-resolution magic angle spinning proton NMR spectroscopic. They showed that muscle dystrophy was

associated with a significantly lower content of anserine, carnosine, and creatine (Sundekilde et al., 2017). These results verify the scalability of NMR metabolomics for chicken health.

1.4. Probiotic and Prebiotic Supplementation to Improve Health

The functioning of the gut mucosal barrier requires a complex network of signals with multiple interactions between the resident microbiota, blood, and epithelial barrier. Nonpathogenic gut bacteria must interact with the epithelial cells to start a network of immune and metabolic functions (Galdeano et al., 2007). Probiotics are live microorganisms that positively impact the host by modulating the gut microbiota and promoting various physiological functions, contributing to overall health and well-being (Hill et al., 2014). Several health-related conditions are associated with the intake of probiotics, including (but not limited to) promoting immunomodulation, reduction of infectious diseases and food allergies, reduction of serum cholesterol, prevention of pathogen colonization, and intestinal inflammation, as well as maintaining mucosal immunity by stabilization of the gut mucosal barrier (Parvez et al., 2006; Galdeano et al., 2007). The gut microbiota can ferment some potential non-digestible oligosaccharides known as prebiotics which act as food for already established microflora to enhance gut microbiota health (Tomar et al., 2015; McLoughlin et al., 2017). Prebiotics stimulate the growth of beneficial microorganisms in the gastrointestinal tract to produce short-chain fatty acids, which are proposed to reduce disease risk and improve general well being by having systemic anti-inflammatory effects (McLoughlin et al., 2017). The reported beneficial effects of prebiotic consumption include growth of beneficial microbes,

inhibition of pathogen colonization, reduction of cancer risk and inflammation, increasing mineral absorption, and reduction of serum total cholesterol as well as LDL-cholesterol and triglycerides (Tomar et al., 2015; Kulshreshtha et al., 2017).

1.4.1. Seaweed

Seaweed refers to several species of marine algae that can be exploited as human and animal food products, for medicinal, agricultural, and industrial purposes (Allen et al., 2001). Seaweeds, potentially excellent sources of highly bioactive compounds such as vitamins, minerals, polysaccharides, polyphenols, and polyunsaturated fatty acids, are also considered to have a prebiotic effect (Holdt & Kraan, 2011). Moreover, these marine organisms have demonstrated remarkable antioxidant, anti-inflammatory, anticancer, and antidiabetic properties (Manilal et al., 2009). Smith (2004) classified seaweeds into three major groups based on their colour and pigmentation: green algae (Chlorophyta), red algae (Rhodophyta), and brown algae (Phaeophyceae). Red seaweed has the highest protein content among these three phyla (Fleurence, 1999).

Different seaweed classes have significant differences in their chemical composition and nutrients. It has been demonstrated that a number of factors may influence the chemical content and nutrient composition of seaweeds, including but not limited to geographical and environmental factors and several ecological conditions such as water temperature, salinity, light, crop cultivar, growing seasons, growing temperature, fertilization, and storage conditions (Jensen, 1993; Dawes, 1998; Manivannan *et al*., 2009). The contents of total protein, peptides, lipids, fatty acids, carotenoids, amino acids, alginates, iodine, vitamins, polyphenols, and polysaccharides were quantified and compared in seaweed

species (Holdt and Kraan, 2011). The choice of seaweed species depends on the applications of seaweed that could potentially be exploited for human and animal health applications.

In recent years, marine algae have garnered attention for their potential in formulating cost-effective and highly nutritious diets for animal food products and supplements. Seaweeds, in particular, serve as exceptional sources of all eight essential amino acids essential for animals, which they are unable to synthesize (Abudabos et al., 2013). Additionally, the polysaccharides found in seaweeds possess prebiotic characteristics, selectively promoting the growth and metabolic activity of beneficial bacterial species while inhibiting the growth of pathogens, ultimately enhancing host health (Xiaolin et al., 2018).

1.4.1.1. Seaweed in Poultry Diets

Many studies have been performed to assess the potential of seaweed as an alternative ingredient that could be partially incorporated into chicken diets (Kumar et al., 2007; Abudabos et al., 2013). Consequently, the effects of seaweed supplementation on growth performance, carcass characteristics, serum constituents, and nutrient retention of broilers were evaluated (Abudabos et al., 2013). Previous studies indicated that supplementation of a chicken's basal diet with seaweed could enhance health, productivity, body weight, performance, and dressing percentage (Gu et al., 1988; Abudabos et al., 2013). Regular seaweed consumption can affect lipid metabolism, mineral absorption, and fatty acid synthesis in broiler chickens (Macfarlane et al., 2008). It has also been demonstrated that inclusion of seaweed in broiler diets can act as a potential prebiotic to improve performance, production, egg quality, and overall gut health by altering chicken immune

status and gut microflora (Wang et al., 2013; Abudabos et al., 2013; Alloui et al., 2013; Kulshreshtha et al., 2014; Makkar et al., 2016). The red seaweed, *Chondrus crispus,* as a rich source of prebiotic fiber can increase the abundance of beneficial gut microbiota with a reduction of pathogenic microbes as well as increase the concentration of SCFAs, promoting colonic growth and elevating immunoglobulin levels (Liu et al., 2015).

Currently, the application of prebiotics in poultry production is under severe scientific and public scrutiny. Although the exact mechanisms of action of prebiotic dietary supplements on chicken health are unclear, it has been proven that consumption of prebiotic supplementation in laying hens can promote layer growth and egg production (Kulshreshtha et al., 2017). Moreover, the administration of prebiotics to chicken has been linked with improving intestinal health and animal performance in the absence of antibiotic growth promoters (Kim et al., 2011; Kulshreshtha et al., 2014). Alternatively, the reduction in pathogen colonization may be due to the detrimental effects of prebiotics on the intestinal epithelium by altering the intestinal microbiota and immune system (Patterson & Burkholder, 2003).

1.5. Role of the Intestinal Epithelium

Epithelial cells are specialized cells lining the external and internal body surfaces, tissues, and organs, such as the skin, respiratory system, gastrointestinal tract, urinary tract, and blood vessels. A single layer of epithelial cells that separates the lumen of the gastrointestinal tract from the underlying lamina propria is called intestinal epithelial cells (Zhang et al., 2015). The absorptive epithelium of the small intestine is ordered into large numbers of self-renewing crypt-villus units. As the most rapidly self-renewing tissue in the

body, they turn over every three to five days (Barker et al., 2007). The epithelium is under continuous renewal from the intestinal epithelial stem cells (Wahn & Sampson, 2015). The estimated number of intestinal stem cells is between four and six per crypt, and they have this ability to specialize into many cell types (Barker et al., 2007).

Intestinal epithelial cells (IECs) are primarily involved in protection, absorption, and mucosal secretion (Edelblum 2015). They serve as a protective barrier and regulatory mechanisms to maintain host-microbial interactions and tissue homeostasis to protect the body from any commensal bacteria and infective agents (Peterson & Artis, 2014). There is evidence that some intestinal epithelial cells function as antigen-presenting cells capable of regulating T-cell responses in the intestinal mucosa (Hershberg & Mayer, 2000). Likewise, they function in immune cell homeostasis through the segregation and regulation of commensal microorganisms as well as possessing immunoregulatory functions that can direct mucosal immune responses toward tolerance or inflammation (Peterson & Artis, 2014; Wahn & Sampson, 2015). The gut-associated lymphoid tissue is also a major part of the body's immune system, comprising Peyer's patches, lamina propria, and intraepithelial lymphocytes of the intestine (Wagner et al., 1996). Intraepithelial lymphocytes, as an important part of the gut-associated lymphoid tissue, can be found along the epithelium at the basolateral surface of enterocytes and have an important role in maintaining the integrity of intestinal mucosa (Cepek et al., 1994). Enterocytes, as a physical barrier against microbes, are directly involved in immune processes and contribute to gut homeostasis (Snoeck et al., 2005).

1.6. Mechanisms of Intestinal Response to Nutritional Supplements

The intestine as a multifunctional organ is the major site for digestion, nutrient absorption, enzyme activation, as well as a defensive barrier against pathogens and dietary-derived mutagens, carcinogens, and oxidants (Aw et al., 2005). The intestine is continuously exposed to dietary nutrients as the stimulators of intestinal maturation and proliferation. Dietary supplementation can affect numerous metabolic functions in one or more of the major intestinal divisions including small (duodenum, jejunum, and ileum) and large intestines. Metabolomics can potentially help to understand the intestinal functionality by revealing metabolic functions and mechanism of action of nutrients (Figure 1.1). A metabolomics approach could reveal those relationships between dietary supplements, intestinal microorganisms, and metabolites in a comprehensive and complex manner (Chung et al., 2018). We reviewed the studies to date examining the metabolome in response to dietary supplements in three highly responsive intestinal compartments including intestinal epithelium, the small intestine itself, and the colon.

1.6.1. Dietary Supplement Impacts on Intestinal Epithelium

The intestinal epithelium is a single layer of cells organized over crypts and villi, consisting of multiple cell types (Allaire et al., 2018). Intestinal epithelial cells line the lumen and play integral roles in the life-sustaining process of food digestion and absorption, as well as a series of activities aimed at protecting of the host body from the external environment and microbial infections (Kong et al., 2018). The epithelium contributes both physical and chemical barriers to separate the gut microbiota in the intestinal lumen from the underlying lamina propria and deeper intestinal layers (Abreu et al., 2010), and thus, among various nutrient supplements that have been studied using metabolomics, selected dietary amino

acids, fatty acids, pre−/probiotics, and polyphenols are suggested to promote the key functions of the intestinal epithelium.

Glutamate and glutamine are two well-studied amino acids that have effective roles in the regulation of epithelial cell function. They serve as the precursor in epithelial enterocytes for the synthesis of bioactive compounds and amino acids (Blachier et al., 2009; Meng et al., 2014). Glutamine and glutamate supplements were found as precursors for synthesis in jejunal enterocytes of mucus-derived sugars such as N-acetylglucosamine and N-acetylgalactosamine that can promote mucin synthesis (Blachier et al., 2009; Kim et al., 2013). N-acetylglutamate is an activator of carbamoyl phosphate synthetase synthesis, which converts ornithine to l-citrulline in the enterocytes, which in turn release it to plasma proportionally to enterocyte mass (Blachier et al., 2009). While plasma citrulline level is difficult to measure accurately, the metabolomics-associated techniques ultra-high-pressure liquid chromatography (UPLC) and mass spectrometry are able to accurately determine plasma citrulline level, thus providing a biomarker of healthy intestinal epithelial cell mass useful in the diagnostics of several diseases affecting intestinal function (Blachier et al., 2009; Jones et al., 2019).

Miaomia and colleagues (Miaomiao et al., 2014) used nuclear magnetic resonance spectroscopy (NMR), in a blood metabolomics study of pigs supplemented with glutamic acid to show that it can alleviate injury induced by intestinal toxicity caused by mycotoxins. NMR spectroscopic measurement revealed that intestinal toxicity induces oxidative stress, energy, lipid, and amino acid metabolism disorders as treatment with mycotoxin resulted in increasing levels of lactate alanine, a metabolite that is a hallmark of energy metabolic disorders, as well as elevating levels of acetate in the glutamate and mycotoxin groups

suggesting the energy metabolism disorders related to an increase in ketone bodies (Miaomiao et al., 2014). Dietary supplementation with glutamate was also shown to decrease oxidative stress and promote intestinal and epithelial cell proliferation in the jejunum and ileum of pigs (Miaomiao et al., 2014). This study and those discussed below suggest, glutamate may help preserve the gut epithelial barrier. Other amino acids including threonine, arginine, glutamine, methionine, and cystine also were found to protect gut barrier functions in the porcine small intestine using non-metabolomic approaches (Yang et al., 2019).

Fatty acid supplementation could also impact intestinal epithelium absorptive and barrier functions by improving their structure leading to thickening mucus and increasing villus height, villus width, and crypt depth. Supplementing medium-chain fatty acids (caprylic and capric acids) were shown to be associated with positive changes in the mucosal epithelial structure in the ileum from 84-day-old piglets, including increased villus height and crypt depth (Hanczakowska et al., 2011). Similar results were observed when 150 new-born piglets were supplemented for 8 days with medium-chain triacylglycerol (Chwen et al., 2013). Moreover, fatty acid supplementation can provide energy to epithelial cells (Robles et al., 2013). Increased ATP due to dietary butyrate intake could be explained by the increased energy consumption required for transport across the intestinal membranes (Robles et al., 2013). This can cause changes in the energy source of enteric cells by decreasing glucose and amino acid oxidation due to butyrate usage as preferred fuel in the colon. The results of other animal studies in pigs have indicated that intestinal tissue uses only 5%-6% of dietary and systemic glucose as an energy source (Van der Schoor et al., 2001; Stoll et al., 2006 31). Butyrate is the major energy source of the colonic epithelium,

while most other cell types use glucose as their primary energy source (Donohoe et al., 2011). Butyrate has also been indicated to improve colonic barrier function in piglets by increasing epithelial cell proliferation and differentiation (Gálfi et al., 1990). As such, fatty acid absorption at the brush border of colonic enterocytes led to the gut cell regeneration, proliferation, and improvement of intestinal epithelium structure and function. Since pigs and humans are omnivores of similar size, these results may have implications for humans, as pigs have been considered a good model to assess human gut function as reviewed by Zhang and colleagues (Zhang et al., 2013).

Metabolomics has been used to evaluate the impact of pre−/probiotic supplementation on intestinal functions using blood, intestine, cecal content, stool, and intestinal epithelium samples (as summarized in Table 1.1). Probiotics not only inhibit pathogenic bacteria and enhance the barrier function of the intestinal epithelium, but also stimulate protective responses from epithelial cells, by modulating gut microbiota or their potential immunomodulatory effects (Alassane-Kpembi et al., 2018). Probiotics suppress intestinal pathogens by competing for epithelial cell binding sites (Ohland et al., 2010). The UPLC-MS method was applied to serum samples from 26 people (14 females and 12 males) with a mean age of 42 years, collected following three-week supplementation with *Lactobacillus rhamnosus*, a probiotic strain with an affinity for intestinal mucosal cells (Kekkonen et al., 2008). Lower concentrations of lysophosphatidylcholines and several glycerophos-phatidylcholines were observed, whereas triglycerides were mainly increased in the probiotic supplemented group (Kekkonen et al., 2008). Previously, a high level of lysophosphatidylcholines was connected to the impaired mucosal barrier function and increased gut permeability, using a rat experimental model with a representative human

microbiota (Tagesson et al., 1985). However, a meta-analysis of the effects of probiotics on human blood lipid levels comparing 30 randomized controlled trials with 1624 participants reported no significant effect of probiotics on HDL cholesterol or triglycerides although total cholesterol and LDL cholesterol were reduced (Cho et al., 2015). The study showed that the long-term probiotic supplementation could confirm the effectiveness of the reduction of cholesterol levels. The effectiveness of probiotics for lowering cholesterol levels relies on various factors such as baseline lipid levels, treatment duration, type of probiotic strains, participant health status, and age (Cho et al., 2015; Ishimwe et al., 2015). Probiotics increase the fermentation of non-digestible carbohydrate, in turn increasing intestinal production of the short-chain volatile fatty acid acetate, butyrate, and propionate, as demonstrated using an in vitro human gut model with a representative human microbiota (Moens et al., 2019; Wang et al., 2019). These short-chain fatty acids are the primary energy source of colonic epithelial cells, promoting their development and barrier function (Wang et al., 2019). Lactic acid levels in digesta were also elevated in response to forty-two-day probiotic intake as observed in turkey (Vahjen et al., 2002). Lactic acid plays an important role in intestinal homeostasis maintenance through its anti-inflammatory actions by the activation of phagocytosis, IgA production, and stimulating the production of pro-inflammatory and anti-inflammatory cytokines (Vodnar et al., 2010; Peterson et al., 2014; Foxall et al., 2016; Peterson et al., 2014; Domingo et al., 2017; Kawashima et al., 2018). Overall, the increase in metabolites such as the short-chain fatty acids and lactic acid validate the role of probiotics in maintaining healthier microbiota and gastrointestinal function in several animal species and humans.

The functioning of the gut mucosal immune system requires a complex network of signals with multiple interactions between the resident microbiota, immune system, and the gut epithelial barrier (Foxall et al., 2016). Intestinal epithelial cells serve as a protective barrier and regulatory mechanism to maintain host-microbial interactions and tissue homeostasis to protect body from any commensal bacteria and infective agents (Peterson et al., 2014). There is evidence that some intestinal epithelial cells function as antigen-presenting cells capable of regulating T-cell responses in the intestinal mucosa (Hershberg et al., 2000). Likewise, they function in immune cell homeostasis through the segregation and regulation of commensal microorganisms as well as possessing immunoregulatory functions that can direct mucosal immune responses toward tolerance (Peterson et al., 2014; Wahn et al., 2015). Recent studies have identified several metabolites involved in regulating adaptive immune cell function, including acetate, propionate, polyamines, retinoic acid, and vitamin B and K groups (Hill et al., 2010; Chinen et al., 2012; Hooper et al., 2012; Wang et al., 2019). The interactions between intestinal immunity and gut bacteria may depend on the production and sensing abilities of metabolites produced either by the gut microbiota or by the metabolism of dietary components (Wang et al., 2019). The gut bacteria are involved in the production, modulation, and degradation of a variety of metabolites that are originated from diet-dependent or diet-independent microbial metabolites (Wang et al., 2019). Among various dietary supplementations, the intake of probiotic nutrients has been investigated for promoting immunomodulation and maintaining mucosal immunity by stabilization of the gut mucosal barrier (Parvez et al., 2006; Galdeano et al., 2007). Maltol that is a gut metabolite was supplemented to broiler chickens and mice as a growth-promoting postbiotic and an alternative to antibiotics, which was found to mediate various

gut physiological functions by enhancement of intestinal immunity, reducing inflammatory responses, and maintenance of epithelial gut integrity (Han et al., 2015; Park et al., 2021). Metabolomic studies have also been directed at understanding the role of polyphenolic supplements in modulating intestinal epithelial cell function and affecting plasma glucose levels. Polyphenols have been shown to inhibit glucose uptake in human intestinal epithelial cells, while these cells have a predominant role in glucose absorption (Harada et al., 2012; Shamloo et al., 2018). Several distinct polyphenols can reduce the intestinal absorption of sugars by inhibiting sugar transporters at the intestinal level in humans and causing a reduction in carbohydrate digestion (Aryaeian et al., 2017; Loureiro et al., 2019). This suggests that polyphenols can cause a reduction in glucose absorbance, which results in lower blood glucose level (Shamloo et al., 2018).

In addition, the modulation of intestinal ecology by polyphenols and their impacts on health-affecting microbial metabolites can improve the profile of human inflammatory markers (Cardona et al., 2013). Conversion of polyphenols to bioactive compounds by gut microbiota can positively impact the human intestinal barrier and epithelium, as well as the host's health and immunity (Cardona et al., 2013).

1.6.2. Nutritional Supplement Impacts on Small Intestine Functions

The small intestine is the longest part of the gastrointestinal tract where most digestion and practically all absorption of nutrients occurs. The duodenum has important roles in digesting foods, while the jejunum and ileum consist of circular folds or villi that greatly increase the amount of surface area for the absorption of nutrients (Kong et al., 2018).

Some of these intestinal functionalities have been assayed by measuring morphological and metabolic changes in response to dietary supplements in animal and human studies. Among feed supplements, fatty acids were found to be associated with changes in intestinal morphology thought to be beneficial. Several studies have clarified the effects of butyrate on animal and human small intestine structure (Gálfi et al., 1990; Kotunia et al., 2004; Manzanilla et al., 2006; Robles et al., 2013; 29, 33, 74, 75). Butyrate can improve human health and animal production by the enhancement of gut development. Besides providing energy to epithelial cells, supplemental butyrate is known to mediate the growth of the small intestine (Kotunia et al., 2004). Butyrate is known as a cellular and molecular mediator of gut by regulating gene expression, revealing anti-inflammatory effects on intestinal mucosal cells, as well as increasing the cholecystokinin release with a significant role in regulating the small intestinal structure and function development (Kotunia et al., 2004; Bedford et al., 2018). The cell growth regulatory effects of butyrate result in cell proliferation and presumably enhancement of the digestion and absorption capacities of the small intestine (Azad et al., 2020). A 14-day butyrate feed supplement resulted in increased jejunum villus height, better nutrient utilization, and a positive influence on body weight gain and feed utilization in early weaned pigs (Manzanilla et al., 2006). Butyrate has also been found to stimulate insulin secretion, which in turn enhanced small intestinal mucosal cell proliferation that permitted epithelial growth as shown by *in vitro* human digestion model (Manns et al., 1967; Charlton et al., 2000). In addition to this, butyrate as a gut metabolite can impact various intestinal functions by maintaining the stability and integrity of colonic epithelium, modulating biological and inflammatory responses, as well as affecting cellular energy metabolism and intestinal homeostasis (Hamer et al., 2008;

Guilloteau et al., 2010; Tan et al., 2014). There is also some evidence to suggest that butyrate from intestinal microbes can modulate the mucosal immune system by regulating the macrophage polarization and expansion of T cells, showing the important role of this gut metabolite in both intestinal health and prevention of metabolic diseases (Ji et al., 2016).

Metabolic changes in duodenum, jejunum, and ileum of 6-week female mice that were fed a probiotic strain of *Lactobacillus paracasei* were observed using NMR spectroscopy (Martin et al., 2009). A high abundance of membrane metabolites and oxaloacetate was observed in the jejunum and ileum. Prebiotic intake also impacts small intestinal function by improving the digestion and absorption capacity of the small intestine and increasing the length of intestinal villi, as well as the number of small intestinal goblets, Paneth, and tuft cells in mice (Rahman et al., 2020).

Zhao and colleagues showed that alginate oligosaccharides with potential prebiotic activity could benefit murine blood metabolites by impacting systemic metabolism and regulating lipid metabolism gene expression, which improve small intestinal function (Zhao et al., 2020). They also suggested that alginate oligosaccharides as a source of prebiotic could regulate transcription factors and improve intestinal cell gene expression by impacting some intestinal marker genes including stem cells and TA cell marker genes (Olfm4 and Scl12a2), enterocyte marker genes (Mttp, Clca, and Fxym3), Rac2 (Tuft cell marker genes in the epithelial lining of the intestines), and goblet and Paneth cell marker genes (Ang4 and Delfa31) (Wang et al., 2006; Zhao et al., 2020, 87). In another study by Zhang et al., (2021), the UHPLC-QE orbitrap/MS was used to determine the small and large intestine metabolome of treated mice with Konjac glucomannan, a valuable prebiotic source. The

metabolomics results revealed that prebiotic supplementation could modulate the expression levels of various key factors involved in phenylalanine, tyrosine and tryptophan biosynthesis, linoleic acid metabolism, and arachidonic acid metabolism signaling pathways (Zhang et al., 2021). The metabolomics approach was also suggested that magnolia bark extract as an antibiotic growth promoter had impacts on chicken intestinal metabolite levels including amino acids, fatty acids, peptides, and nucleosides (Zhnag et al., 2021). These gut metabolites were demonstrated to maintain intestinal homeostasis within epithelial or immune cells, which could enhance the chicken growth and overall gut health.

Polyphenolic compounds seem to have beneficial effects on small intestinal function, since about 10% of the total dietary polyphenol intake is absorbed in the human small intestine and the remaining is stored unchanged in the colon (Valdes et al., 2015). High-resolution NMR spectroscopy was used to evaluate the impacts of olive pomace as a source of polyphenols and confirmed a huge dose-related perturbation of intestinal cell metabolome (Di Nunzio et al., 2018). Polyphenol-rich food could impact glucose metabolism and glycolytic pathway and inhibit glucose transporter activity (Di Nunzio et al., 2018). Other studies have demonstrated that the concentration of intestinal glucose regulates the secretion of gut hormones including cholecystokinin, glucagon-like peptide, and peptide tyrosine (Wren et al., 2007; Ishii et al., 2008; He et al., 2009; Rodacka et al., 2010; Lei et al., 2012; Sun et al., 2017; Keating et al., 2018).

It is well demonstrated that structural, hormonal, enzymatic, digestive, and absorptive functions of the small intestine are affected by food composition. Nevertheless, the metabolic pathways that are associated with dietary differences are not fully understood,

and advances in metabolomics offer sensitive tools to further study small intestinal metabolic function and the molecular mechanisms behind the effects of diet.

1.6.3. Nutrient Supplement Impacts on Colon Microbial Functions

Intestinal functions are intensely affected by gut microorganisms that live mostly in the large intestine as well as part of the small intestine. Diet composition can profoundly affect intestinal microbiota, which in turn leads to metabolome changes that continue to be documented to better understand the linkages between diet, gut microbiome, and health. The use of protein and amino acid supplements can be considered as humans' first attempt for ergogenic assistance and the microbial responses are the most studied using metabolomic techniques. Using NMR to determine the effects of 46-day arginine supplementation on porcine metabolism, He and colleagues (He et al., 2009) observed a reduction in 120-day-old porcine serum concentrations of intestinal bacterial metabolites, including formate, ethanol, and the short-chain fatty acids such as acetate and propionate, as well as nitrogenous products such as methylamine and dimethylamine. The gas chromatography mass spectrometry (GC-MS) analysis revealed metabolic changes in pig colonic digesta, including enrichment of xanthine, hypoxanthine, leucine, glutamic acid, and tyrosine following long-term consumption (150 days) of a diet lower in protein (12% crude protein) than the control diet (16% crude protein). The exact relationship between these metabolites and gut microbiome was not clear, but there were potential impacts of the changes in gut microbiota and their fermented metabolites on host health (Zhou et al., 2016).

Branched chain fatty acids (BCFA) can be also produced through protein degradation by microbiota. The BCFAs that do not originate from diet are thought to be the result of microbial digestion of proteins in the large intestine that are inaccessible or indigestible in the small intestine (Zhou et al., 2016). Using the microbial composition and metabolomic profile of cecal and colonic digesta, these researchers reported that a low protein diet decreased the amounts of isobutyrate, isovalerate, and BCFA proportions in pig cecum, suggesting lower availability of indigestible dietary protein for degradation by cecal microbial proteases (Zhou et al., 2016).

Trimethylamine N-oxide (TMAO) is a metabolite, which is generated by both diet and action of intestinal microbiota from choline (Janeiro et al., 2018). The 8-week supplementation with whey protein, a rich source of BCAAs, was found to increase urinary levels of the TMAO metabolite in 27 healthy human adults (male and female), using NMR and LC-MS analyses (Cronin et al., 2018). These results were in accordance with noted targeted studies of protein supplementation on adult metabolomic patterns (Piccolo et al., 2015; Barton et al., 2018). These studies show the value of GC-MS, UPLC-MS, and NMR in tracing the specific pathways of diet ingredients through the digestive tract, on the microbiome and ultimately plasma metabolism.

A large number of genomic and metabolomic studies have shown that prebiotics and probiotics positively affect intestinal microbial metabolism. The fecal bacterial metabolic changes, measured by NMR spectroscopy, showed an improvement in the human gut ecosystem in response to 20 g a day, one-week fructooligosaccharide prebiotic supplementation (Kato et al., 2014). This study demonstrated a positive correlation between increased fecal immunoglobulin A levels and beneficial fecal metabolites

including butyrate, phenylalanine, lysine, and tyramine. The polymerase chain reaction (PCR) products containing bacterial 16S rRNA gene in the fecal DNA samples were analyzed and the microbe-metabolite correlation analysis suggested that some bacterial strains were directly or indirectly involved in the metabolism of above-noted intestinal metabolites (Kato et al., 2014). An NMR-based metabolomics approach was also used to demonstrate increased levels of beneficial pimelate and butyrate in the fecal contents of *Bifidobacterium longum* supplemented mice for 14 days with a single gavage of 15 species of a predominant human gut-derived microbiota cocktail (Sugaharan et al., 2015). *Allium hookeri* as a prebiotic feed supplement, and a source of polyphenol was found to have health benefits in broiler chickens by modulating gut microbiome and changing metabolic compositions (Lee et al., 2020). The absorbable polyphenols reaching the colon get digested by the gut microbiome and alter metabolite composition by generating intermediates including aglycones that promote various aromatic acid metabolites (Lee et al., 2020). The effects of a diet containing wholegrain rye, wheat, pulses, and barley-milled grain flours as natural sources of prebiotics in humans were studied by NMR, using an *in vitro* 3-stage continuous culture system simulating the human colon (Maccaferri et al., 2012). Pulse flour contains oligosaccharides that are thought to be metabolized by the gut microbiota and potentially improve intestinal health (Selma et al., 2009). Pulse flour intake resulted in an increase in *Bacteroides* and *Prevotella* species in the large intestine along with increased acetate and proprionate levels (Janeiro et al., 2018). Propionate is a beneficial short-chain fatty acid microbial fermentation product of the human intestine that is absorbed and converted to glucose in the liver and thought to lower lipogenesis and

serum cholesterol levels (Hosseini et al., 2011). Changes in microbial metabolites indicated that prebiotic intervention could modify both host carbohydrate and lipid metabolism.

Additional NMR studies support the concept of positive modulations of the microbiota composition and higher levels of acetate and propionate in the human colon in response to addition of pre- and probiotics. Barley flour consumption was shown by *in vitro* human colon model to modulate the gut microbiota and increase the production in the colon of acetate and other short chain fatty acids (Maccaferri et al., 2012). Similarly, wholegrain oat consumption was associated with increased bifidobacterial populations as well as increased acetate and propionate levels in human fecal samples as shown by an *in vitro* assay using human microbiota (Hughes et al., 2008). Moreover, a comprehensive NMR analysis revealed that prebiotic galactosyl-oligosaccharides could alter the metabolome in plasma, urine, adrenal glands, liver, pancreas, kidney, and feces of female germ-free mice that received a mix of human baby microbiota bacteria at 8 weeks of age (Martin et al., 2008). Integrating the data from both microbial community analysis and metabolomics data from various sample types has provided important insight into the physiological mechanisms through which pre- and probiotic supplements support health.

Appropriate mineral supplementation is associated with intestinal health by supporting the diversity of gut microbiota and elevating the beneficial anti-inflammatory bacterial metabolites such as butyrate (Gîlcă-Blanariu et al., 2018; Shannon et al., 2019). Studies of the gut microbial metabolome have shown that both iron deficiency and iron supplementation influence the microbiome and result in metabolic activity change in humans and animals (Dostal et al., 2014; Dostal et al., 2014; Kortman et al., 2016; Dostal et al., 2018). Notably, more studies reported the negative effects of excess oral iron

supplementation on intestinal health, gut metabolome, and disease progression of humans and rats (exacerbation and neurodegenerative disease) (Chen et al., 2007; Kortman et al., 2014; Prentice et al., 2017). 1H-NMR spectroscopy and GC-MS techniques showed that a rich iron condition (250 μmol/L ferrous sulfate and 250 μmol/L ferric citrate) is associated with a decrease in metabolites that were derived from carbohydrate including lactate, formate, ethanol, and succinate in the *in vitro* model of the human large intestine (Kortman et al., 2016). However, protein fermentation-related metabolites such as ammonia, BCFAs, and valerate were increased in high iron conditions resulting in an increased proteolytic activity using a colonic *in vitro* system (Dostal et al., 2018). These explorations indicated that in response to high levels of iron supplementation proteolytic activity is stimulated and the metabolome changes from a saccharolytic to a proteolytic profile (Kortman et al., 2016). Proteolytic activity converts proteins and peptides to BCFAs and ammonia, so their levels increase significantly, with high iron intake.

The gut microbiota and their metabolites directly affect the host metabolome, resulting in a wide range of responses to food interventions. The best characterized microbial products affected by the nutrient supplements are the volatile short-chain fatty acids involved in colonic enterocyte nutrition, amino acid metabolism, and energy-related metabolic pathways. More research on potentially beneficial microbial profiles using advanced metabolomics tools enabling more comprehensive nutrient assessment should be conducted for better insight into the molecular and physiological basis of nutrient and supplement effects.

1.7. Blood Metabolome Role to Determine Intestinal Mechanisms

As the major transportation system for absorbed nutrients, blood is extremely important for detecting metabolic perturbations under different conditions. Moreover, blood metabolomics analysis is an important part of the gut study since intestinal epithelium has the main role in nutrient absorption and transporting products of digestion into the blood so that blood metabolome can be used to investigate changes across multiple physiological pathways. Metabolomic studies illustrated that supplementary glutamate increased concentrations of glutamate and other amino acids in swine plasma including arginine, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, valine, alanine, glycine, proline, serine, tyrosine, and ornithine (Blachier et al., 2009; Meng et al., 2014). The reduction in the serum concentrations of very-low-density lipoprotein and lipids due to 46-day arginine treatment was linked to increased catabolism of fat and amino acids in 120-day-old pigs (He et al., 2009). In this regard, some products of fatty acid oxidation in the liver such as acetoacetate and b-hydroxybutyrate can be used as a useful marker of the mitochondrial function (Wu et al., 1991). An increase in acetoacetate rat serum concentration was observed, while there was a decrease in b-hydroxybutyrate concentration in the arginine treatment diet (120). This enhanced ratio suggested a more oxidized state in cells by intensive oxidation of fatty acids. Furthermore, NMR assay showed a reduction in serum levels of some metabolites including very-low-density lipoprotein, triglycerides, glycerophosphorylcholine, choline, citrate, 1-methylhistidine, glycoprotein, dimethylamine, and TMAO, whereas there were increased serum concentrations of 3-methylhistidine, betaine, pyruvate, and ketone bodies in the arginine-supplemented group. These changes in lipids and related metabolites, proposed amino acid

supplementation has an important role in the metabolism of energy substrates (He et al., 2009).

The combination of NMR with GC-MS strategies investigated the metabolic responses to dietary supplementation with lactosucrose, a functional trisaccharide, on the serum metabolome and intestinal luminal content in infected colitic rats (Rezaei et al., 2013; Ruan et al., 2013). Tricarboxylic acid cycle metabolites, including succinate, citrate, and fumarate, were affected by lactosucrose supplementation. An increased concentration of succinate, was associated with reduction of some amino acids (glutamate, glutamine, histidine, isoleucine, methionine, arginine, ornithine, proline, and valine), showing that there were protein breakdown and catabolism in humans, pigs, and rats (Rezaei et al., 2013; Ruan et al., 2013; Sun et al., 2017). Oligosaccharide supplementation also resulted in lower concentrations of all serum amino acids except glutamate and glutamine. Several possible metabolic mechanisms have been proposed to explain the mechanism of amino acid reduction. First, it is suggested that lactosucrose could increase amino acid catabolic rates of rats because of stimulating the activity of intestinal microbiota (Ruan et al., 2013). This can be supported by high levels of TMAO, which is related to higher microbial activity in the lumen of the human small intestine (Janeiro et al., 2018). Some other gut bacteria can deplete TMAO and reduce the level in blood to form dimethylamine (Janeiro et al., 2018). Lactosucrose was suggested to reduce the digestion and absorption of amino acids in the small intestine by shortening the transit time of digesta through the gut (Kong et al., 2018). In summary, metabolomic analyses have been widely used and shown to be effective to trace various dietary supplements including amino acid, carbohydrates, and pre/probiotics through to the blood metabolome level, often with improved use if biomarkers indicate

whether the changes are direct from the diet or from intestinal microbiota. However, future studies are needed to advance more knowledge on metabolome variations by applying more targeted metabolomic techniques and separation protocols on more functional food extracts. This would likely lead to the detection of more comprehensive metabolites and demonstrate a mechanistic rationale that explains the association between dietary supplements and intestinal metabolic pathways.

1.8. Current Challenges and Limitations in the Nutrimetabolomics Area

While the popularity of nutrimetabolomics as one of the latest directions in nutrition development and the omics branch has grown rapidly in the last decade, it is not without its challenges or difficulties. When nutrimetabolomics is properly performed, it can routinely lead to the detection of mechanisms that are influenced by nutrients, which can help to develop targeted diet-based disease treatments, although experimental data are limited up until now. Metabolomic results are still experimental, and they have to overcome many challenges in order to be fully implemented in several existing domains and industries. There are four issues that continue to challenge nutrimetabolomics, including sample selection, sample size, sample extraction, and metabolite detection and/or quantification. It seems obvious that a single biological sample is not sufficient to elucidate all the nutritional characteristics. The overall picture of the dietary intake and nutritional status of the experimental subjects could be measured by some biological samples, such as saliva, blood, urine, feces, and gut tissue. The rationale for selecting a particular tissue or biofluid for nutrient analysis will be essential for the appropriate physiological and biological interpretation of the observed metabolome (Ulaszewska et al., 2019).

Sample selection and sample size have always been other challenging factors to avoid nutrimetabolomics study bias and generate high-quality metabolomic data. Ideally, the best route to deal with population variation is to increase the sample size to obtain a sufficient number of biological replicates. However, metabolomics studies with high dimensional data have different sample size criteria than classical statistical methods since it is often not known which metabolites will change during nutritional studies (Nyamundanda et al., 2013). There are some proposed methods, one of which was developed by Nyamundanda et al., and is known as MetSizeR, to estimate sample size in metabolomic experiments whilst controlling the false discovery rate even when experimental pilot data are not available (Nyamundanda et al., 2013). Moreover, detailed characteristics of the population, including age, gender, body mass index, illness status, physical activity, dietary habits, medication use, and smoking habits, can hugely affect metabolomic results (Rist et al., 2017). These pre-analytical factors can greatly affect metabolomic results. Thus, it is important to capture as much "meta-data" as possible about all of the variables and carefully consider their metabolic effects.

Regarding sample extraction or separation, there continue to be a number of challenges in nutrimetabolomics-based analysis. The complexity of the sample extraction leads to low attention to new protocol development for nutritional metabolomics (Ulaszewska et al., 2019). Most laboratories also do not have access to optimized and roboticized protocols developed for biological sample extractions. Instead, they depend on the least expensive manual methods and decades-old extraction techniques, which can lead to variable results in replicate analysis. Additionally, metabolomic techniques will have to become far simpler

and cheaper and also far more 'kit' oriented to gain increasing popularity and significance in the life sciences (Wishart, 2016).

Among the most important issues, compound identification and quantification can highly influence the validity and usefulness of diet supplemented results (Rist et al., 2017). Biological samples in response to feed additives may consist of several hundred thousand different metabolites, spanning a variety of chemical classes that cannot be detected using a single method. Instead, researchers should rely on combinations of quantitative metabolomic assays to detect nutrient compounds and prove all aspects of feed supplementary effects. Detection of metabolites also requires very extensive databases of compound structures, and a continuing challenge is obtaining appropriate referential mass or NMR spectra. Therefore, significant investments in money, time, and a large number of metabolite standards are needed to provide an insight into the nutritional characteristics of samples. Although this seems obvious, the issue of instrument diversity or platform availability is much less of a challenge these days. There have been significant developments in highly standardized machines, software, and databases that facilitate high throughput chemical characterization as well as datasets suitable for data mining and sharing.

1.9. Thesis Objectives

The overreaching goal of this thesis was to investigate the utility and feasibility of metabolomics in understanding chicken biological and physiological responses to environmental factors and genetic background. Thus, this research used studies of prebiotics, probiotics, and heat stress treatments in chickens of different genetic backgrounds (two laying hen and one broiler strain) and different life stages to provide information on the nature and responsiveness the poultry metabolome.

The following three animal studies were used to accomplish the indicated objectives:

- A short-term (21-day) study to evaluate the effects of the seaweed supplement *Chondrus crispus* on laying hens of two commercial genetic lines beginning at week 34 of age. Specific objectives were to determine effects on performance, hematological

and blood chemistry (Chapter 2), as well as plasma and intestinal epithelial cell metabolomes (Chapters 3-4);

- Collaboration in an ongoing long-term study to evaluate the effects of seaweed supplementation with either *Chondrus crispus* or *Ascophyllum nodusum* for 45 weeks that included a four-week heat stress period in late stage lay in laying hens of two commercial genetic strains. Specific objectives were to assess experimental effects on hematological and blood biochemical indicators (Chapter 2) and the plasma and intestinal epithelial cell metabolomes (Chapter 3-4);
- Collaboration in an ongoing grower trial evaluating the response to three routes of in-ovo and oral probiotic administration in growing broiler chickens. The specific objectives were to assess experimental probiotic effects on the broiler plasma metabolome (Chapter 5).

1.10. Thesis Hypotheses

The specific hypotheses addressed in my thesis research are as follow:

- Seaweed supplementation, genetic strain, and heat stress will impact laying hen blood health indicators and performance traits, including egg production, feed intake, feed efficiency, and body weight.
- 1H NMR spectroscopy will reveal environmental and genetic impacts on the chicken plasma and epithelial cell metabolome.

- Untargeted metabolomics can effectively differentiate the delivery route of probiotic supplementation in broilers by identifying key metabolic changes and pathways that serve as indicators of responsiveness or significance.

1.11. Thesis Outline

- This thesis consists of six chapters. This introductory chapter (Chapter 1) provides the background information, motivation, hypotheses, and objectives for my PhD research program. It also provides a detailed literature review regarding poultry metabolomics and pre- and probiotic supplementation to improve health. This chapter also provides background material on the mechanisms of blood and intestinal responses to nutritional supplements. This chapter offers the foundation for later chapters of this thesis (Chapters 2-5) that describes my research work on blood chemistry, hematology, and metabolomic characterization of plasma and epithelial cells in chickens that were fed pre- and probiotic-supplemented diets.
- The second chapter investigates the effects of seaweed supplementation, genetic strain, heat stress, and their interactions on laying hen performance, blood chemistry, and hematology. These include measuring the performance and productivity of birds through feed intake, growth rate, egg production, and feed efficiency, as well as examining liver enzyme levels. In particular, the effects of 3% red seaweed *Chondrus crispus* and 0.5% brown seaweed *Ascophyllum nodosum* supplements on the heat stress responses in two genetic strains of laying hens, Lohmann LSL-Lite (White) and Lohmann Brown-Lite (Brown) are explored in the

long-term trial. Additionally, the short-term trial examines the effects of the 3% red seaweed supplement on these two genetic strains of laying hens. The results provides evidence of seaweed suitability for use in layer hens by focusing on the plasma health indicators.

- Chapters 3 and 4 provide the results of the quantitative metabolomic characterization of seaweed supplement, heat stress, and genetic strain in plasma and epithelial cells of laying hens in two trials. Several metabolites and metabolic pathways are examined through NMR spectroscopy for assessing bird responses to management interventions and identifying the key biomarkers and pathways linked to chicken health.
- The fifth chapter investigates the impact of the probiotic *Bacillus subtilis* delivery route (in-ovo, in-water, and in-feed probiotic intake) on the plasma metabolome of broilers. The differentially expressed metabolites are further used to identify the biological pathways associated with chicken performance, welfare, and health traits.
- Chapter 6 consists of a summary, a general discussion, and general conclusions drawn from the preceding data analysis. The chapter is closed by proposing some knowledge gaps and future directions that would guide researchers in advancing future studies in this field.

1.12. Preface

This thesis is an original work by myself, Shima Borzouie, under the supervision of Dr. Leslie MacLaren and Dr. Bruce Rathgeber at Dalhousie University. As detailed in the

following, a modified longer version of Chapters 1 of this thesis has been published in CABI Reviews, Chapter 2 has been published in Animals, Chapter 3 is ready for submission to the Canadian Journal of Animal Science, Chapter 4 and 5 are being prepared for submission to the Journal of Poultry Science.

All the studies were approved by the Dalhousie University Faculty of Agriculture Animal Care & Use Committee (Protocol number 2018-031; approved 31.5.2018; Protocol number; approved 1.9.2016) and followed the Canadian Council of Animal Care guidelines on the care and use of farm animals in research (CCAC, 2009).

Most of Chapter 1 of this thesis has been published as Borzouie, S., Rathgeber, B. M., and MacLaren, L. A. (2022). Application of metabolomics to assess the intestinal response to dietary supplementation. CABI reviews. 17, 004. doi: 10.1079/cabireviews202217004. I contributed to writing the original draft preparation. MacLaren, L. A., and Rathgeber, B. M., supervised and conceptualized the study and contributed to review and editing. All authors read and approved the final manuscript.

Chapter 2 of this thesis has been published as Borzouie, S., Rathgeber, B. M., Stupart, C.M., MacIsaac, J., and MacLaren, L. A (2020). Effects of dietary inclusion of seaweed, heat stress, and genetic strain on performance, plasma biochemical and hematological parameters in laying hens. Animals. 10, 1570. doi:10.3390/ani10091570. I wrote the original draft of the paper and performed the data analyses in this study. MacLaren, L. A., and Rathgeber, B. M., supervised the study, reviewed, and revised the paper, and were the principal investigators of the project, and participated in project management. MacLaren, L. A., Rathgeber, B. M., Stupart, C. M., MacIsaac, J., and I helped with experimental design, data collection, and provided

information on animals. All authors read, commented, and approved the final manuscript.

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MacLaren, L. A., Rathgeber, B. M., Stupart, C. M., and I helped with experimental design, data collection, and provided information on animals. Ian Burton ran samples on NMR.

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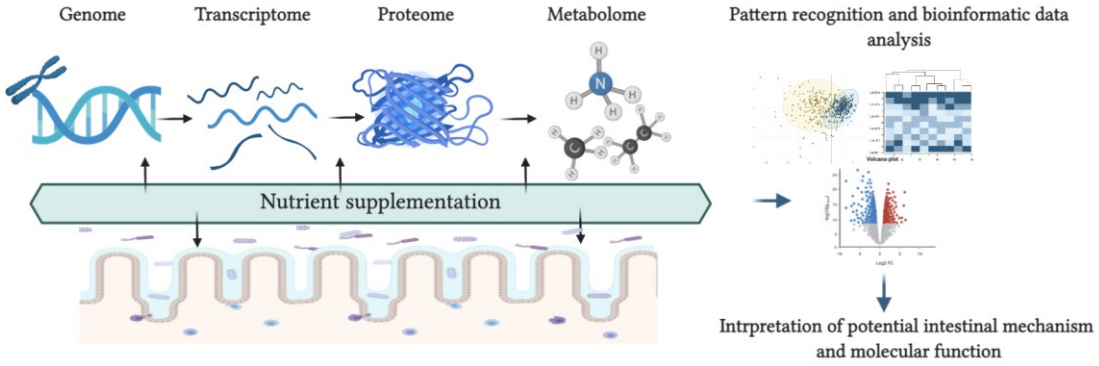
Table1.1. Pre/probiotic supplementation impacts on host metabolites.

Sample Type	Impacted Metabolites	Metabolomic Assay	References
Human blood	lysophosphatidylcholines ↓ sphingomyelins ↓ glycerophosphatidylcholines ↓ triglycerides ↑	UPLC/MS [3]	Cho et al., 2015
Human blood	triglycerides ↑ HDL cholesterol - triglycerides -	Meta-analysis	Moens et al., 2019
Murine epithelial tissue from distal colon	myoinositol ↑ scylloinositol ↑ cytosine ↓	NMR	Chinen et al., 2012
Murine epithelial tissues from jejunum and ileum	taurine ↑ glutathione ↑ creatine ↑ oxaloacetate ↑ choline ↑	NMR	Chinen et al., 2012
Murine fecal sample	isovaleric acid ↑ pentanoic acid ↑ hexanoic acid ↑ heptadecanoic acid ↑ hexadecanoic acid ↑ nonadecanoic acid ↑ tetradecanoic acid ↑ endotoxin ↓	GC-MS [2]	Parvez et al., 2006
Murine cecal content	acetate ↓ butyrate ↓ valerate ↓ propionate ↑ iso-butyrate ↑ isovalerate ↑	NMR [1]	Galdeano et al., 2007
Human colon content	indoles ↓ phenols↑ ammonia ↓	GC-MS	Park et al., 2021
Human blood	alanine ↑ isoleucine ↑ leucine ↑ 2-hydroxyisovalerate ↑ 2-oxoisocaproate ↑	NMR	Han et al., 2015
Goat ileum and jejunum	malondialdehyde ↓ superoxide dismutase ↑ glutathione peroxidase ↑	commercial kit	Harada et al., 2012

Lamb blood	cholesterol ↓ urea ↓	commercial kit	Shamloo et al., 2018
Broiler chicken blood	cholesterol - LDL[4] - VLDL [5] -	spectrophotometer	Aryaeian et al., 2017
Lamb blood	urea - creatinine -	commercial kit	Loureiro et al., 2019

[1] NMR: Nuclear magnetic resonance, [2] GC-MS: Gas chromatography-mass spectrometry, [3] UPLC/ MS: Ultra performance liquid chromatography/mass spectrometry, [4] LDL: Low density lipoproteins, [5] Very low-density lipoproteins (VLDL). ↑: increased levels, ↓: decreased level, -: no change.

Figure1.1. A schematic illustration of how nutrient supplementation are connected to metabolites and biological functions by showing connectivity of genome, transcriptome, proteome, and metabolome. Metabolomics can potentially help to understand the intestinal functionality by revealing metabolic functions and mechanism of action of nutrients. The effects of dietary supplementation can be evaluated at cellular and molecular levels as the consequence of metabolome change.



CHAPTER 2. EFFECTS OF DIETARY INCLUSION OF SEAWEED, HEAT STRESS, AND GENETIC STRAIN ON PERFORMANCE, PLASMA BIOCHEMICAL, AND HEMATOLOGICAL PARAMETERS IN LAYING HENS

2.1. Introduction

Interest in the use of prebiotics for poultry production is high as the need for alternatives to conventional antibiotics to promote better animal health and welfare grows. The reported beneficial effects of prebiotic consumption include the growth of beneficial microbes, inhibition of pathogen colonization, reduction of cancer risk and inflammation, improved mineral absorption and a reduction of total cholesterol, as well as low-density lipoprotein protein (LDL) cholesterol and triglycerides in blood serum (Tomar, et al., 2015; Kulshreshtha et al., 2017). Seaweed polysaccharides (alginate oligosaccharides and neoagarooligosaccharides) with prebiotic properties hold considerable interest as natural, low-cost, and highly nutritive additives for poultry (Gudiel-Urbano et al., 2002; Kulshreshtha et al., 2017; Chen et al., 2018; Cherry et al., 2019). Seaweed can be considered as an excellent source of bioactive compounds and dietary fiber, with strong antioxidant, anticoagulant, antiviral, anti-inflammatory, anticancer and antidiabetic properties (Manilal et al., 2009; Souza et al., 2012). Studied species reportedly contain highly bioactive metabolites such as vitamins, minerals, carotenoids, polysaccharides, polyphenols, and polyunsaturated fatty acids (Holdt et al., 2011). Red species such as *Chondrus crispus* are high in proteins, with an amino acid profile relatively high in glutamic acid, aspartic acid, glycine, alanine, arginine, and proline (Cerna et al., 2011).

We and others have identified seaweeds as a prebiotic source due to their low digestibility, fermentability and ability to enhance the growth of beneficial bacteria and reduce the

population of pathogenic bacteria (Abudabos et al., 2013; Kulshreshtha et al., 2017; Chen et al., 2018). The supplementation of chickens' basal diets with seaweed has been shown to enhance gut health, productivity, and dressing percentage (Abudabos et al., 2013; Evans et al., 2014; Kulshreshtha et al., 2014; Makkar et al., 2016). Although many studies on the beneficial effects of seaweed on chickens have focused on broilers (Carrillo et al., 1990; El-Deek et al., 2011; Evans et al., 2014), the inclusion of seaweed in the diets of laying hens showed improvements in antimicrobial resistance, egg quality, feed conversion ratio and overall gut health by altering chicken immunity (Carrillo et al., 2008; Kulshreshtha, et al., 2014). Collectively, these studies support the consideration of seaweed primarily as a prebiotic, recognizing that many seaweeds also are high-quality protein supplements. It is expected that the use of seaweed in diets will continue to increase, although more work is needed to understand how long-term seaweed supplementations impact the blood metabolism and leukocyte function, as well as overall health and performance.

Chickens respond to heat stress by generating a combination of behavioral, biochemical, physiological, and immunological changes. Heat stress causes significant decreases in chicken performance and productivity through reducing the feed intake, growth rate, egg production, egg quality and feed efficiency (Sahin et al., 2009). Heat stress can reduce the immune response of birds by suppressing the production of antibodies (Lebman et al., 1988; Savi et al., 1993; Zulkifi et al., 2000). The exposure of chickens to heat can also increase plasma corticosterone and decrease mineral levels, as well as cause a reduction in the activity and performance of lymphoid organs and total leukocytic count (El husseiny, et al., 1981; Hu et al., 2019). Examining the heat blood parameters and leukocyte numbers may provide useful clinical indicators of how birds respond to prebiotic supplements and

heat stress and their interactions in long-term production settings. How these vary with genetic strain is also of interest, since genetic backgrounds are expected to influence the resistance of laying hens to heat stress (Mack et al., 2013).

Thus, the present study was undertaken (1) to evaluate the effects of seaweed and thermal heat stress for two commercial laying hen lines on performance, hematological and blood biochemical determinations and (2) to evaluate the interaction effects of seaweed, heat stress and bird strain on the performance, hematological and biochemical parameters.

2.2. Materials and Methods

2.2.1. Birds, Housing and Diets

Two strains of commercial laying hens, Lohmann LSL-Lite (White) and Lohmann Brown-Lite (Brown), were housed at the Atlantic Poultry Research Institute (APRI) of the Dalhousie Agricultural Campus in Truro, Nova Scotia, Canada. For the short-term trial, 50 White and 50 Brown laying hens at 55 weeks of age and 10 moths of laying cycle, were supplemented with 0 or 3% red seaweed *Chondrus crispus* (CC) for 21 days prior to sampling using a completely randomized design with bird strain and seaweed as the factors (n = 100, 20 cages, 5 hens/cag and 5 cages of each strain-seaweed treatment combination, so that cage is the experimental unit). The long-term trial utilized 240 hens at 31 weeks of age and 3 months of laying cycle, in a factorial design testing the effects of bird strain, seaweed supplementation and heat stress. White or Brown hens (n = 240, 48 cages, 5 hens/cage and 4 cages of each treatment combination) were assigned to 0% or 3% (wt/wt) CC or to 0.5% (wt/wt) brown seaweed *Ascophyllum nodosum* (AN) (Tasco®, Acadian Seaplants Ltd., Dartmouth, Nova Scotia, Canada) from weeks 31 to 72. At 68 weeks of

age, two birds from each cage (per strain and per seaweed treatment) were transferred to mobile battery cage units in a separate room for a heat-stress evaluation, where temperatures gradually rose from 25 °C to 33 °C from 11 a.m. to 6 p.m. for 28 days.

For all trials, the lighting program used was the continuous lighting program of 16-h light and 8-h darkness per day. Basal diets were formulated based on the commercial requirements for Lohmann LSL-Lite provided by Lohmann Tierzucht GmbH, Cuxhaven, Germany (Table 2.1). Diet components were adjusted for the major nutrient contributions of the seaweeds to ensure diets were isocaloric and isonitrogenous. The poultry research unit is compliant with the Chicken Farmers of Canada's On-Farm Food Safety Assurance Program, and the use of both CC and AN were approved by the Canadian Food Inspection Agency. Birds were housed in conventional battery cages with ad libitum access to food and water in temperature-controlled rooms at 25 °C, except for the heat stress treatment, as indicated above. All experimental procedures were approved by the Dalhousie University Faculty of Agriculture Animal Care and Use Committee (Protocol number 2018-031; approved 31.5.2018) and carried out in accordance with the 3R principles and the Council of Animal Care Guidelines on the care and use of farm animals for research, testing and teaching (CCAC, 2009).

2.2.2. Seaweed Preparation

Cultivated *Chondrus crispus* were grown on land in saltwater by Acadian Seaplants Limited, Dartmouth, NS, Canada. It was dried at room temperature for 48 h in an environmentally controlled room at Atlantic Poultry Research Centre and manually turned every few hours to allow for uniform drying. Following drying, the seaweed was ground

through a 0.4-mm screen of a micro-Wiley mill (model 3; Arthur H. Thomas Co., Philadelphia, PA, USA) to be mixed into the diet. Tasco®, a commercially available prebiotic supplement for livestock and poultry, was also obtained from Acadian Seaplants Limited and was received harvested and solar-dried. For CC and AN, crude protein, crude fat, calcium, total phosphorus, potassium, magnesium, sodium, copper, manganese, and zinc were analyzed at the Nova Scotia Department of Agriculture Analytical Lab, Truro, NS, Canada. The specific amino acid analyses were provided by Acadian Seaplants Limited. Analyses were conducted by Silliker Canada Co., Markham, ON, Canada.

2.2.3. Layer Performance

Eggs were collected daily at the same time at 10 a.m., and the hen-day egg production percentage was recorded throughout the experiment. The egg production (%) was calculated on a per cage basis over the entire trial period. The total eggs laid per cage was divided by the number of days, which was multiplied by the number of hens (hen-day) on trial. The resulting value was then multiplied by 100. Individual feed intake and body weight for each cage were recorded throughout the trials to ascertain the variation. Feed intake (grams of feed per bird per day) was reported by determining the difference between the total daily feed given to each cage with the feed refusals collected and weighted. Eggs were collected daily, and body weights were determined on the first and last days of the trial to measure the body weight gain by subtracting the final weight from the initial weight. The weight gain and feed/egg were calculated based on the data recorded. All birds remained healthy during the whole trial.

2.2.4. Collection of Blood Samples and Chemical Analysis

At the end of the trial (55 weeks of age in the short-term trial and 72 weeks of age in the long-term trial), one bird from each cage was euthanized, and blood samples were collected for analysis in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, US) containing sodium heparin. Approximately 2 mL of blood plasma was isolated from birds by centrifugation at 2000× *g* at 4 °C for 10 min. The separated plasma was stored overnight at 4 °C and then analyzed at Diagnostic Services, Atlantic Veterinary College, University of Prince Edward Island (Charlottetown, PEI, Canada). The concentration of total plasma proteins, albumins, globulins, glucose, cholesterol with the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP) and glutamate dehydrogenase (GLDH) in the blood plasma were determined using a Roche Cobas 6000 analyzer (Roche Diagnostics, Mannheim, Germany). Duplicate samples were submitted, and results were averaged for each bird.

2.2.5. Hematology Sample Preparation

Blood samples collected at euthanasia were used immediately to create smears on a standard microscope slide. The smeared specimens were air-dried at room temperature for 24 h before being fixed and stained by Fisher HEMA 3 Stat pack TM (Fisher Diagnostics, Middle-town, VA, USA). Dried samples were dipped into Hema 3 solutions, including HEMA 3 fixative, eosinophilic staining solution (HEMA 3 solution I) and basophilic solution (HEMA 3 solution II), followed by dipping in distilled water five times in a row for 1 s each step. Stained blood smear samples were then stored at room temperature for

white blood cell count. The white blood cell count was performed manually in all samples. Hematological values were examined for differences due to strain, seaweed level and heat stress using a three-way Analysis of Variance (ANOVA) by the general linear model (GLM) procedure of the SAS software version 9.4 (SAS Institute Inc., Cary, NC, US).

2.2.6. Statistical Analyses

Each cage of five birds was considered as an experimental unit. A completely randomized design was used to analyze the effects of the strain, seaweed intake and heat stress and their interactions. Data were analyzed by three-way Analysis of Variance (ANOVA) using the general linear model (GLM) procedure of the SAS software version 9.4 (SAS Institute, Cary, NC, USA). Some data sets, including AST, ALT, GGT and GLDH, in both trials were log-transformed to convert to a normal distribution prior to ANOVA. The obtained results were subjected to statistical calculations of the least square mean and standard errors of the mean values. Significance was declared at $p \leq 0.05$, and where effects were significant, Tukey's test method was used to separate the means.

2.3. Results

2.3.1. Layer Production Performance

The hen production performance data for the short-term trial is presented in Table 2.2. Our data showed that the feed intake and weight gain were not significantly affected by the strain of bird ($p > 0.05$). However, the body weight of the Brown strain was clearly higher than that of the White strain ($p < 0.05$). The White hens displayed a higher egg production and lower feed/egg ratio for the entirety of the trial compared to the Brown birds ($p < 0.05$).

The feed intake, weight gain and feed/egg were significantly lower ($p < 0.05$) in laying hens with a short-term dietary supplementation of 3% CC compared with control birds. However, the short-term inclusion of red seaweed in laying hen diets had no effect on egg production ($p > 0.05$). There was no significant interaction between the short-term dietary inclusion of the seaweeds and the strain of bird ($p > 0.05$). The long-term trial layer production performance data is reported elsewhere, but briefly, White hens displayed consistently higher egg production, lower body weight and improved feed conversion compared to Brown hens ($p < 0.05$) (Stupart et al., 2019). Long-term seaweed intake and heat stress did not have a notable effect on any production parameters (Stupart et al., 2019).

2.3.2. Blood Plasma Biochemistry

The effects of the experimental treatments on the blood biochemistry is summarized in Table 3.5. The short-term seaweed supplementation elevated the globulin and protein levels in White birds but not in Brown. The remaining tested plasma biochemical parameters were not significantly ($p > 0.05$) influenced by the short-term seaweed supplementation (Table 2.3). The White birds fed with 0.5% AN seaweed had the highest globulin levels. Significant differences ($p < 0.05$) were noted between the long-term seaweed intake and control groups for albumin, ALT and GGT (Table 2.4). The statistical evaluation showed a significant increase of GGT in AN-supplemented hens versus control and CC-supplemented hens ($p < 0.05$; Figure 2.1D). Albumin and ALT levels were significantly reduced due to CC intake, while the Tasco® (AN) diet caused an elevation of their levels ($p < 0.05$). We noticed an increase of AST and decrease of GLDH in seaweed-supplemented birds; however, the observed differences were not significant ($p > 0.05$).

Interestingly, in the short-term trial, the two strains of bird had similar enzyme profiles. However, the strain effect was more dominant in the long-term trial and expressed more, since significant differences in the biochemistry parameters were found between the two strains for the total protein, globulin, ALP, ALT and GGT ($p < 0.05$) (Table 2.4). These parameters were higher in the White strain, except for ALT and GGT, which were higher in the Brown strain (Figure 2.1). The White hen strain had higher plasma protein and globulin levels in both trials. As Table 2.5 demonstrates, the heat stress was associated with reduced protein, globulin and glucose and increased cholesterol and GGT levels ($p < 0.05$). There was, however, a significant interaction between bird strain and heat stress on the total protein, globulin, glucose, cholesterol and GLDH ($p < 0.05$). This interaction was also observed between the bird strain and seaweed for the effects on cholesterol ($p < 0.05$). Among the blood parameters studied, GGT was affected the most by the heat-stress treatment. As shown in Table 2.5, the only instance that the glucose level increased in the White strain was when both the heat stress and Tasco were applied, and a decrease in the GGT level happened only when both the 3% CC and heat stress were applied. Seaweed did not affect a heat stress-associated blood response

2.3.3. Hematology Analysis

The effects of three different treatments on the differential white blood cell counts and heterophil-to-lymphocyte ratio (H/L) were examined in this study. Basophils, eosinophils, and monocytes accounted for less than 1% of the total leukocytes, roughly 23% for heterophils and about 76% for lymphocytes. The white blood cell count was not statistically associated with the seaweed intake, with no interactions with other treatments

($p > 0.05$). However, there was a significant effect of the strain on the values of the heterophil and lymphocyte counts, as the Brown strain had higher heterophil and lower lymphocyte counts and a higher H/L ratio ($p < 0.05$) (Figure 2.2). Furthermore, the blood eosinophil counts were associated with heat stress. After heat stress, the eosinophil number in Brown hens reduced from 0.51 to 0.27 (eosinophils/100 leukocytes) and, in White hens, from 0.83 to 0.17 ($p < 0.05$). The basophil number was not affected by any treatments in the study ($p > 0.05$).

A significant interaction was detected between the heat stress and strain for effects on the H/L ratio (Figure 2.2C). The H/L ratio increased in response to the heat stress in Brown, but not White, hens independent of the seaweed treatment ($p < 0.05$).

2.4. Discussion

2.4.1. Layer Performance

Our study indicated significant effects of short-term red seaweed intake on laying hen performances. The reduction in feed intake decreased the feed/egg ratio, but the egg production remained at consistent levels throughout the experiment. Birds fed red seaweed lost an average of 14% of their body weight (about 130 g) during the 21-day trial. However, in the long-term trial, the feed intake, feed/egg and body weight were not affected over the long term by the seaweed levels (Stupart et al., 2019) which may be associated with an improvement of gut health and/or adaptation to the change in palatability of the diets. Similarly, the 21-day supplementation of broilers with 0.3% and 0.4% CC led to an increased productive efficiency, with significant decreases in the feed intake and feed conversion ratio (Martínez et al., 2019). However, the body weights of broilers did not

show significant differences among broilers at 1, 21 and 32 days with CC supplementation (Martínez et al., 2019). This was in accordance with another 30-day feeding trial with no significant effect on the feed intake or body weight of laying hens fed on the 0.5%, 1% and 2% CC diets (Holdt et al., 2011). Several researchers reported similar performance results with other seaweeds. Ventura et al., studied the effect of inclusion of *Ulva rigida* seaweed on chicken performance, which showed that, as the level of seaweed increased, the feed intake and body weight gain decreased (Ventura et al., 1994). This shows a good correlation with these results. Ross and Dominy also reported that blue-green algae, *Spirulina platensis*, does not affect the growth performance and egg production of layer chickens (Ross et al., 1990). Another study demonstrated that a supplementation of prebiotics had no impact on the feed intake of broilers in a 42-day trial (Mookiah et al., 2014). In other avian species, it was previously reported that the dietary supplementation of another red seaweed (*Polysiphonis SPP*) did not have any effect on the growth performance and carcass quality in ducks (El-Deek et al., 2009). Conversely, there were some studies that reported the body weight gain, feed intake and feed conversion of broilers were increased upon a prebiotic intake (Bednarczyk et al., 2016; Nikpiran et al., 2013).

2.4.2. Blood Biochemistry

The blood enzyme activity appears to be a suitable diagnostic method to evaluate the hepatic function and reflect the degree of hepatocellular damage and leakage, tissue damage or organ dysfunction in birds (Jaensch et al., 2000). As such, liver damage can be indicated by analyzing the plasma levels of ALT, AST, ALP, GGT and GLDH as the most frequently used biomarkers in clinical practice (Jaensch et al., 2000; Bona et al., 2018).

The plasma levels of ALT and AST reflect the integrity of hepatocytes, while ALP and GGT have limited roles in the diagnosis of hepatic diseases in birds (Ritchie et al., 1994; Jaensch et al., 2000). Among all the blood parameters, the AST level is considered as the most specific and sensitive enzyme for detecting avian liver and muscle damage (Jaensch et al., 2000), and its level was not modified in response to the supplementation with seaweed, heat stress or among strains. Generally, the seaweed intake did not significantly affect the plasma enzyme levels, although the CC supplementation was associated with numerically decreased levels of ALT enzyme activity in White layers. It may be that a larger-scale study might show a modest beneficial effect. Conversely, the AN supplementation was associated with elevated GGT levels. Overall, the enzyme values were within the ranges observed previously in layers (Neijat et al., 2014).

The genetic strain and heat stress had more widespread impacts on these indicator enzymes; for example, both GLDH and ALT were increased in response to heat stress in Brown, but not White, birds, indicating genetic differences in heat-stress susceptibility. Regardless of the strain, the plasma GGT level was elevated, over three times higher than normal level, when heat stress was applied. A high blood GGT level can be associated with an inflammatory response or indicate avian liver and biliary compromises (Ikeda et al., 1995; Harrison et al., 2005). Overall, our results are consistent with previous works in broiler. Chickens and rats that showed that the liver is susceptible to oxidative stress during acute heat exposure in broiler chickens (Lin et al., 2006; Bloomer et al., 2008).

The blood serum protein can reflect changes in the condition of an animal associated with inflammation, liver, or kidney damage, as well as other internal and external factors. That heat stress was associated with decreased protein and globulin levels in Brown, but not

White, birds further illustrate the former's increased sensitivity to heat stress. Genetic differences in heat-stress responses have been observed by others (Mack et al., 2013). The elevated plasma cholesterol and decreased glucose levels observed in Brown hens in response to heat stress were not predictable based on the studies of others, but certainly, similar changes have been observed, as discussed by El Kholy and colleagues (El-Kholy, et al., 2017).

Similarly, Kulshreshtha et al., reported that serum concentrations of the total protein, glucose and AST of layer hens supplemented with CC seaweed meal for 28 days were close to those of the control group (Kulshreshtha, et al., 2014). Al-Harthi and colleagues found no significant changes in the ALP contents of plasma in laying hens after a brown seaweed intake (Weber et al., 2013), and others found no significant differences in the globulin and AST when a brown seaweed treatment was administered in laying hens (Choi et al., 2013). Evenni et al., and Rizk et al.'s findings indicated that albumin levels were elevated in layers fed diets containing brown seaweed, which is in a good agreement with our results (Evenni, et al., 2016; Rizk et al., 2017). Albumin is associated with the functionality of the liver (Evenni et al., 2016). Different results were observed in other animal species, as rats fed brown seaweed resulted in a significant decrease of AST (Evenni et al., 2016).

2.4.3. Hematological Parameters

The H/L ratio can provide an indicator of stress in birds, reflecting changes in the cellular and humoral immune system responses. High H/L ratios have been associated with increased stress and mortality, as well as weaker antibody responses in broiler chickens (Krams et al., 2012). There are limited leucocyte studies in farm animals fed various

seaweed species (Archer et al., 2007; Sotoudeh, et al., 2015), and very little is known about the effect of the inclusion of seaweed on the white blood cell count and H/L ratio when fed to laying hens. Our earlier study showed reduced total leukocyte counts at a 2% CC supplementation and elevated total leukocyte counts at a 4% CC supplementation, so perhaps the current outcome of no observed impact at a 3% CC on the neutrophil, heterocyte or lymphocyte counts is not surprising. Similarly, Kang et al., reported that a supplementation of green algae *Chlorella vulgaris* had no impact on the blood leukocyte counts of broiler chickens, including heterophils, lymphocytes, monocytes, eosinophils, or basophils (Kang et al., 2013). However, supplemental *Ascophyllum nodosum* tended to increase ($p \leq 0.10$) the white blood cell counts, eosinophils, and lymphocytes in lambs (Archer et al., 2007). In contrast, a reduction in the total leukocyte counts was observed in mice following an intake of red seaweed *Gracilaria birdiae* (Vanderlei et al., 2011). The difference between our results and some reports can be mainly due to the different animal species or seaweed types.

Our results also confirmed that the level of neutrophils was not different ($p > 0.05$) among the seaweed dietary treatments and control birds, which was similar to Sotoudeh and Jafari's results in rainbow trout fed with red seaweed (Sotudeh et al., 2015). In contrast to these results, Vetvicka and Oliveria and Archer et al.'s findings indicated significant increases in the blood neutrophils of pigs and lambs (Archer et al., 2005; Vetvicka et al., 2014).

That heat stress increased the blood H/L ratio in Brown layer hens was consistent with our plasma biochemistry observations that the Brown strain was more susceptible to the adverse effects of heat stress than the White strain. An elevated H/L ratio is expected in

stressed hens, including in heat-stressed birds, and is thought to be a consequence of acute inflammatory responses to infectious, as well as noninfectious, causes (Hu et al., 2019). The type and intensity of the immune response depends on the environmental and genetic factors, as well as the physiological status of the animal. This increased H/L ratio correlated with increased numbers of heterophils, and decreased numbers of lymphocytes has been reported as a reliable indicator of life-threatening stressors in chickens (Gross et al., 1983; Mashaly et al., 2004).

2.5. Conclusions

Overall, White laying hens displayed a higher egg production and feed efficiency and were less susceptible to the adverse effects of heat stress, as indicated by the production, plasma components.

and liver enzyme profiles and white blood cell data. A long-term supplementation with *Chondrus crispus* or *Ascophyllum nodosum* seaweed did not negatively affect the plasma health indicators. *Chondrus crispus* supplementation at 3% transiently increased the feed efficiency in the short term, providing further evidence of its suitability for use in layer hens.

Table 2.1. Diet formulation (g/kg) and calculated composition (as the fed basis) of the laying diet phases.

Ingredients	Phase 1			Phase 2			Phase 3		
	0%	3% CC	0.5% AN	0%	3% CC	0.5% AN	0%	3% CC	0.5% AN
Ground Corn	532.56	497.47	524.5	538.45	503.37	530.47	549.13	513.17	541.13
Canola Meal	100	100	100	100	100	100	100	100	100
Wheat	100	100	100	100	100	100	100	100	100
Soybean Meal	143.59	136.64	144.28	135.57	126.62	136.26	123.35	116.42	124.05
Limestone	45.15	44.96	45.07	46.43	46.26	46.35	47.7	47.55	47.61
Shell Mix	22.58	22.49	22.53	23.23	23.13	23.17	23.85	23.77	23.81
Oyster Shell	22.58	22.49	22.53	23.21	23.13	23.17	23.85	23.77	23.81
Animal/Vegetable Fat	11.82	27.47	14.7	11.93	27.57	14.81	11.42	27.09	14.31
Dicalcium Phosphate	11.18	11.39	11.21	10.8	11.01	10.83	10.44	10.66	10.48
Vitamin/Mineral Premix [1]	5	5	5	5	5	5	5	5	5
Salt	3.73	0.14	3.33	3.61	0.02	3.21	3.63	0.04	3.22
Mehionine Premix [2]	1.8	1.91	1.75	1.77	1.88	1.72	1.62	1.74	1.58
Tasco	0	0	5	0	0	5	0	0	5
Ground *Chondrus crispus*	0	30	0	0	30	0	0	30	0
Calculated Composition (%)									
Metabolizable Energy (kCal/kg)	2800	2800	2800	2800	2800	2800	2800	2800	2800
Protein (%)	16.04	16.04	16.04	15.71	15.71	15.71	15.22	15.22	15.22
Calcium (%)	3.73	3.73	3.73	3.82	3.82	3.82	3.91	3.91	3.91
Available Phosphorus (%)	0.4	0.4	0.4	0.39	0.39	0.39	0.38	0.38	0.38
Sodium (%)	0.17	0.17	0.17	0.16	0.16	0.16	0.16	0.16	0.16

[1] Vitamin and mineral mixture (g/kg of premix): vitamin A (retinol), 1.56 g; vitamin D3 (cholecalciferol), 480.00 g; vitamin E (dl-alpha tocopheryl acetate), 8.00 g; vitamin K (menadione sodium bisulphate), 1.80 g; thiamine, 0.40 g; riboflavin, 1.90 g; pantothenic acid (as calcium pantothenate), 3.20 g; biotin, 32.00 g; folic acid, 4.40 g; vitamin B12, 2.30 g; niacin, 6.16 g; pyridoxine, 0.80 g; manganous oxide, 23.40 g; zinc oxide, 22.22 g; copper sulphate, 20.00 g; selenium premix, 14.86 g; ethoxyquin, 16.66 g; ground corn, 46.66 g and limestone, 100 g. [2] Methionine premix is composed of 50% wheat middlings and 50% DL methionine. CC, *Chondrus crispus* and AN, *Ascophyllum nodosum*.

Table 2.2. Effects of strain and seaweed intake on the means and standard errors of layer hen performances in the short-term trial.

Parameters	Brown Strain		White Strain		SEM	P-Value Strain	P-Value Seaweed
	0% CC	3% CC	0% CC	3% CC			
Egg production (%)	85.1 ^{b}	81.3 ^{b}	88.2 ^{a}	87.1 ^{a}	0.16	0.002	NS [1]
Feed Intake (g/bird/day)	95.1 ^{a}	85.9 ^{b}	90.6 ^{ab}	87.1 ^{b}	29.5	NS	<0.001
Feed/egg	108.0 ^{a}	96.7 ^{bc}	96.7 ^{b}	95.2 ^{c}	1.7	0.02	0.02
Body Weight (g)	2113.3 ^{a}	2083.9 ^{ab}	1798.4 ^{b}	1743.6 ^{b}	36.3	<0.001	NS
Weight gain (g)	98.2 ^{ab}	−119 ^{b}	135.8 ^{a}	−142.4 ^{b}	42.8	NS	<0.001

^{a,b,c} Least squares means within the same row with different superscripts were different according to the Tukey means comparison test ($p < 0.05$). [1] NS: no significant differences detected (p-value > 0.05). Note: No significant interaction was observed between the treatments.

Table 2.3. Genetic strain and seaweed effects on the plasma chemistry (least squares mean ± standard error) in the short-term trial.

Plasma Parameter	Units	Brown Strain		White Strain		P-Value Strain	P-Value Seaweed
		0% CC	3% CC	0% CC	3% CC		
Protein	g/L	52.9 ± 3.4 [b]	52.3 ± 1.8 [b]	54.3 ± 0.9 [ab]	64.0 ± 3.1 [a]	0.02	NS
Albumin	g/L	18.1 ± 0.7	19.3 ± 1.1	17.4 ± 0.6	18.1 ± 0.4	NS	NS
Globulin	g/L	34.8 ± 2.7 [b]	33.0 ± 0.9 [b]	36.9 ± 1.3 [ab]	46.0 ± 3.5 [a]	<0.01	NS
Glucose	mM/L	14.2 ± 0.3	13.7 ± 0.4	13.5 ± 0.3	13.2 ± 0.3	NS	NS
Chol [2]	mM/L	1.1 ± 0.2	0.9 ± 0.07	0.8 ± 0.07	0.8 ± 0.08	NS	NS
ALP [3]	U/L	246.9 ± 47.2	206.8 ± 49.2	226.5 ± 44.5	202.3 ± 27.6	NS	NS
AST [4]	U/L	194.9 ± 19.1	167.8 ± 8.3	181.0 ± 4.9	227.2 ± 32.7	NS	NS
ALT [5]	U/L	4.6 ± 2.8	3 ± 0.3	2.7 ± 0.5	2.8 ± 0.3	NS	NS
GGT [6]	U/L	24.3 ± 3.1	32.9 ± 5.7	21.7 ± 1.9	22.3 ± 2.3	NS	NS
GLDH [7]	U/L	2.6 ± 0.4	2.4 ± 1.2	3.2 ± 1.6	1.3 ± 0.2	NS	NS

[a,b,c] Least squares means within same row with different superscripts were different according to the Tukey means comparison test ($p < 0.05$).[1] NS: no significant differences detected (p-value >0.05). [2] Chol: Cholesterol, [3] ALP: alkaline phosphatase, [4] AST: aspartate aminotransferase, [5] ALT: alanine aminotransferase, [6] GGT: gamma-glutamyl transferase and [7] GLDH: glutamate dehydrogenase. Note: No significant interaction was observed between the treatments.

Table 2.4. *P*-values from the ANOVA test results from the long-term trial, showing the effects of the genetic strain, seaweed intake and heat stress and their interaction effects on the plasma chemistry in layer hens.

Plasma Parameter	Units	P-Value Strain	P-Value Seaweed	P-Value Heat Stress	Strain × Seaweed	Seaweed × Heat Stress	Strain × Heat Stress	Strain × Seaweed × Heat Stress
Protein	g/L	<0.01	NS [1]	<0.01	NS	NS	0.02	NS
Albumin	g/L	NS	0.04	NS [1]	NS	NS	NS	NS
Globulin	g/L	<0.01	NS	<0.01	NS	NS	0.02	NS
Glucose	mM/L	NS	NS	<0.01	NS	NS	0.01	NS
Chol	mM/L	NS	NS	<0.01	0.01	NS	<0.01	NS
ALP	U/L	<0.01	NS	NS	NS	NS	NS	NS
AST	U/L	NS	NS	NS	NS	NS	NS	NS
ALT	U/L	0.01	NS	<0.01	NS	NS	<0.01	NS
GGT	U/L	0.02	0.02	0.04	NS	NS	NS	NS
GLDH	U/L	NS	NS	NS	NS	NS	<0.01	NS

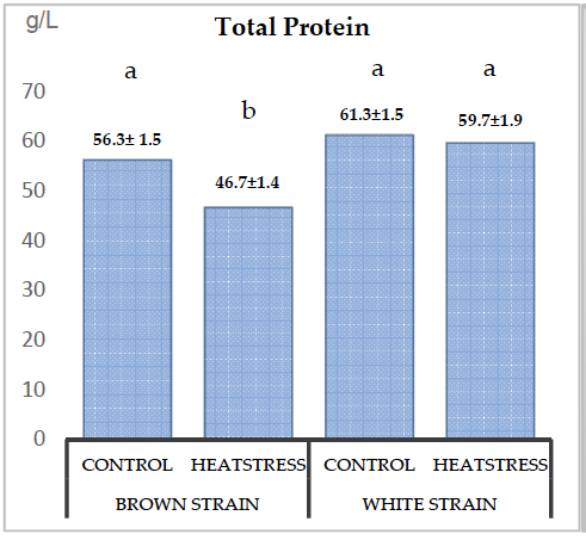
[1] NS: no significant differences detected (p-value >0.05) Note: No significant interaction was observed between the strain and seaweed treatments, except for cholesterol.

Table 2.5. Effects of the long-term seaweed intake and a 28-day heat stress on the plasma biochemical analytes in two genetic strains of layers. Values shown as least squares means ± standard error.

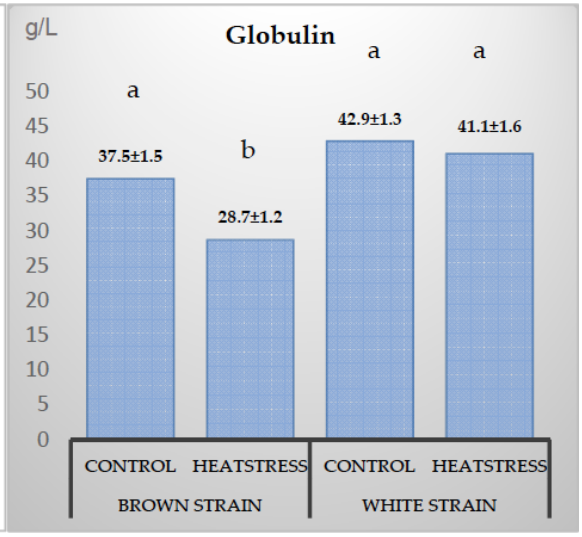
	0% CC		3% CC		0.5% AN	
Brown Strain						
Plasma Parameter	**Control**	**Heat Stress**	**Control**	**Heat Stress**	**Control**	**Heat Stress**
Protein	59.7 ± 1.4 ^{abc}	45.2 ± 1.9 ^{d}	53.5 ± 3.2 ^{abcd}	47.4 ± 3.4 ^{cd}	55.9 ± 2.2 ^{abcd}	47.6 ± 2.5 ^{bcd}
Albumin	19.5 ± 0.5	18.1 ± 0.4	17.5 ± 0.7	17.4 ± 1.1	19.5 ± 0.9	18.6 ± 0.4
Globulin	40.5 ± 1.1 ^{abc}	27.1 ± 2.1 ^{d}	35.7 ± 3.8 ^{abcd}	30.0 ± 2.5 ^{bcd}	36.4 ± 2.1 ^{abcd}	29.0 ± 2.2 ^{cd}
Glucose	14.1 ± 0.3 ^{ab}	11.9 ± 0.6 ^{b}	14.1 ± 0.3 ^{ab}	12.5 ± 0.8 ^{b}	14.4 ± 0.8 ^{ab}	11.8 ± 0.4 ^{ab}
Chol	1.4 ± 0.3 ^{c}	3.7 ± 0.4 ^{a}	1.7 ± 0.2 ^{bc}	3.4± 0.5 ^{ab}	2.4 ± 0.3 ^{abc}	3.8 ± 0.6 ^{a}
ALP	156.7 ± 31.6	190.5 ± 59.8	195.4 ± 39.6	191.6 ± 66.3	171.9 ± 18.1	131.7 ± 28.1
AST	169.2 ± 7.9	156.125 ± 10.5	153.6 ± 13.7	198.1 ± 36.9	218.5 ± 30.1	194.4 ± 12.2
ALT	4.0 ± 0.7 ^{bc}	16.0 ± 4.5 ^{a}	3.5 ± 0.9 ^{c}	14.7 ± 2.6 ^{ab}	3.5 ± 1.5 ^{c}	12.2 ± 5.6 ^{abc}
GGT	32.5 ± 1.2	65.6 ± 30.9	42.2 ± 9.5	115.5 ± 48.6	49.4 ± 7.6	133.8 ± 56.3
GLDH	2.7 ± 2.3 ^{b}	26.6 ± 8.4 ^{a}	1.1 ± 0.6 ^{b}	10.7 ± 7.7 ^{ab}	6.6 ± 3.1 ^{ab}	9.7 ± 5.1 ^{ab}
White strain						
Protein	59.5 ± 2.1 ^{abc}	58.5 ± 2.1 ^{abcd}	61.5 ± 3.5 ^{ab}	58.4 ± 5.1 ^{abcd}	62.9 ± 2.0 ^{a}	62.4 ± 2.4 ^{a}
Albumin	18.0 ± 0.4	18.6 ± 0.7	18.7 ± 0.5	17.2 ± 0.6	18.4 ± 0.9	20.0 ± 1.1
Globulin	41.5 ± 2.4 ^{abc}	39.9 ± 1.6 ^{abc}	42.7 ± 3.1 ^{a}	41.1 ± 4.5 ^{abc}	44.5 ± 1.7 ^{a}	42.4 ± 1.8 ^{ab}
Glucose	13.7 ± 0.5 ^{ab}	12.5 ± 0.4 ^{ab}	13.7 ± 0.3 ^{ab}	12.7 ± 0.3 ^{ab}	14.0± 0.6 ^{ab}	14.8 ± 0.7 ^{a}
Chol	2.8 ± 0.3a ^{bc}	3.5 ± 0.3 ^{ab}	3.4 ± 0.4 ^{ab}	2.2 ± 0.1 ^{abc}	1.6 ± 0.2 ^{bc}	2.5 ± 0.6 ^{abc}
ALP	347.6 ± 46.9	263.6 ± 47.8	218.6 ± 31.4	264.7 ± 55.9	233.8 ± 9.1	219.9 ± 27.6
AST	190.1 ± 14.9	161.4 ± 9.4	176.5 ± 11.7	225.6 ± 31.9	199.6 ± 13.5	202.9 ± 27.3
ALT	6.4 ± 2.9 ^{abc}	3.7 ± 0.8 ^{c}	2.4 ± 0.5 ^{c}	1.6 ± 0.7 ^{c}	5.1 ± 1.5 ^{abc}	6.4 ± 1.3 ^{abc}
GGT	26.1 ± 4.5	28.0 ± 4.9	26.9 ± 6.9	20.4± 0.2	35.0 ± 1.8	139.4 ± 59.5
GLDH	9.4 ± 7.2 ^{ab}	1.7 ± 0.7 ^{b}	2.8 ± 0.8 ^{b}	2.5 ± 0.5 ^{b}	7.5 ± 2.9 ^{ab}	3.6 ± 1.9 ^{b}

^{a,b,c,d} Least squares means within the same row with different superscripts were different according to the Tukey means comparison test ($p < 0.05$).[1] NS: no significant differences detected (p-value >0.05).

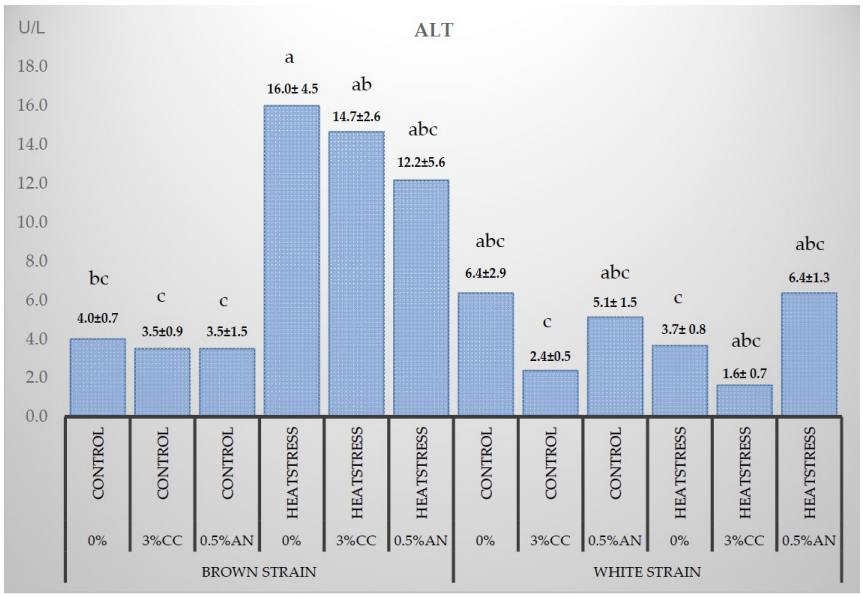
Figure 2.1. Effects of the genetic strain, long-term seaweed intake and heat stress on the total protein (**A**), globulin (**B**), alanine aminotransferase (ALT) (**C**) and gamma-glutamyl transferase (GGT) (**D**) in layer hens (p-value <0.05). Values are least squares means ± standard error. a,b,c: Bars with different letters were different according to the Tukey means comparison test ($p < 0.05$). CC: *Chondrus crispus.* AN: *Ascophyllum nodosum.*



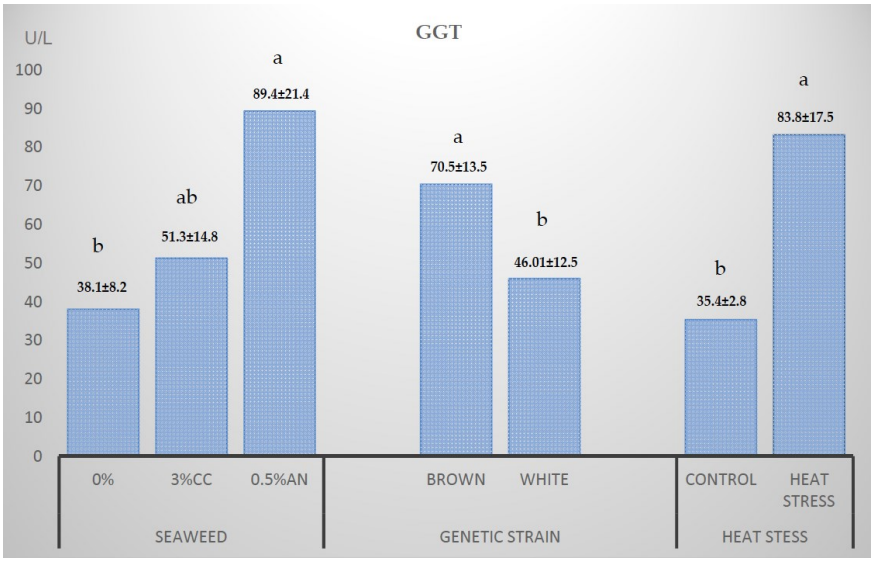
(A)



(B)

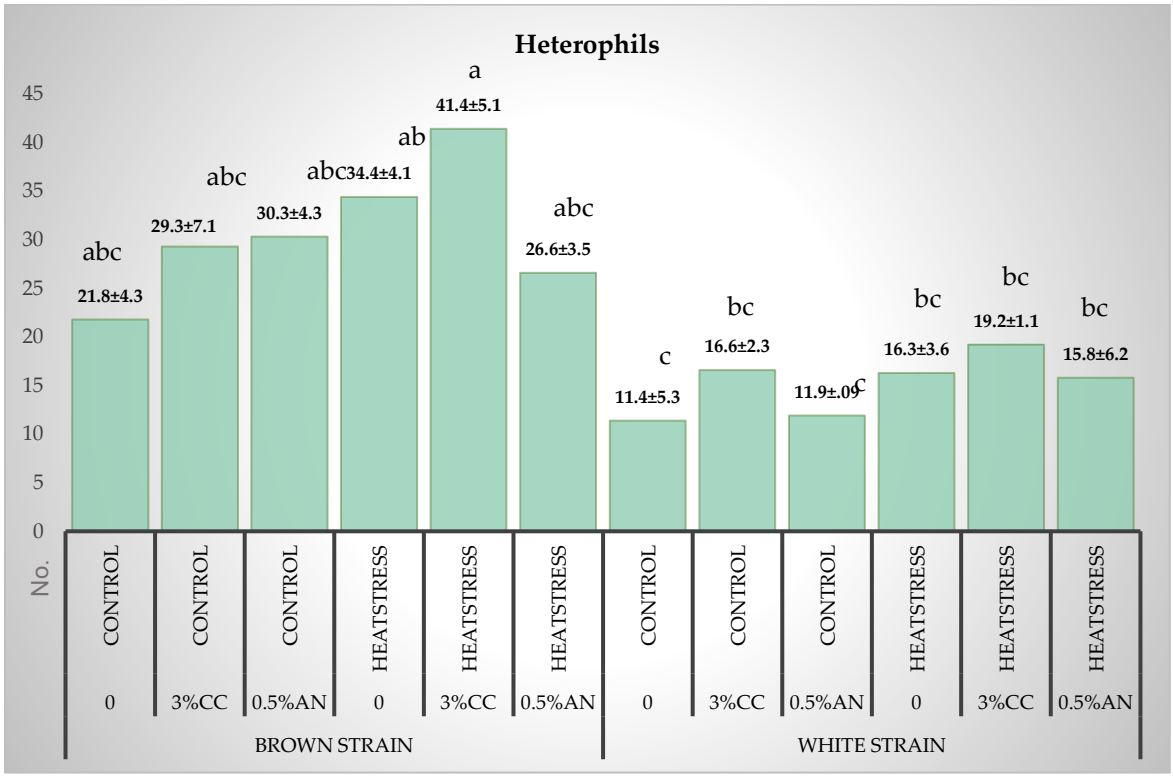


(C)

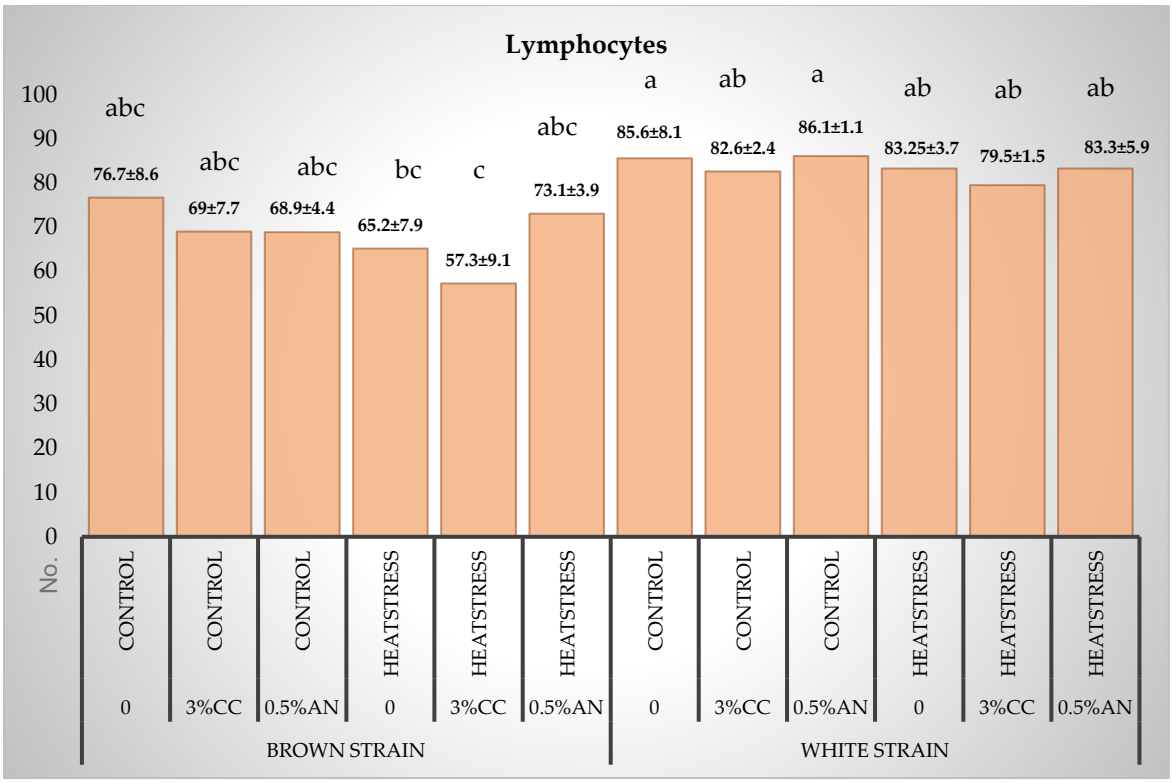


(D)

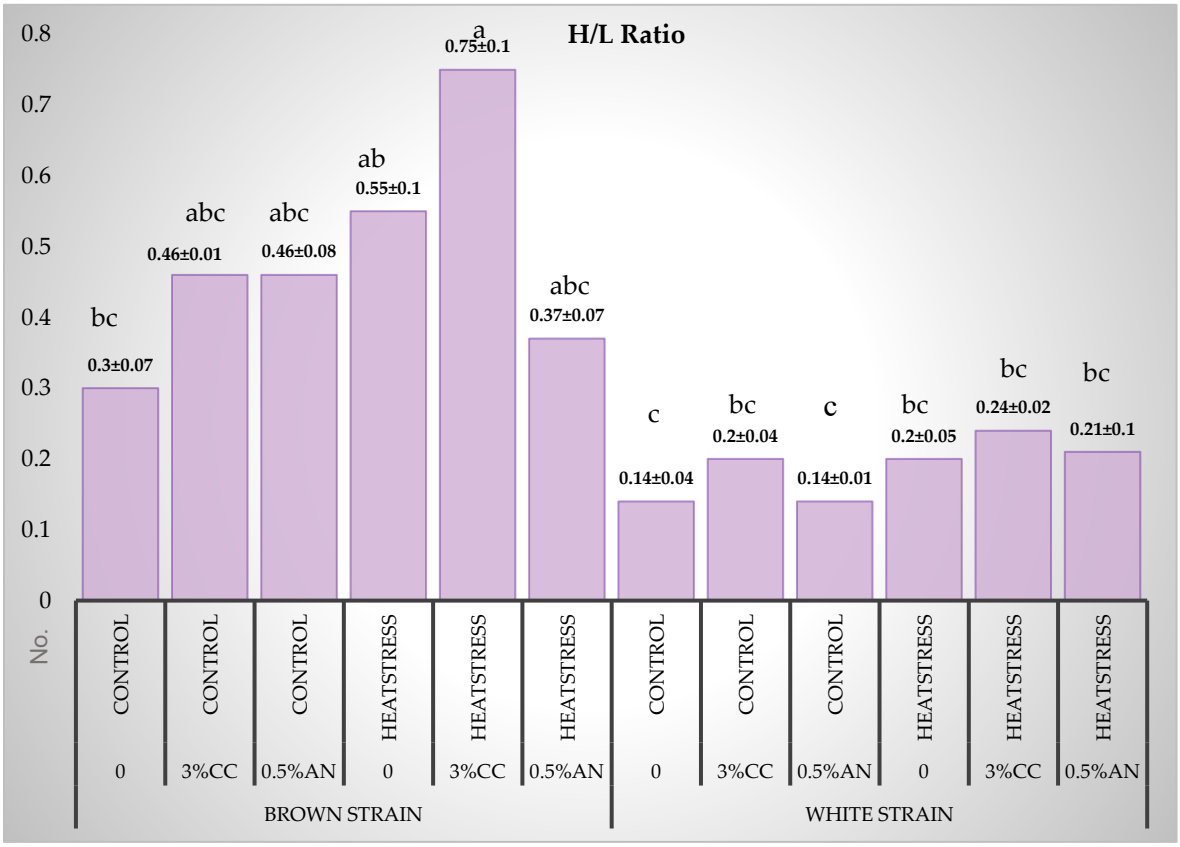
Figure 2.2. Heterophil number (**A**), lymphocyte number (**B**) and Heterophil/lymphocyte ratio (**C**) in White and Brown layer hens subjected to 4-week heat stress following the long-term feeding of seaweed supplements (means ± standard error). a,b,c: Bars with different letters were different according to the Tukey means comparison test ($p < 0.05$). CC: *Chondrus crispus.* AN: *Ascophyllum nodosum.*



(A)


Lymphocytes
100
90
80
70
60
50
40
30
20
10
0
No.
abc
76.7±8.6
abc
69±7.7
abc
68.9±4.4
bc
65.2±7.9
c
57.3±9.1
abc
73.1±3.9
a
85.6±8.1
ab
82.6±2.4
a
86.1±1.1
ab
83.25±3.7
ab
79.5±1.5
ab
83.3±5.9
CONTROL
CONTROL
CONTROL
HEATSTRESS
HEATSTRESS
HEATSTRESS
CONTROL
CONTROL
CONTROL
HEATSTRESS
HEATSTRESS
HEATSTRESS
0
3%CC
0.5%AN
0
3%CC
0.5%AN
0
3%CC
0.5%AN
0
3%CC
0.5%AN
BROWN STRAIN
WHITE STRAIN

(B)


H/L Ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
No.
bc
0.3±0.07
abc
0.46±0.01
abc
0.46±0.08
ab
0.55±0.1
a
0.75±0.1
abc
0.37±0.07
c
0.14±0.04
bc
0.2±0.04
c
0.14±0.01
bc
0.2±0.05
bc
0.24±0.02
bc
0.21±0.1
CONTROL
CONTROL
CONTROL
HEATSTRESS
HEATSTRESS
HEATSTRESS
CONTROL
CONTROL
CONTROL
HEATSTRESS
HEATSTRESS
HEATSTRESS
0
3%CC
0.5%AN
0
3%CC
0.5%AN
0
3%CC
0.5%AN
0
3%CC
0.5%AN
BROWN STRAIN
WHITE STRAIN

(C)

CHAPTER 3: PLASMA 1H-NUCLEAR MAGNETIC RESONANCE METABOLOMICS REVEAL AVIAN RESPONSES TO SHORT AND LONG-TERM SEAWEED SUPPLEMENTATION IN TWO GENETIC LINES OF *GALLUS GALLUS DOMESTICUS*

3.1 Introduction

As an increasingly popular "Omics" approach, metabolomics differs from other "Omics" fields by focusing on smaller, often neglected molecules with molecular weights less than 1500 Daltons, which are smaller than most proteins, DNA, and other cellular macromolecules (Wishart 2008; Zhang et al., 2010). Like genomics, transcriptomics, and proteomics, this field has experienced tremendous advancements in analytical technologies for the separation, identification, and absolute quantification of compounds. To date, there have been few nuclear magnetic resonance (NMR) spectroscopy studies on chicken biofluids and/or tissue samples that resulted in both the identification and quantitation of specific chemical compounds (Beauclercq et al., 2016; Fotakis et al., 2017; Sundekilde et al., 2017), although several NMR studies reported data only on compound detection (Beauclercq et al., 2016; Niemuth et al., 2017; Xiao et al., 2019; Wang et al., 2020; Wang et al., 2020b). Blood metabolomics provides us with quantitative measurement of the end products of genetic, epigenetic, and environmental interactions, as well as a sensitive phenotype characterization than is characteristic of many performance measures (Nicholson et al., 1999; Oresic et al., 2011).

Seaweeds as feed additives in poultry have been previously determined to improve, productivity, feed conversion ratio, dressing percentage, and overall gut health (Oresic et al., 2011; Niemuth et al., 2017; Sundekilde et al., 2017; Borzouie et al., 2020). Seaweeds are highly bioactive, containing vitamins, minerals, sterols, polysaccharides, polyphenols,

and polyunsaturated fatty acids that have been observed to have antioxidant, anti-viral, anti-microbial, anti-tumor, and anti-inflammatory properties (Holdt et al., 2011; Souza et al., 2012). Red seaweeds are rich in protein and unique carbohydrates, whereas brown seaweeds tend to have higher minerals and dietary fiber, and both contain various bioactive polysaccharides such as laminarin, fucoidan, alginate, and some unique sulfated galactans, such as agar, agarose, and carrageenan (Usov 2011, Okolie et al., 2017). Seaweed polysaccharides have prebiotic properties that selectively stimulate the growth and metabolic activity of some beneficial bacterial species, inhibit the growth of pathogenic bacteria, and improve host health (Xiaolin et al., 2018). Unfortunately, there is little known regarding the impact of these prebiotics on blood chemical composition, with limited metabolomic studies on the impacts of gut bacteria on blood profile (Wikoff et al., 2009; Chen et al., 2018; Visconti et al., 2019).

Heat stress is an environmental stressor that can adversely affect chicken productivity by reducing feed intake, weight gain, egg production, and egg quality (Sahin et al., 2009). It has been shown to lead to oxidative stress and immune suppression, as well as increase the incidence of respiratory disease and mortality (Jaiswal et al., 2017). Heat stress triggers physiological responses in chickens to regulate body temperature and minimize negative effects. These responses include panting, increased respiration, and reduced feed intake (Lara and Rostagno, 2013). Insufficient protein intake during heat stress may decreases protein metabolism and synthesis due to impaired digestion and absorption of dietary proteins (Star et al., 2007). Heat stress may also impair the digestive processes responsible for breaking down and absorbing dietary proteins, limiting the availability of amino acids, the building blocks of proteins, for protein synthesis (Star et al., 2007). Furthermore, the

disruption of hormonal balance during heat stress, including reduced secretion of growth hormone and other anabolic hormones, can contribute to decreased protein synthesis and overall protein metabolism (Yahav et al., 2005). Additionally, chickens respond to heat stress by increasing the activity of proteolytic enzymes, which results in increased protein degradation or catabolism (Lara and Rostagno, 2013). These combined factors can contribute to the inhibition of poultry protein metabolism under heat stress conditions, compromising the maintenance and synthesis of proteins essential for various physiological functions.

Heat stress impacts on blood metabolite profile have not been studied, although we have shown effects on plasma proteins, glucose, and cholesterol (Borzouie et al., 2020). The role of bird genetic strain is also of interest, since genetic background is known to affect heat stress response and is expected to influence the metabolomic profile of the host (Chan et al., 2010; Borzouie et al., 2020). Two strains of Lohmann LSL-Lite and Brown laying hens are among the most efficient in most markets of the world at converting feed into egg mass, high output, and good egg quality (Preisinger 2000).

As the body's primary transport and major communication system, we hypothesize that a robust metabolic profiling of blood metabolites will lead to a better understanding of bird responses to management interventions and help us identify useful markers of host health and production. In the present study, we applied an untargeted NMR-based metabolomics approach to investigate the utility of plasma metabolome in understanding chicken physiological responses to environmental changes and genetic background by using seaweed supplement and heat stress, with laying hens of different genetic background. We

hypothesized that internal and external stimuli can modulate the chicken plasma metabolome.

3.2. Materials and Methods

3.2.1 Experimental Overview

Two trials, one short-term and one long-term, were conducted at the Atlantic Poultry Research Centre of the Dalhousie Agricultural Campus in Truro, Nova Scotia, Canada. The experimental methods have been previously reported in detail (Borzouie et al., 2020). The schematic experimental overview is shown in Figure 3.1. Briefly, the short-term experiment was a 2 x 2 factorial trial in a completely randomized design with seaweed inclusion level (0 and 3% red seaweed *Chondrus crispus* (CC)) and strain of hen Lohmann LSL-Lite (White) and Lohmann Brown-Lite (Brown) as the main effects, with no heat challenge. The study utilized 100 laying hens, 50 White and 50 Brown, in 20 cages with 5 hens per cage thus the cage was the experimental unit. At 55 weeks of age the birds were supplemented with 0 or 3% CC for 21 days prior to sampling one bird from each cage (thus 5 replicates per treatment). The long-term trial was designed as a 2 × 2 × 3 factorial arrangement with the main effects of the strain of laying hens (White and Brown), exposure to heat stress and level of seaweed in the feed. It consisted of 240 hens, 120 White and 120 Brown, in 48 cages, with 5 hens per cage assigned to 0% seaweed, 3% CC or 0.5% *Ascopyllum nodosum* (AN) (Trademark name Tasco®, Acadian Seaplants Ltd., Dartmouth, Nova Scotia, Canada), from weeks 31 to 72. Beginning at week 68, two birds from each cage were randomly transferred by mobile battery cage units to a heat stress room for 28 days, where the temperature was gradually raised from 25°C to 33°C from 11 a.m. to 6

p.m. daily. Birds were euthanized at the end of the trial, and one bird per cage was sampled to provide four replicates of each treatment combination.

In both trials, birds were provided 16-h light and 8-h darkness per day in temperature-controlled housing at 25°C (except for the heat stress treatment) and received *ad libitum* access to food and water. Basal diets were formulated to meet the commercial requirements for Lohmann LSL-Lite provided by Lohmann Tierzucht GmbH, Cuxhaven, Germany. The CC seaweed was received semi-moist and dried for two nights at 32°C. The AN seaweed was received harvested and solar-dried, ground and mixed into the other ingredients. The diets were accordingly adjusted for the seaweed major nutrient composition to ensure birds were fed isocaloric and isonitrogenous diets.

All birds remained healthy during the entire experimental period, and their health was monitored daily. The studies were approved by the Dalhousie University Faculty of Agriculture Animal Care and Use Committee (Protocol number 2018-031; approved 31.5.2018; Protocol number; approved 1.9.2016) and followed the Canadian Council of Animal Care guidelines on the care and use of farm animals in research (CCAC 2009; Chicken Farmers of Canada 2014).

3.2.2 Blood Plasma Sample Collection and Preparation for NMR Spectroscopy

At the end of the trial, when birds were at 55 weeks of age in the short-term trial and 72 weeks of age in the long-term trial, one bird from each cage was randomly chosen for euthanasia and sampling. Blood samples were drawn into Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing sodium heparin as an anticoagulant. Samples were centrifuged for 10 min at 2000 x g at 4° C. The plasma was decanted into

microcentrifuge tubes and processed immediately for the targeted analysis to minimize any possible metabolite degradation. NMR sample preparation, storage, and analysis were prepared according to standard procedures (Beckonert et al., 2007; Ghini et al., 2019; Vignoli et al., 2019). Standard operating procedures were strictly followed for all the blood samples to ensure that the NMR assay reflected the metabolome in the original sample and not an artificial profile generated during the pre-analytical workflow. Approximately 2 mL of blood plasma was subjected to deproteinization using ultrafiltration to remove plasma proteins (Amicon centrifugal filter devices 10 kDa cut-off, Millipore, Billerica, USA). A 570 µL aliquot of filtered blood plasma was transferred to a 1.5 ml Eppendorf tube followed by the addition of 70 µL deuterium water and 60 µL of a standard NMR buffer solution (11.667 mM DSS (disodium-2,2 dimethyl 2-silapentane-5-sulphonate), 730 mM imidazole, and 0.47% NaN3 (Sigma-Aldrich, Mississauga, ON). The samples were transferred into a standard thin walled, 5 mm cylindrical glass NMR tube (Wilmad, Buena, NJ, USA) for subsequent NMR spectral analysis.

3.2.3 NMR Spectroscopy

All ^{1}H-NMR spectra were collected on a 700 MHz a Bruker Avance III (Bruker Biospin, Fällanden, Switzerland) using the first transient of the noesy-presaturation pulse sequence, which was chosen for its high degree of quantitative accuracy (Saude et al., 2006). The spectrometer equipped witha 5 mm HCN Z-gradient pulsed-field gradient cryoprobe with automatic tuning and matching and a z-axis gradient amplifier and digital lock. Tuning and matching to 50 Ω resistive impedance as well as shimming were applied under automation to each sample while the receiver gain was held constant for all samples. All free induction

decays were zero-filled to 250 k data points. The methyl singlet produced by a known quantity of DSS was used as an internal standard for chemical shift referencing (set to 0 ppm), quantification, and alignment of NMR signals. Following manual zero- and first-order phasing, manual baseline correction, as well as solvent signal removal using TopSpinTM 2.1 (Bruker Biospin), the resultant spectra were calibrated. All processed ^{1}H-NMR spectra were analyzed, and quantifications were carried out using the advanced version of Bayesil software tool (University of Alberta, Canada) as a tool to automatically determine the metabolites of 1D ^{1}H NMR spectra of the complex biofluids (Ravanbakhsh et al., 2015). Bayesil itself performed all the spectral processing functions, including smoothing, chemical shift referencing, and reference deconvolution. The NMR peaks were aligned across all samples based on their chemical shift (ppm) values, within the Bayesil software, using targeted profiling algorithms to ensure that the correct molecules were quantified (Weljie et al., 2006; Ravanbakhsh et al., 2015).

3.2.4 Statistical Analyses

Both univariate and multivariate analyses were performed, using the entire NMR spectra. Analysis of variance (ANOVA) followed by the Tukey's multiple means comparison test using the General Linear Model (GLM) procedure of the SAS software version 9.4 (SAS Institute, Cary, NC) was performed on individual metabolite concentrations to examine genotype and treatment effects on. Statistical significance was determined at the 95 % probability level ($P < 0.05$).

For multivariate statistical analysis, metabolite concentrations were imported to the MetaboAnalyst 5.0 web server according to previously published protocols (Xia et al.,

2009; Xia and Wishart 2010). Prior to data analysis, a data integrity check, normalization of data sets, and Pareto scaling transformation (mean-centered and divided by the square root of the standard deviation of each variable) were performed to scale the data to reduce inter-individual variation. Data were analyzed then using principal components analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). PCA is a non-supervised method to explore the sample variance in a dataset without referring to the class label by reducing the dimensionality or complexity of the data, while PLS-DA is a supervised regression technique that maximizes the separation between predefined classes to better reveal group structure. The PLS-DA was then validated using cross-validation and the *P*-value, obtained from permutation testing to ensure those models were not overfitted. In addition, in the PLS-DA model, the variable importance in the projection (VIP) was used to rank the importance of each variable for the classification of the metabolic profiles, while *P-value*s less than 0.05 were considered statistically significant. To interpret the metabolomic data, multiple metabolites were selected following the statistically significant ANOVA test ($P < 0.05$) and significant permutation test ($n = 1{,}000$; $P < 0.05$).

Enrichment Analysis and Pathway Analysis were performed to explore the associated metabolic pathways for all the potential biomarkers using a module within the MetaboAnalyst software. Statistical significance pathways were defined by a *p*-value less than 0.05 and the pathway impact of more than 0.1 for those KEGG pathways that hit two or more metabolites in each pathway.

3.3. Results

3.3.1 Treatment Effects on Plasma Metabolite Identification and Concentration

Representative high-resolution NMR spectra of plasma from a healthy Brown and White hens are shown in Figure 3.2. Typically, 95% of all visible peaks were assigned resulting in identification and quantification of an average of 57 compounds in laying hen plasma including sugars, alcohols, amino acids and organic acids.

Seaweed supplementation affected 40 metabolite concentrations in the short-term trial and 50 metabolite values in the long-term trial ($P < 0.05$) (Table 3.1). In both trials, a significant increase in levels of citric acid, creatinine, malonate and urea were observed due to 3% CC seaweed intake (see Table 3.1). Metabolites that were decreased by CC treatments compared to the control group included acetic acid, propylene glycol, succinate, glutamic acid and aspartate ($P<0.05$). Increasing proportions of AN seaweed was associated with increased concentration of 33 blood metabolites (Table 3.1). Most metabolites detected in the short-term trial were at higher levels in control birds (35 compounds) while seaweed intake increased the concentration of several compounds in long-term trial (33 for AN intake and 17 for 3% CC intake).

Heat stress affected 40 metabolite values ($P<0.05$), as shown on Table 3.2. The prominent changes of NMR detected compounds due to heat exposure included an increased level of 2-hydroxybutyrate, creatine, choline, glycerol, hypoxanthine, tyrosine, mannose, acetylcarnitine, pyruvic acid, succinate, 2-hydroxyisovalerate, creatinine, ketoleucine, 3-hydroxyisovaleric acid, isopropanol, methanol, propylene glycol.

Significant changes in the levels of corresponding metabolites due to hen strain were observed including 41 metabolites in the short-term trial and 30 metabolites in the long-term study ($P< 0.05$), the majority of which were amino acids and carbohydrates; the minority of which were carboxyl acids and hydroxyl acids (Table 3.3).

Among all treatments, the main signals in terms of the highest concentrations were for glucose, lactic acid, serine, glutamine, and betaine respectively. A total of 54 of the 57 identified metabolites were affected by one or more of seaweed, heat stress or strain ($P< 0.05$). Genetic strain induced the least metabolic change (30 compounds), while seaweed supplementation induced the greatest changes (50 compounds) ($P< 0.05$). For many of the metabolites, there were interactions between the main effects (Table 3.4).

3.3.2 Multivariate Analyses of Treatment Effects

The unsupervised PCA scores plots showed that seaweed intake modulated plasma metabolites in the 21-day trial (Fig. 3.3A), with two well-separated clusters explaining 79.3% of the variation. Figure 3.3A indicated the presence of four clusters that were separated along the PC2 and along with the second latent variable which corresponded to the White and Brown birds with no overlapping region between the samples (marked by circles in Fig. 3.2A). The control diet showed a tighter cluster compared to the CC diet. This suggests more homogeneous metabolic composition for control diet birds than seaweed supplemented diet. Similarly, PLS-DA, a supervised pattern recognition method, showed a clear shift in key metabolites between samples from control and 3% CC-supplemented hens (Fig. 3.3B). In the cross-validation testing, Q2 represents the cross-validated, R2 represents the sum of squares captured by the model, accuracy represents the prediction accuracy, and red star indicates the best component number in building the mode. The cross-validation indicated that the PLS-DA models had a very high accuracy and prediction (Fig. 3.3C) with successful permutation tests ($Q2 > 0.9$, $p < 0.05$). The VIP was constructed from the loading plots of PLS-DA to rank the metabolites based on their

importance in discriminating seaweed intake group from the control group of birds (Fig. 3.3D). The VIP values greater than one are significant and VIP values greater than two are highly significant in the separation of samples, so metabolites with the highest VIP values are the most powerful group discriminators. Eleven metabolites accounted for most of the observed separation with a significant VIP score (higher than 1) including D-glucose, L-lactic acid, L-proline, L-glutamine, 3-hydroxybutyric, L-lysine, L-alanine, L-threonine, methionine, L-carnitine, L-serine. All these metabolites also had significant statistical values as found by ANOVA analysis ($p < 0.05$). The heat map on the right side of the VIP plots (Fig. 3.3D) indicates that three metabolites were enhanced in birds supplemented with seaweed for a short-term, including, D-glucose, L-glutamine, and methionine.

Seaweed supplement effects on plasma were different for the long-term trial. Figure 3.4A shows that PC1 vs PC3 could separate control diet class from the AN and CC diet classes well, covering 38.6% of the variance. The score plot summarizes the distribution of the principal component scores for the 3 different diets with clear separation of control, 3% CC and 0.5% AN spots in different PCs, confirming the differential modulation of these selected spots (Fig. 3.4A). The relative contributions and the relationships between the metabolites can be seen in the loadings plot of Fig. 3.4B, showing the plasma metabolites contributing the most to the separations between the groups (PC1 vs PC3). Loading plots describe how much each metabolite contributes to a particular principal component. Positive or negative large loadings indicate that the treatment strongly influences the variable, so the metabolite value has a strong relationship to a particular principal component. To maximize the differences between the groups and confirm the metabolites that contribute to discrimination, the PLS-DA was further employed. Figure 3.4C showed

the comparative PLS-DA analysis of samples in the long-term trial. The responses of hens fed control diet were clustered further apart from those corresponding to diets 3% CC, whereas the cluster of birds with 0.5% AN diet was located between the clusters corresponding to control diet and 3%CC (Fig. 3.4C). Cross-validation (Fig. 3.4D) indicated that the PLS-DA model had good quality (Q2 comparable to R2, Q2>0.9) with successful permutation test of PLS-DA model for PC1 vs PC2 ($p < 0.05$). Comparing. Furthermore, a VIP plot in a PLS-DA model was used to evaluate those metabolites that contributed most to the differentiation of seaweed from the non-seaweed diet. Using this VIP score from PLS-DA together with ANOVA, we were able to select 13 plasma metabolites, all with 95% probability level ($p < 0.05$) and VIP score higher than one which included L-carnitine, isopropanol, L-alpha-aminobutyric acid, oxoglutarate, dimethyl sulfone, L-asparagine, L-aspartate, malonate, acetic acid, acetone, hypoxanthine, and propylene glycol (listed from high to low VIP scores). Among these compounds, the concentration of only three metabolites were higher in control diet including, L-aspartate, acetic acid propylene glycol, while the rest of the metabolite concentrations were increased by 3% CC or AN seaweed intake. These metabolites accounted for most of the observed separation between three groups in the long-term study. Cross-validation indicated that the PLS-DA model had good quality (Q2 comparable to R2, Q2>0.9) with successful permutation test of PLS-DA model for PC1 vs PC2 ($p < 0.05$). Comparing only control and 3% CC-supplemented hens yielded similar results (data not shown) to the analysis of the 0% control, 3%CC and 0.5% AN data.

Consistent with the ANOVA results, PCA and PLS-DA analyses showed an impact of heat stress on many of the metabolites. Figure 3.5A shows the score plot of the first two

principal components extracted from heat stress and control groups that could partially separate classes into distinct clusters, while Fig. 3.5B shows that PLS-DA separated the treatments into two distinct clusters. Cross-validation indicated that the PLS-DA model had modest quality (Q2 was somewhat smaller than R2, indicating slight training, Q2~ 0.7, $p < 0.05$). The VIP plot was used to rank the most promising metabolites according to the orders of their contributions to the separation of clustering including 3-hydroxyisovaleric acid, isopropanol, L-ornithine, L-alpha-aminobutyric acid, 2-hydroxybutyrate, L-asparagine, L-proline, dimethyl sulfone, 2-hydroxyisovalerate, creatine and L-threonine that all except L-alpha-aminobutyric acid had significant ANOVA statistical values ($p < 0.05$).

Comparing the White and Brown strains, PCA did not discriminate between metabolites of two strains, while the supervised PLS-DA model (Fig. 3.6A) could separate classes into two distinct clusters with good quality and good prediction ability. The VIP plot (Fig 3.6B) showed that dimethylglycine, 2-hydroxy butyric, dimethyl sulfone, L-threonine, L-carnitine, 1-methylhistidine, hypoxanthine, L-ornithine, isopropanol, L-serine, acetone, and acetoacetate were the strongest discriminating metabolites for separating the two strains. The heat map on the right side of the VIP plot shows that dimethyl sulfone, L-carnitine, L-serine and acetoacetate were enhanced in Lohmann White birds.

3.3.3 Pathway Analysis

We performed Enrichment and Pathway Analyses using the chicken (*Gallus gallus*) library from the KEGG database of the metabolites identified as affected by our treatments. Metabolites with a *p*-value less than 0.05 and VIP score higher than 1 from PLS-DA, were

included. Figure 3.7 summarizes the differentiating pathways along with the fold enrichment and *P- values* for each of the main effects in the long-term trial. Total of 10, 4 and 2 potentially affected metabolic pathways that were significantly altered in the plasma samples from seaweed supplemented group, strain effect and heat-stressed groups respectively.

As shown on Table 3.5, enrichment analysis suggested that short-term seaweed supplementation affected nine different metabolic pathways, primarily related to amino acid metabolism. The short-term seaweed supplement trial resulted in more affected pathways than long-term intake, but both impacting alanine, aspartate and glutamate metabolism, as well as glutamine and glutamate metabolism. In the long-term trial, heat stress was associated with metabolite changes indicative of changes in valine, leucine and isoleucine biosynthesis, aminoacyl-tRNA biosynthesis, arginine and proline metabolism and glycine, serine and threonine metabolism. Genetic strain potentially affected two pathways, including synthesis and degradation of ketone bodies and glycine, serine and threonine metabolism. The total metabolites involved in each biological pathway, the number of metabolites hit in the biological pathway, as well as the statistical significance of metabolic pathways were all noted in Table 3.5.

3.4. Discussion

3.4.1 The plasma metabolome of chicken

There have been few quantitative studies of the metabolomic profiles of chicken plasma. Serum amino acids, biogenic amines, acylcarnitine, sphingomyelin, lysophosphatidylcholines and diacyl- and acyl-alkyl-glycerophospholipid profiles of

broiler chickens were quantified in the study of Metzler-Zebeli et al., (2019), showing that quantitative feed restrictions and the residual feed intake rank cause distinct metabolomic profiles (Metzler-Zebeli et al., 2019). Our short-term quantitative results for glycine, tyrosine, proline, asparagine, isoleucine, histidine, serine and valine were similar to those reported by Zebeli in their *ad libitum*-fed chicken trial, while levels of leucine and tryptophan were substantially lower in our experiment. This might be due to impacts of age, strain or basal diets of birds. Another 21-day study quantified the chicken plasma concentrations of free amino acids and biogenic amines in response to dietary crude protein intake (Hofmann et al., 2019). Their reported values of branched chain amino acids, histidine, alanine, glutamic acid, glutamine, aspartic acid and proline were in close range with the values of our 21-day trial.

The majority of previous studies mainly focused on metabolome detection, not quantification, to investigate the relationship between diet and metabolome. Flavonoids were used as dietary additives in broiler production to promote chicken meat quality in an NMR study of the metabolic fingerprint of chicken plasma; 37 metabolites were detected of which 24 were also detected in our study, including isoleucine, leucine, valine, 3-hydroxybutyric acid, lactic acid, alanine, lysine, arginine, acetic acid, proline, glutamic acid, glutamine, succinic acid, citric acid, dimethylglycine, creatinine, betaine, creatine, glucose, glycine, mannose, tyrosine, phenylalanine, formic acid (Fotakis et al., 2017). Twenty-three metabolites were identified in the serum of broiler chickens supplemented with grape seed extracts (Cao et al., 2020). Chen et al., (2020) reported a list of 20 serum metabolites from chicken fed with dietary plant essential oils (Chen et al., 2020).

In reviewing these studies, we note that fewer than 40 metabolites in total were identified in chicken plasma. While most of those studies were carried out in broilers, we quantified 57 compounds laying hen plasma. Approximately half of these metabolites (27 out of 57) have been reported previously while our study added 30 compounds to the blood metabolome of chicken (the complete list can be seen on Tables 3.1-3.3). As sample preparation and NMR methods continue to advance with improved software capability and more complete databases with larger numbers of standards and reference spectra, metabolomic data will provide a sensitive tool to understand biological impacts of various stressors and management interventions.

3.4.2 Literature-reported biomarkers of hen seaweed intake

We identified a series of plasma metabolites including alanine, aspartate, glutamate, glycine, serine, threonine, cysteine, and methionine, as significantly impacted by seaweed supplemented birds. Moreover, aminoacyl-tRNA biosynthesis involves a total of 48 metabolites, we found seven of these metabolites impacted by short term seaweed intake and two by long term seaweed intake (Table 3.5). Aminoacyl-tRNA biosynthesis pathway is involved in protein biosynthesis including threonine, serine, glycine, and glutamate (Ibba and Soll 2000). All these metabolite levels were reduced by the short/long term seaweed intake, indicating that this pathway was highly activated by prebiotic intake. Previously, probiotic supplementation was found to be correlated with aminoacyl-tRNA biosynthesis and amino acid metabolism in mice and pigs (Vemuri et al., 2018; Alassane-Kpembi et al., 2020). We obtained similar results in chicken here. Moreover, we observed increased in lysine levels in both short and long-term seaweed trials, which may result from increased

activity of the lysine degradation. Arginine can be converted into urea and ornithine, and ornithine can be further metabolized to proline through ornithine aminotransferase (Caldwell et al., 2015). We also observed reduction in the levels of ornithine and proline that could be due to an improved bioavailability of arginine. In general, amino acid metabolism pathways were noticeably altered in the plasma of seaweed supplemented layers, implying the prebiotic impact on protein metabolism.

The impacts of seaweed supplements on chicken metabolism are not well understood. Recently, the short- and long-term seaweed supplement impacts on several chicken blood parameters and enzyme activities indicated changes in plasma proteins and enzymes due to seaweed prebiotic intake (Kulshreshtha et al., 2008; Borzouie et al., 2020). Similarly, non-metabolomic methods have shown significant changes in several minerals and compounds including glucose, calcium, phosphorus, zinc, iron, copper, total protein, cholesterol, and triglyceride in response to different prebiotic sources (Mateova et al., 2012; Yalçinkaya et al., 2012; Alagan et al., 2020).

Contrary to expectations, layers fed with seaweed exhibited significantly lower levels of amino acids in their blood, including glycine, alanine, proline, glutamic acid, aspartic acid, and serine, typically found in higher concentrations in red seaweed (Sullivan and Ikawa, 1973). Possible explanations for this observation include inefficient metabolism or utilization of seaweed-derived amino acids, degradation or modification during digestion, interference from other dietary components or factors, as well as potential influences from genetic factors, gut microbiota composition, or physiological differences affecting amino acid processing and utilization in chickens. Seaweed's nutrient and anti-nutrient properties can impact amino acid metabolism in poultry. Bioactive peptides present in seaweed can

interact with enzymes and receptors involved in amino acid metabolism, influencing their utilization, absorption, or synthesis (Santos-Silva et al., 2020). However, seaweed may also contain anti-nutrients such as phytic acid, tannins, and enzyme inhibitors that bind to amino acids or hinder enzyme activity in amino acid metabolism. These anti-nutrients indirectly affect amino acid metabolism by binding to essential minerals or interfering with protein digestion and absorption (Reddy et al., 1982; Hagerman et al., 1998; Dunaief et al., 2012). Further research is needed to fully understand how these interactions impact amino acid homeostasis in seaweed-fed chickens.

The reduced levels of the gluconeogenic precursors glucose, alanine, glutamate, lactate and histidine suggest that gluconeogenesis may be reduced in response to seaweed intake. Our pathway analysis results support these observations, indicating altered glycolysis/gluconeogenesis pathways in short-term seaweed supplemented birds. Interestingly, the glucose levels were reduced after short-term treatment. Similarly, Guerreiro et al., (2015) observed decreased gluconeogenesis in fish fed fructooligosaccharides and xylooligosaccharide as prebiotic supplements (Guerreiro et al., 2015). Previously, transcriptome analysis of the Pacific white Shrimp muscle revealed the association between the glycolysis/gluconeogenesis pathway and residual feed intake (Dai et al., 2017). Our previous study reported the significant reduction of feed intake and improvement of feed/egg efficiency in the short-term seaweed supplemented birds. Thus, the present study suggests that the feed efficiency of layers might be linked to the glycolysis/gluconeogenesis pathway.

Moreover, a study of rodent maternal prebiotic supplementation effects on offspring showed changes in host microorganisms thought to decrease blood glucose by rescuing

impaired glycolysis (Zhang et al., 2019). We observed lower glucose only in short-term CC seaweed supplementation. We observed differences between two trials for glucose, suggesting there may some adaptation over the longer 41-week trial. A larger investigation on glucose response to prebiotic intake may help to fully elucidate the differences between the short and long-term impacts.

Several previous studies have identified possible associations between metabolome and host response to various types of prebiotic supplements in other species. Fish serum composition was investigated after 46-day feeding of diets containing the prebiotic mannan oligosaccharide (4 g/kg), using the Jaffe colorimetric method, a non-metabolomic technique (Akrami et al., 2013). The reduced glucose serum and increased levels of creatinine due to short-term prebiotic intake were similar to our observed results with short-term *Chondrus crispus* seaweed supplement in hens. Verbeke and colleagues (2015) reviewed human *in vitro* studies of prebiotic impacts on microbiota and found that short chain fatty acid proportions vary between individuals and were particularly sensitive to the type of prebiotic carbohydrate diet, altering propionic, acetic and butyric acids (Verbeke et al., 2015). Our NMR results showed that plasma levels of formic, isobutyric and acetic acids were reduced in both trials due to seaweed intake. We also observed reductions in the plasma levels of fermentation intermediates, lactic and succinic acids (lactic acid in short-term trial and succinic acid in both trials) due to seaweed intake. These observations support the idea that seaweed is acting as a prebiotic in laying hens, affecting microbial fermentation and subsequently plasma short-chain fatty acid composition. The closer analysis of the NMR spectral regions and quantitative interpretation of blood variables may help us better track downstream effects of prebiotic supplements not only in chicken but

also in other species. To our knowledge, these results are a new contribution to the literature.

3.4.3 Literature-reported biomarkers of hen heat stress impact

Our results indicate that heat stress significantly reduces plasma levels of valine, leucine, isoleucine, glycine, arginine, proline, threonine, ornithine, and methionine in the heat stress group. This suggests that heat stress inhibits poultry protein metabolism by affecting specific amino acid pathways, including valine, leucine, and isoleucine biosynthesis, as well as arginine and proline metabolism, and glycine, serine, and threonine metabolism. While studies on metabolic changes in response to heat stress in livestock have primarily focused on dairy cows, only a limited number of studies have been published on poultry (Wheelock et al., 2010; Gao et al., 2017). Azad et al., (2010) investigated the effects of chronic heat treatments on plasma compounds in broiler chickens using enzymatic and electrode methods to measure potassium, sodium, chloride, albumin, glucose, triacylglycerol, and uric acid. Additionally, Lu et al., (2018) identified 78 metabolites in heat-stressed broiler serum, although quantification data were not provided (Lu et al., 2018).

The study conducted by Quirk et al., (1989) served as one of the initial NMR investigations on epithelial cells, revealing that fasting stress could significantly elevate the levels of pyruvate and succinate. Interestingly, these findings align with our observations induced by heat stress in layers. These findings may suggest that chickens possess metabolic adaptations that prioritize anaerobic pathways, specifically glycolysis, to maintain energy production and overcome challenges to cellular respiration caused by elevated

temperatures (Elia et al., 2022). Succinate, being both a byproduct of the TCA cycle and a key component of aerobic respiration, can also accumulate during heat stress, as heat stress leads to the disruption of TCA cycle (Elia et al., 2022).

Our results indicate that heat stress-induced changes in amino acid and protein metabolism do not necessarily result in immediate negative impacts on bird performance and production. It appeared that the identified pathways may not have influenced the parameters linked to production and egg quality, suggesting that the observed pathway changes in birds could be attributed to their inherent resilience. These findings suggest that birds have the ability to overcome the adverse effects of heat stress, highlighting their adaptability and capacity to maintain satisfactory levels of performance and production.

Furthermore, it is noteworthy to consider environmental factors such as ventilation, shade, and access to cool water, as they can also contribute to mitigating the impact of heat stress. However, it is crucial to recognize that prolonged or severe heat stress, particularly at higher temperatures, can have a more pronounced influence on metabolic pathways and imbalances, potentially compromising production and performance in the long run.

Disruption in the balance of protein metabolism can have detrimental effects on muscle tissue, where inhibition or compromise of protein synthesis prompts muscles to act as a reservoir of proteins for amino acid utilization in energy production. Thus, the inhibition of protein metabolism in heat stress conditions indicates potential muscle breakdown as chickens adapt to limited protein availability and heightened energy demands, suggesting that heat stress can divert energy towards muscle catabolism. Similarly, two previous studies showed that muscle protein deposition was decreased in broilers exposed to heat stress (Yunianto et al., 1997; Zuo et al., 2014). Amino acid metabolism pathway was also

found to be impacted by heat stress in laying hens (Zhu et al., 2019). Additionally, Suryawan et al., (2008) demonstrated that leucine blood level could activate muscle protein synthesis in a pig model (Suryawan et al., 2008). Therefore, it could be hypothesized that the reduced concentration of leucine in heat stressed chickens might lead to muscle damage and cause muscle protein degradation. Similarly, previous experiments have explored that acute heat exposure could affect metabolic characteristics and reduce protein deposition in the skeletal muscles resulting in oxidative damage to skeletal muscle as well as protein muscle breakdown in chickens (Azad et al., 201; Qaid et al., 2021). Moreover, methionine as an essential amino acid for layers that improves egg production is suggested for alleviating the negative effects of heat stress on birds (Bunchasak 2009). The decreased blood levels of methionine in heat stressed layers might contribute to the increased methionine requirement under heat exposure.

Our previous blood plasma biochemistry study indicated that heat stress was associated with reduced protein, globulin and glucose and increased cholesterol and gamma-glutamyl transferase levels that similar results were observed here for glucose, the detectable compound by NMR (Borzouie et al., 2020). As shown on Table 3.2, the plasma glucose levels of heat stress birds reduced from 13051.5 to 12407.2 showing the effect of stress response on glucose metabolism, similar to the findings of the stress response to feed/water deprivation in turkey hens (Zadworny et al., 1985). Our results suggest that stressed birds increased glycolysis to generate energy under heat stress conditions. This metabolic adaptation can be explained by Baumgard and Rhoads (2013) study showing the glucose-sparing strategy when animals were in negative energy balance during heat stress (Baumgard et al., 2013).

The plasma concentration of 3-hydroxybutyrate was significantly reduced in heat stressed birds. The levels of 3-hydroxybutyrate which is generated from lipolysis is often used as an index of lipid metabolism (Akram 2013). This metabolite is known to increase as a result of β-oxidation of lipids when glucose supply is limited to serve it as an energy source (Akram 2013). Lower levels of 3-hydroxybutyrate might indicate the inhibitory effect of heat stress on chicken lipolysis, similar to the research results of heat stressed cows (Yue et al., 2020). Glucose involvement in energy decomposition and increased glycolysis rate can support the hypothesis that lipolysis may not be an energy-generating process for layers during heat stress condition.

To our knowledge, this is the most comprehensive quantitative metabolomics study to measure metabolite levels that were expressed differently in the plasma of heat-stressed chickens.

3.4.4 Literature-reported biomarkers of hen strain impact

Our research findings revealed that genetic strain played a significant role in the hens' metabolome compared to seaweed intake and heat stress. The short-term trial demonstrated a stronger strain effect, leading to the expression of a higher number of metabolites (Table 3), possibly influenced by age or the stage of laying. These key metabolites were associated with pathways that provide valuable insights into the relationship between quantitative metabolic data and genetic variation in chickens. Notably, the hen's metabolome undergoes changes throughout the laying cycle, impacting nutrient requirements, energy, amino acid, lipid, and mineral metabolism, which are crucial for eggshell formation and embryonic development (Gloux et al., 2019; Brown et al., 2022). Confirming the impact of genetic

strain on metabolome levels, studies on cows and mice have also been conducted. An NMR study demonstrated distinct urinary concentrations of metabolites between two mouse strains, indicating variations in metabolic patterns and pathway activities (Gavaghan et al., 2000). Likewise, another study observed differences in plasma metabolite concentrations of leptin and glucose in two cow strains, reflecting variations in the energetic and metabolic status of the dairy cow (Kay et al., 2009). These studies reinforce the notion that genetic strain has a significant influence on the metabolome in different animal species, including chickens.

As our previous study showed that White strain had higher egg production, feed efficiency and resistance to heat stress with higher levels of protein, globulin and alkaline phosphatase while the body weight of the Brown strain was higher than White, with significant higher liver enzyme in alanine aminotransferase and gamma-glutamyl transferase (Borzouie et al., 2020). Therefore, the higher levels of proteins identified in the White strain might be associated with higher amino acid metabolism (Table 3.3), most amino acids including glutamine, glutamate, alanine, threonine, ornithine, arginine and tryptophan had higher concentrations in White strain. Similarly, Wadood et al., (2021) reported the association between amino acid synthesis and follicle development in chickens, as the follicle ovulatory process has a major impact on egg production (Wadood et al., 2021). Therefore, we speculate that the amino acid metabolism pathways could be associated with chicken egg production.

The synthesis and degradation of ketone bodies metabolic pathway was also impacted by strain. In Brown group, we observed increased levels of 3-hydroxybutyrate as an energy-rich ketone body that can transport energy to body tissues (Laffel 1999). The reduced levels

of blood glucose in the Brown strain (in short-term trial) may indicate that the synthesis and degradation of ketone bodies could provide energy when there are low glucose levels in the blood.

Our findings indicate that the observed impacts on the metabolome were primarily due to genetic and dietary factors rather than age and laying cycle. Although there was a 16-week age difference between the hens before slaughter, this time gap is unlikely to have significantly influenced the metabolome compared to the factors we investigated, such as genetics and prebiotic supplementation. Additionally, our analysis revealed no shared pathways between seaweed and genetic strains in the long-term trial. This suggests that the differentially expressed pathways we observed were predominantly influenced by the treatments themselves, rather than other factors such as age, laying cycle, or seasonal variations. Thus, it is apparent that the treatments played a primary role in shaping the observed differences in pathway expression, while other factors played a lesser role in comparison.

3.5. Conclusions

Overall, the NMR spectrum of healthy laying hen plasma provided quantitative data on 57 plasma metabolites that can provide a convenient, centralized resource to advance the fields of quantitative metabolomics. This study demonstrated that chicken plasma metabolome was sensitive to respond to internal and external stimuli, including pre- and probiotic supplementation, heat stress, and genetic strain. These findings suggested that metabolomics could be successfully used for metabolic biomarker discovery to address the challenge of identifying pathways and mechanisms linked to chicken productivity and

health traits. Future work applying various metabolomics assays and focusing on other chemical compounds can help to expand the repertoire of quantitative assays available for blood composition analysis to define more biological functions and molecular mechanisms.

Table 3.1. Concentrations (micromolar) of metabolites impacted by short-term and long-term seaweed supplementation in laying hen plasma, as measured by NMR.

		Short-Term Trial [a]				Long-Term Trial				
	Metabolites	**0% CC**	**3% CC**	**SEM**	***P-value***	**0% CC**	**3% CC**	**0.5% AN**	**SEM**	***P-value***
1	1-Methylhistidine	13.2	16.1	0.34	<0.01	14.7	13.3	14.7	0.44	0.05
2	2-Hydroxybutyrate	5.8	7.0	0.60	NS	5.1	7.2	7.1	0.52	0.01
3	Acetic acid	65.4	44.0	1.9	<0.01	64.3	47.7	50.2	2.4	<0.01
4	Betaine	510.1	456.1	20.7	NS	537.1	520.4	582.3	17.5	0.05
5	Acetoacetate	1.6	2.0	0.11	0.02	1.7	1.4	1.9	0.09	<0.01
6	L-Carnitine	187.2	87.2	5.7	<0.01	20.0	134.9	114.4	3.0	<0.01
7	Creatine	56.3	55.1	2.4	NS	66.7	61.9	72.2	2.7	0.03
8	Dimethylglycine	21.7	17.2	0.69	<0.01	17.4	22.0	21.8	0.84	<0.01
9	Citric acid	88.9	101.1	3.4	<0.01	112.9	122.7	106.7	3.6	0.01
10	Choline	4.3	4.4	0.12	NS	16.8	17.9	19.8	0.44	<0.01
11	Ethanol	11.3	17.4	0.70	<0.01	29.7	29.2	29.2	4.0	NS
12	D-Glucose	13408.8	12514.8	132.9	<0.01	12189.3	12217.0	13781.7	237.4	<0.01
13	Glycine	457.3	370.8	13.8	<0.01	373.0	349.7	448.7	11.1	<0.01
14	Glycerol	227.6	238.5	8.4	NS	285.3	262.2	281.6	8.9	NS
15	Formate	60.9	63.2	1.9	NS	51.2	44.8	55.5	1.4	<0.01
16	L-Glutamic acid	123.9	92.4	2.5	<0.01	143.1	115.4	120.5	6.1	<0.01
17	Hypoxanthine	6.4	5.7	0.30	NS	7.2	5.7	7.9	0.31	<0.01
18	Tyrosine	118.2	95.2	3.5	<0.01	91.4	94.9	97.6	3.2	NS
19	L-Phenylalanine	106.3	87.6	1.6	<0.01	80.0	88.5	91.9	2.2	<0.01
20	L-Alanine	435.3	314.0	5.9	<0.01	301.8	283.8	366.4	10.7	<0.01
21	L-Proline	483.5	247.9	6.6	<0.01	227.8	219.8	308.7	9.8	<0.01
22	L-Threonine	279.0	189.6	5.2	<0.01	184.8	174.5	266.2	10.5	<0.01
23	L-Asparagine	125.9	73.6	1.3	<0.01	54.7	63.9	76.4	3.4	<0.01
24	D-Mannose	31.9	23.3	1.1	<0.01	43.8	45.4	36.5	2.0	0.01
25	Isoleucine	90.6	66.4	3.8	<0.01	49.9	53.0	63.5	1.6	<0.01
26	L-Histidine	13.2	16.1	0.34	<0.01	109.1	106.1	133.8	2.5	<0.01
27	L-Lysine	203.4	122.1	5.0	<0.01	150.6	127.8	215.9	12.2	<0.01
28	L-Serine	780.1	613.7	12.9	<0.01	558.2	500.7	577.7	10.9	<0.01
29	L-Lactic acid	3610.0	2494.7	35.1	<0.01	2652.2	2769.1	2680.0	237.5	NS
30	L-Aspartate	39.6	32.4	0.74	<0.01	32.3	24.1	28.9	2.6	<0.01
31	L-Acetylcarnitine	6.6	6.3	0.14	NS	6.6	6.0	6.4	0.19	NS
32	Oxoglutarate	14.8	16.1	0.26	NS	9.2	13.9	7.6	0.79	<0.01
33	L-Ornithine	82.2	49.6	1.8	<0.01	67.4	50.3	76.3	3.2	<0.01
34	Pyruvic acid	124.1	125.7	5.6	NS	90.8	111.8	98.5	4.2	<0.01
35	Succinate	15.8	11.2	0.37	<0.01	17.2	13.5	11.4	0.53	<0.01

36	Sarcosine	8.9	10.7	0.40	<0.01	9.8	8.0	11.3	1.7	<0.01
37	Urea	37.5	45.8	1.5	<0.01	41.5	55.9	52.1	2.5	<0.01
38	3-Hydroxybutyric acid	442.9	264.5	5.1	<0.01	297.6	261.1	332.3	12.9	<0.01
39	2-hydroxyisovalerate	1.0	1.2	0.05	0.02	1.3	1.1	1.5	0.08	<0.01
40	L-Alpha-aminobutyric acid	1.9	1.5	0.25	NS	0.3	0.9	0.2	0.11	0.05
41	3-Methyl-2-oxovaleric acid	5.3	4.4	0.38	NS	4.6	5.2	5.7	0.16	<0.01
42	L-arginine	218.3	210.2	11.9	NS	194.3	212.4	209.8	4.7	0.02
43	Creatinine	3.2	5.0	0.27	<0.01	3.2	3.4	2.6	0.33	<0.01
44	L-Glutamine	583.7	683.5	5.0	<0.01	512.2	534.1	559.4	16.7	<0.01
45	L-Leucine	90.6	66.4	3.8	<0.01	131.0	129.8	165.1	3.0	<0.01
46	Malonate	6.7	9.1	0.60	0.01	8.1	11.0	8.3	0.45	<0.01
47	Ketoleucine	13.1	12.8	0.22	NS	12.6	14.2	12.7	0.50	NS
48	Methionine	90.9	118.2	2.3	<0.01	78.9	93.0	112.9	3.5	<0.01
49	3-Hydroxyisovaleric acid	1.0	0.5	0.08	<0.01	1.9	2.7	1.9	0.23	0.04
50	Isopropanol	4.0	4.8	0.22	0.03	4.1	7.6	10.1	0.36	<0.01
51	Valine	207.0	156.9	5.4	<0.01	124.8	138.0	166.7	3.3	<0.01
52	Tryptophan	6.9	7.2	0.20	NS	7.3	8.2	6.3	0.31	<0.01
53	Acetone	19.8	10.5	0.61	<0.01	12.5	9.5	14.5	0.57	<0.01
54	Isobutyric acid	10.1	6.7	0.13	<0.01	5.8	6.1	9.3	0.33	<0.01
55	Methanol	17.4	18.1	0.74	NS	80.7	87.3	69.4	7.6	NS
56	Propylene glycol	5.4	5.8	0.23	NS	16.1	11.2	13.2	0.78	<0.01
57	Dimethyl sulfone	81.6	62.1	4.4	<0.01	74.3	38.8	100.2	5.56	<0.01

[a] Mean concentration in micromolar

Table 3.2. Concentrations (micromolar) of laying hen plasma metabolites impacted by heat stress, as measured by NMR.

		Long-Term Trial [a]			
	Metabolites	**Control**	**Heat Stress**	**SEM**	***P-value***
1	1-Methylhistidine	14.1	14.3	0.4	NS
2	2-Hydroxybutyrate	4.9	8.1	0.4	<0.01
3	Acetic acid	53.5	54.6	2.0	NS
4	Betaine	538.5	554.8	14.3	NS
5	Acetoacetate	1.6	1.7	0.1	NS
6	L-Carnitine	101.5	78.0	2.5	<0.01
7	Creatine	56.7	77.2	2.2	<0.01
8	Dimethylglycine	21.9	18.9	0.7	<0.01
9	Citric acid	119.9	108.3	2.9	0.01
10	Choline	17.5	18.9	0.4	0.01
11	Ethanol	26.0	32.7	3.2	NS
12	D-Glucose	13051.5	12407.2	193.8	0.02
13	Glycine	412.9	368.1	9.0	<0.01
14	Glycerol	264.1	288.6	7.3	0.02
15	Formate	53.1	47.9	1.1	<0.01
16	L-Glutamic acid	131.5	121.2	4.9	NS
17	Hypoxanthine	6.4	7.5	0.2	0.01
18	Tyrosine	90.8	98.4	2.6	0.04
19	L-Phenylalanine	89.7	83.9	1.8	0.03
20	L-Alanine	332.0	302.7	8.8	0.03
21	L-Proline	306.7	197.5	8.0	<0.01
22	L-Threonine	225.3	191.6	8.6	0.01
23	L-Asparagine	86.7	43.3	2.7	<0.01
24	D-Mannose	37.9	45.9	1.6	<0.01
25	Isoleucine	61.9	49.0	1.3	<0.01
26	L-Histidine	125.1	107.5	2.1	<0.01
27	L-Lysine	168.6	160.9	9.9	NS
28	L-Serine	591.0	500.1	8.6	<0.01
29	L-Lactic acid	2500.9	2900.0	193.9	NS
30	L-Aspartate	30.9	26.0	2.1	NS
31	L-Acetylcarnitine	6.1	6.5	0.16	NS
32	Oxoglutarate	11.3	9.2	0.65	NS
33	L-Ornithine	87.3	42.0	2.7	<0.01
34	Pyruvic acid	93.2	107.5	3.4	0.01
35	Succinate	12.3	15.8	0.44	<0.01
36	Sarcosine	10.6	8.7	1.3	NS

37	Urea	53.9	45.7	2.0	0.01
38	3-Hydroxybutyric acid	328.9	265.2	10.5	<0.01
39	2-hydroxyisovalerate	1.0	1.5	0.06	<0.01
40	L-Alpha-aminobutyric acid	0.18	0.79	0.09	NS
41	3-Methyl-2-oxovaleric acid	5.1	5.2	0.13	NS
42	L-arginine	219.6	191.5	3.9	<0.01
43	Creatinine	2.8	3.4	0.27	0.05
44	L-Glutamine	562.8	507.7	13.5	0.01
45	L-Leucine	156.1	127.8	2.5	<0.01
46	Malonate	8.8	9.5	0.37	NS
47	Ketoleucine	12.5	13.9	0.41	0.02
48	Methionine	98.8	91.1	2.8	NS
49	3-Hydroxyisovaleric acid	1.0	3.4	0.19	<0.01
50	Isopropanol	5.4	9.2	0.29	<0.01
51	Valine	157.2	129.1	2.6	<0.01
52	Tryptophan	7.0	7.4	0.25	NS
53	Acetone	13.1	11.2	0.47	0.01
54	Isobutyric acid	7.5	6.6	0.27	0.02
55	Methanol	69.8	88.5	6.2	0.04
56	Propylene glycol	12.6	14.5	0.63	0.04
57	Dimethyl sulfone	87.2	55.1	4.7	<0.01

Table 3.3. Concentrations (micromolar) of laying hen plasma metabolites impacted by strain, as measured by NMR.

		Short-Term Trial				Long-Term Trial			
	Metabolites	**Brown strain**	**White strain**	**SEM**	***P-value***	**Brown strain**	**White strain**	**SEM**	***P-value***
1	1-Methylhistidine	16.2	13.2	0.34	<0.01	16.0	12.4	0.36	<0.01
2	2-Hydroxybutyrate	3.0	9.7	0.60	<0.01	8.0	5.0	0.42	<0.01
3	Acetic acid	44.5	64.9	1.9	<0.01	56.4	51.8	0.10	NS
4	Betaine	438.0	528.2	20.7	<0.01	514.3	578.9	14.3	<0.01
5	Acetoacetate	1.8	1.8	0.11	NS	1.6	1.7	0.07	NS
6	L-Carnitine	165.6	108.8	5.7	<0.01	79.3	100.2	2.5	<0.01
7	Creatine	56.8	54.6	2.4	NS	70.1	63.8	2.2	0.05
8	Dimethylglycine	23.9	15.0	0.69	<0.01	27.1	13.6	0.68	<0.01
9	Citric acid	86.1	103.9	3.4	<0.01	119.4	108.8	2.9	0.01
10	Choline	4.4	4.3	0.12	NS	18.5	17.9	0.36	NS
11	Ethanol	13.8	14.9	0.70	NS	21.7	37.0	3.2	<0.01
12	D-Glucose	12706.2	13217.4	132.9	0.01	13302.8	12155.9	193.8	<0.01
13	Glycine	395.8	432.4	13.8	NS	395.7	385.2	9.0	NS
14	Glycerol	214.4	251.8	8.4	<0.01	258.3	294.4	7.3	<0.01
15	Formate	58.1	66.0	1.9	<0.01	50.9	50.1	1.1	NS
16	L-Glutamic acid	76.8	139.6	2.5	NS	125.0	127.7	4.9	NS
17	Hypoxanthine	7.4	4.6	0.30	<0.01	7.7	6.2	0.25	NS
18	Tyrosine	109.9	103.5	3.5	NS	90.7	98.5	2.6	0.04
19	L-Phenylalanine	100.0	93.9	1.6	0.01	92.3	81.3	1.8	<0.01
20	L-Alanine	363.4	386.0	6.8	0.04	318.5	316.2	9.0	NS
21	L-Proline	358.9	372.5	6.6	NS	255.8	248.4	8.0	NS
22	L-Threonine	223.6	244.9	5.6	0.01	248.1	168.8	8.6	<0.01
23	L-Asparagine	115.8	83.7	1.3	<0.01	73.0	57.0	2.7	<0.01
24	D-Mannose	28.4	26.8	1.1	NS	44.2	39.6	1.6	0.05
25	Isoleucine	78.1	78.8	3.8	NS	57.0	53.9	1.3	NS
26	L-Histidine	143.2	124.2	5.0	0.02	118.0	114.6	2.1	NS
27	L-Lysine	169.2	156.3	5.0	NS	165.6	164.0	9.9	NS
28	L-Serine	600.2	793.6	12.9	<0.01	495.5	595.6	8.6	<0.01
29	L-Lactic acid	3000.9	3103.8	35.1	0.05	2767.2	2633.8	193.9	NS
30	L-Aspartate	34.9	37.1	0.74	0.05	27.7	29.2	2.0	NS
31	L-Acetylcarnitine	7.6	5.3	0.14	<0.01	7.0	5.6	0.16	<0.01
32	Oxoglutarate	14.3	16.7	0.26	<0.01	9.1	11.4	0.65	NS
33	L-Ornithine	45.6	86.1	1.8	<0.01	69.2	60.1	2.7	0.01
34	Pyruvic acid	141.2	108.6	5.6	<0.01	98.5	102.2	3.4	NS
35	Succinate	14.9	12.0	0.37	<0.01	14.3	13.8	0.44	NS

36	Sarcosine	11.2	8.4	0.40	<0.01	8.4	10.9	1.3	NS
37	Urea	36.4	47.0	1.5	<0.01	49.2	50.5	0.65	NS
38	3-Hydroxybutyric acid	366.6	340.7	5.1	<0.01	310.8	283.2	10.5	NS
39	2-hydroxyisovalerate	1.2	1.0	0.05	0.02	1.4	1.2	0.09	NS
40	L-Alpha-aminobutyric acid	0.7	2.7	0.25	<0.01	0.4	0.6	0.09	0.03
41	3-Methyl-2-oxovaleric acid	3.7	6.0	0.38	<0.01	4.9	5.4	0.13	<0.01
42	L-arginine	182.3	246.1	11.9	<0.01	197.3	213.8	3.9	<0.01
43	Creatinine	3.3	5.0	0.27	<0.01	2.9	3.3	0.26	NS
44	L-Glutamine	478.9	788.2	5.0	<0.01	502.1	568.4	13.1	<0.01
45	L-Leucine	78.1	78.8	3.8	NS	142.0	141.9	2.5	NS
46	Malonate	8.4	7.5	0.60	NS	9.6	8.7	0.37	NS
47	Ketoleucine	10.5	15.4	0.22	<0.01	11.7	14.7	0.41	<0.01
48	Methionine	107.8	101.3	2.3	NS	101.3	88.6	2.8	<0.01
49	3-Hydroxyisovaleric acid	1.0	0.4	0.08	<0.01	2.3	2.1	0.19	NS
50	Isopropanol	4.4	4.4	0.22	NS	8.1	6.5	0.29	<0.01
51	Valine	171.8	192.1	5.4	0.02	148.9	137.5	2.6	<0.01
52	Tryptophan	7.0	7.1	0.20	NS	6.8	7.7	0.25	0.02
53	Acetone	17.3	13.0	0.61	<0.01	13.3	11.0	0.47	<0.01
54	Isobutyric acid	6.6	10.2	0.13	<0.01	7.2	6.9	0.27	NS
55	Methanol	15.0	20.5	0.74	<0.01	65.4	92.9	6.2	<0.01
56	Propylene glycol	6.3	5.0	0.23	<0.01	13.6	13.5	0.63	NS
57	Dimethyl sulfone	48.7	95.0	4.4	<0.01	57.7	84.6	4.5	<0.01

Table 3.4. Interactions between three treatments in both trials (concentrations in micromolar).

	Short-term Trial	Long-term Trial			
	Strain × Seaweed	**Strain × Seaweed**	**Seaweed × Heat-stress**	**Strain × Heat-stress**	**Strain × Seaweed × Heat-stress**
1-Methylhistidine	NS [a]	0.05	0.05	NS	NS
2-Hydroxybutyrate	NS	<0.01	NS	<0.01	NS
Acetic acid	NS	<0.01	NS	<0.01	NS
Betaine	NS	NS	NS	NS	<0.01
Acetoacetate	<0.01	NS	NS	0.00	NS
L-Carnitine	<0.01	<0.01	<0.01	<0.01	<0.01
Creatine	NS	0.02	NS	0.00	NS
Dimethylglycine	0.03	<0.01	NS	0.00	NS
Citric acid	<0.01	0.03	NS	0.02	0.05
Choline	<0.01	<0.01	NS	0.00	<0.01
Ethanol	NS	<0.01	0.01	NS	NS
D-Glucose	NS	NS	<0.01	0.03	<0.01
Glycine	NS	0.03	NS	NS	0.01
Glycerol	<0.01	NS	0.01	0.05	NS
Formate	<0.01	<0.01	NS	<0.01	0.01
L-Glutamic acid	NS	0.01	NS	0.03	0.01
Hypoxanthine	<0.01	NS	0.02	<0.01	0.01
Tyrosine	NS	NS	NS	NS	NS
L-Phenylalanine	0.03	<0.01	0.01	NS	0.01
L-Alanine	<0.01	NS	NS	NS	<0.01
L-Proline	NS	<0.01	NS	NS	NS
L-Threonine	<0.01	NS	NS	0.01	NS
L-Asparagine	NS	<0.01	NS	<0.01	<0.01
D-Mannose	0.02	NS	0.04	NS	NS
Isoleucine	<0.01	NS	NS	NS	NS
L-Histidine	NS	0.01	NS	NS	<0.01
L-Lysine	NS	NS	NS	NS	NS
L-Serine	NS	NS	<0.01	<0.01	<0.01
L-Lactic acid	<0.01	NS	NS	NS	NS
L-Aspartate	<0.01	NS	NS	NS	NS
L-Acetylcarnitine	<0.01	NS	<0.01	<0.01	<0.01
Oxoglutarate	<0.01	<0.01	<0.01	0.04	<0.01
L-Ornithine	<0.01	0.02	NS	<0.01	NS
Pyruvic acid	NS	NS	0.01	NS	0.01
Succinate	<0.01	<0.01	<0.01	<0.01	<0.01
Sarcosine	NS	0.05	NS	NS	NS
Urea	<0.01	0.05	0.04	<0.01	NS
3-Hydroxybutyric acid	NS	<0.01	0.01	<0.01	NS
2-hydroxyisovalerate	<0.01	NS	NS	<0.01	<0.01
L-Alpha-aminobutyric acid	NS	NS	NS	NS	NS

3-Methyl-2-oxovaleric acid	0.02	NS	<0.01	<0.01	<0.01
L-arginine	0.03	<0.01	NS	NS	<0.01
Creatinine	0.03	<0.01	<0.01	NS	NS
L-Glutamine	0.05	NS	NS	NS	NS
L-Leucine	<0.01	0.02	0.01	NS	0.02
Malonate	NS	0.01	0.04	<0.01	NS
Ketoleucine	NS	0.03	NS	NS	0.02
Methionine	NS	NS	0.02	<0.01	NS
3-Hydroxyisovaleric acid	0.01	NS	NS	<0.01	NS
Isopropanol	<0.01	0.01	NS	<0.01	0.05
Valine	<0.01	0.01	NS	NS	NS
Tryptophan	<0.01	<0.01	<0.01	<0.01	<0.01
Acetone	0.05	0.02	<0.01	NS	<0.01
Isobutyric acid	<0.01	0.03	NS	0.02	NS
Methanol	<0.01	0.01	NS	0.00	<0.01
Propylene glycol	NS	NS	NS	<0.01	0.01
Dimethyl sulfone	<0.01	0.01	NS	NS	0.01

[a] NS: no significant differences detected (*p-value > 0.05*).

Table 3.5. KEGG metabolic pathways significantly impacted by seaweed intake, hen strains and heat-stress as revealed by Metabolic Pathway Analysis. In the short-term trial, the effects of red seaweed were assessed, while both red and brown seaweed were examined in the long-term trial.

Treatment	Pathway	Hits [a]	*P*-value [b]
Short seaweed intake	Aminoacyl-tRNA biosynthesis	7/48	<0.001
	Glycolysis / Gluconeogenesis	2/26	0.01
	Alanine, aspartate and glutamate metabolism	2/28	0.02
	Glyoxylate and dicarboxylate metabolism	2/32	0.02
	Cysteine and methionine metabolism	2/33	0.02
	Glycine, serine and threonine metabolism	2/34	0.02
	Synthesis and degradation of ketone bodies	1/5	0.04
	D-Glutamine and D-glutamate metabolism	1/6	0.04
	Nitrogen metabolism	1/6	0.04
Long seaweed intake	Alanine, aspartate and glutamate metabolism	3/28	<0.001
	Arginine biosynthesis	2/14	0.005
	D-Glutamine and D-glutamate metabolism	1/6	0.04
	Aminoacyl-tRNA biosynthesis	2/48	0.04
Heat stress	Valine, leucine and isoleucine biosynthesis	3/8	<0.001
	Aminoacyl-tRNA biosynthesis	5/48	<0.001
	Arginine and proline metabolism	3/38	0.004
	Glycine, serine and threonine metabolism	2/33	0.03
Genetic Strain	Glycine, serine and threonine metabolism	3/33	0.002
	Synthesis and degradation of ketone bodies	1/5	0.04

[a] Number of metabolites hit in the biological pathway/ total metabolites involved in each biological pathway.
[b] *p*-Value calculated from data.

Figure 3.1. A schematic overview of the experimental methods used.

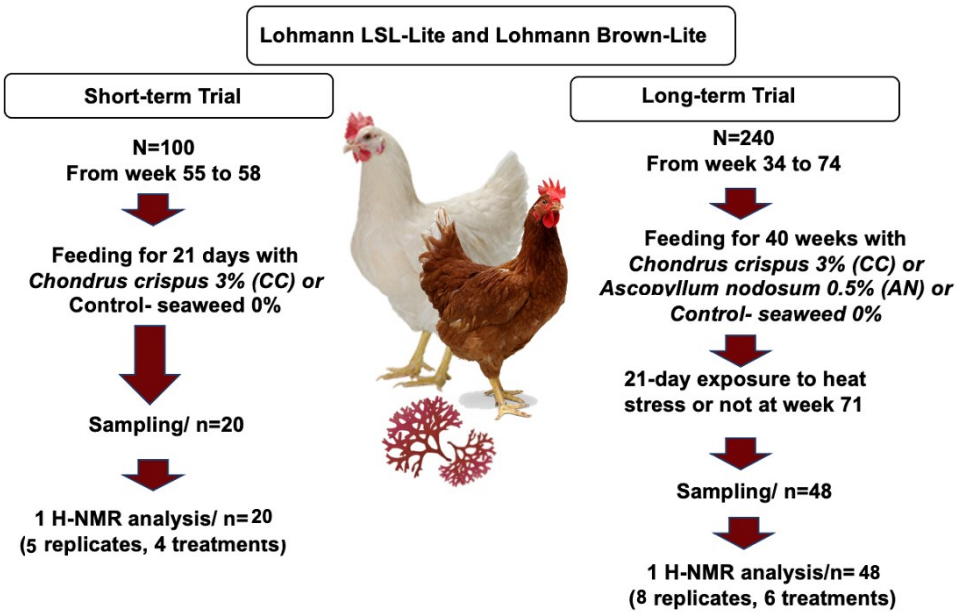


Figure 3.2. Typical 500 MHz ^{1}H-NMR spectrum of healthy hen plasma in White strain (A) and Brown strain (B). The full assignment of metabolites is given in Table 3.1.

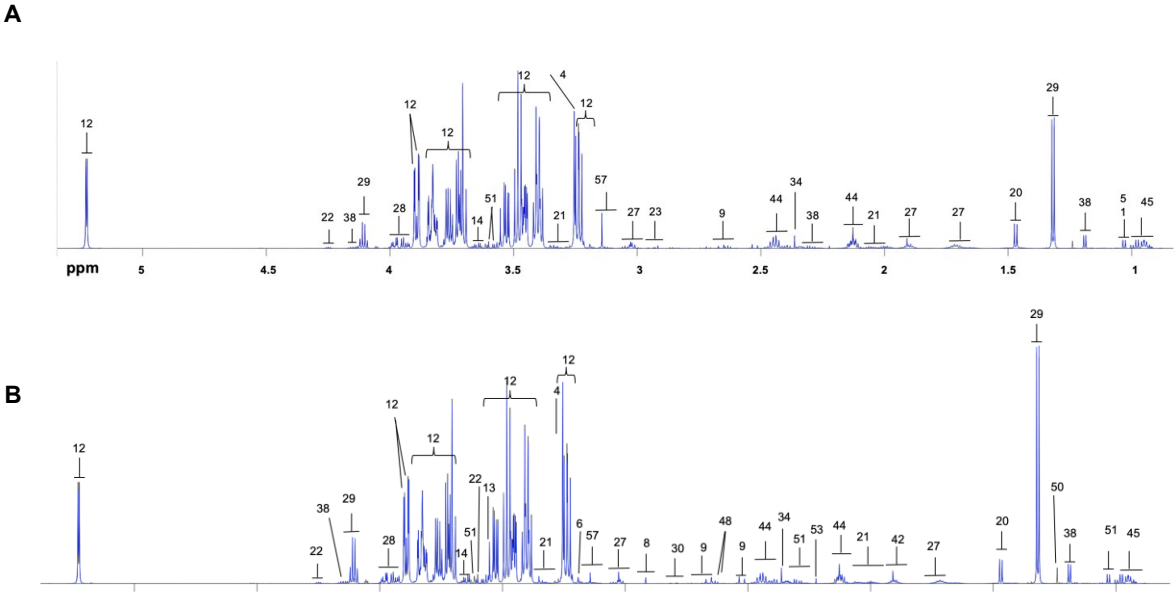


Figure 3.3. Multivariate analysis of the data to discriminate between metabolites of hen blood plasma fed with 0% seaweed (C) and 3% *Chondrus crispus* (CC) in the short-term trial. (A) PCA allows for the separation of the variation into principal components. (B) Partial least squares-discriminant analysis (PLS-DA) score plot distinguishes the metabolic profiles of plasma in hens fed 0% and 3% CC in the diet. (C) The cross validation test for PLS-DA model. (D) The variable importance in projection plot (VIP) ranking markers for their contribution to separation of two groups.

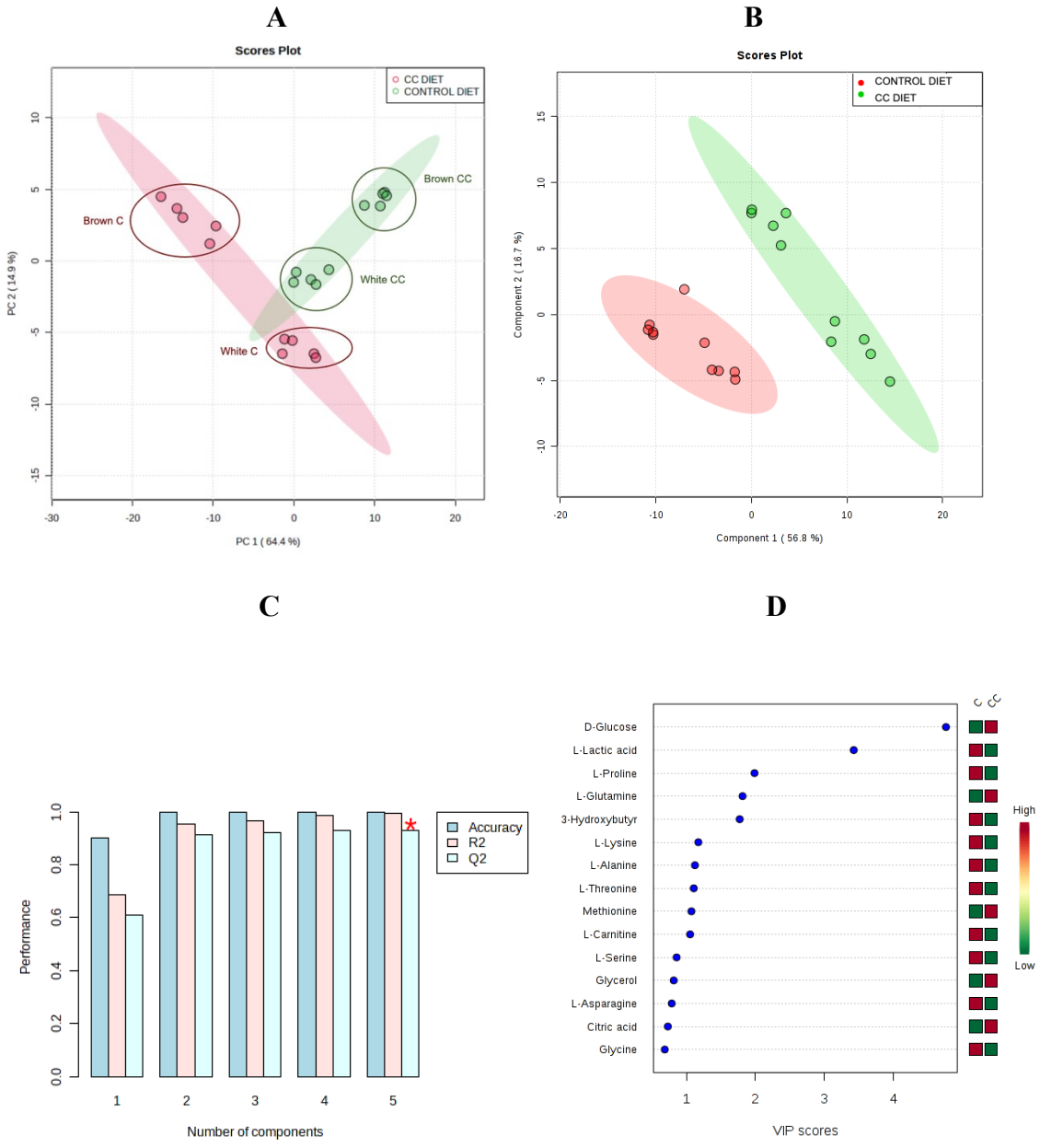
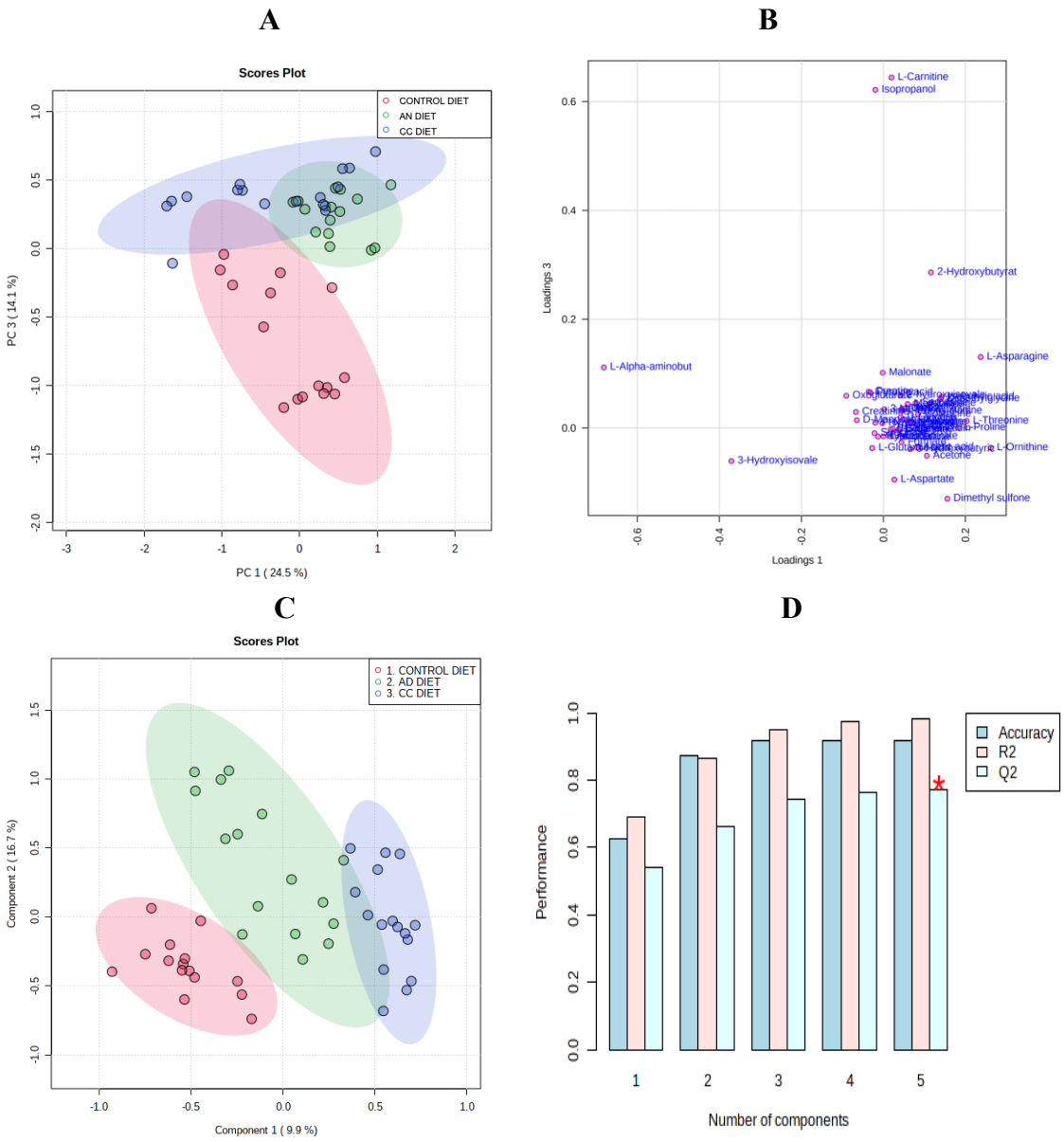


Figure 3.4. Multivariate analysis of the data to discriminate between metabolites of hen blood plasma fed with 0% seaweed (C), 3% *Chondrus crispus* (CC) and 0.5% *Ascopyllum nodosum* (AN) in the long-term trial. (A) PCA score plots derived from NMR spectra of plasma samples allows for the separation of the variation between three groups. (B) Loading plot demonstrating the differentiating metabolites between groups. (C) The PLS-DA showing three clusters. (D) The VIP plot to identify the metabolites showing maximum abundance change between 3 diets.



E

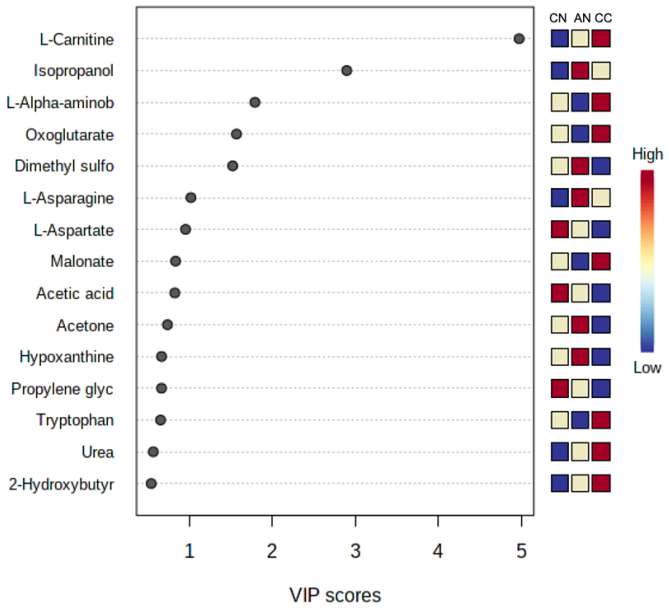


Figure 3.5. Summary of multivariate statistical analysis allows for the separation of the variation between samples. (A) PCA of heat stressed and control birds. (B) Partial least squares-discriminant analysis showing two clusters for two groups.

A **B**

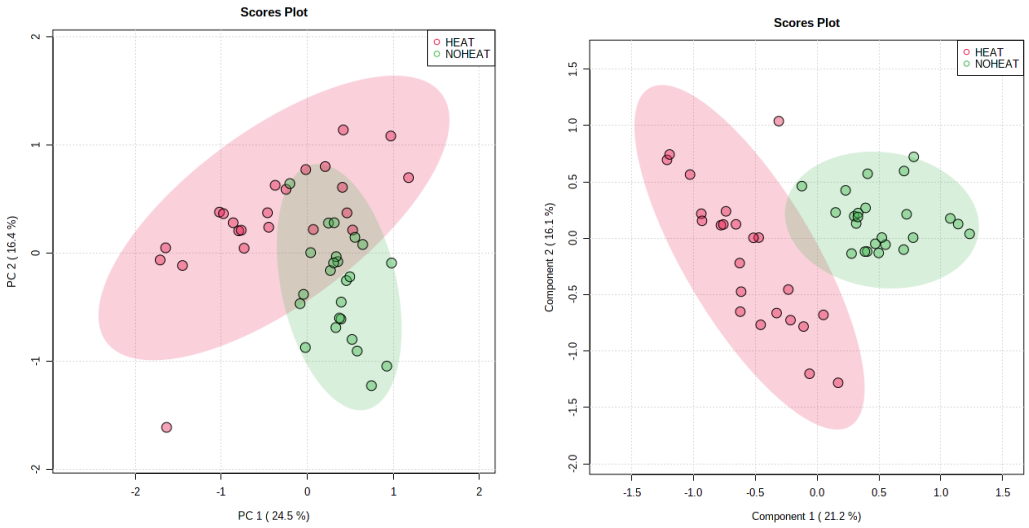


Figure 3.6. Multivariate analysis of the data to discriminate between metabolites of Lohmann LSL-Lite (White) and Lohmann Brown-Lite (Brown). (A) The PLS-DA model could separate classes into two distinct clusters. (B) The VIP ranking metabolites for their contribution to separation of two groups.

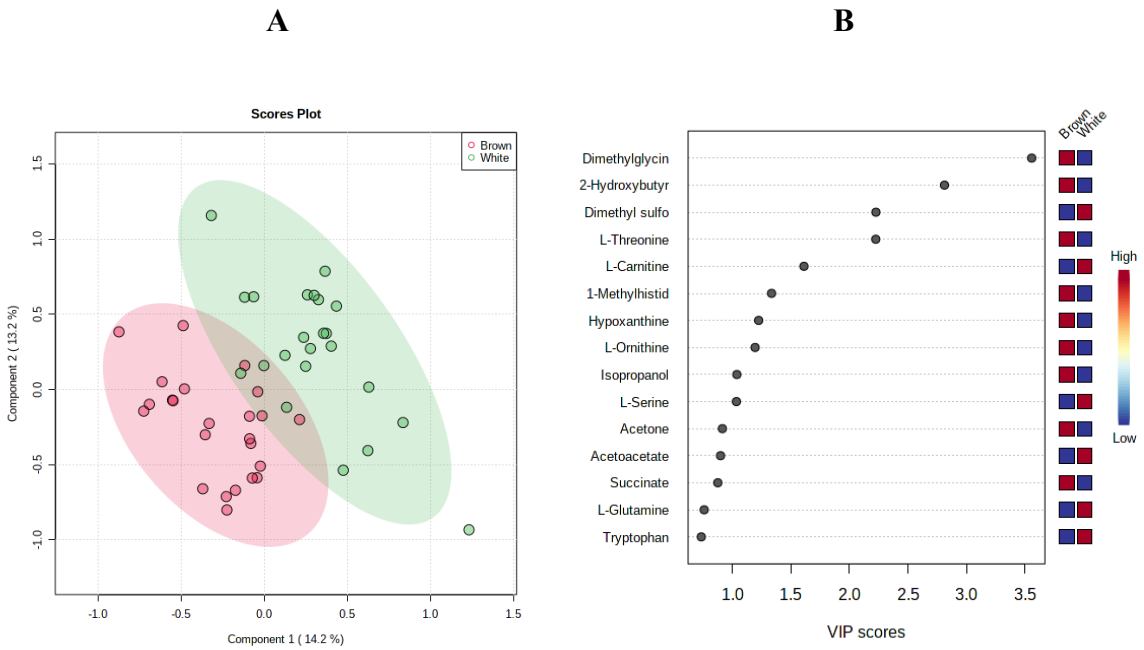
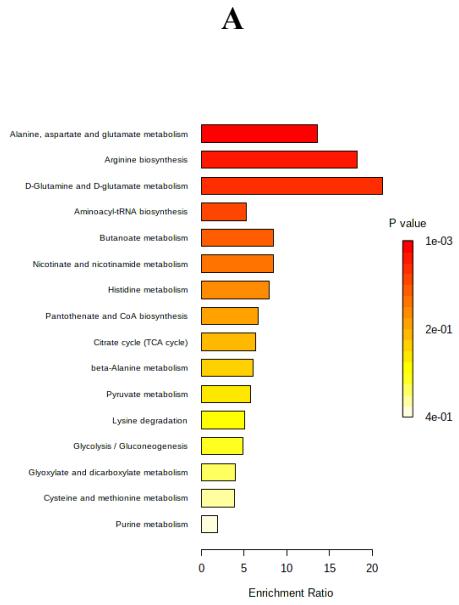
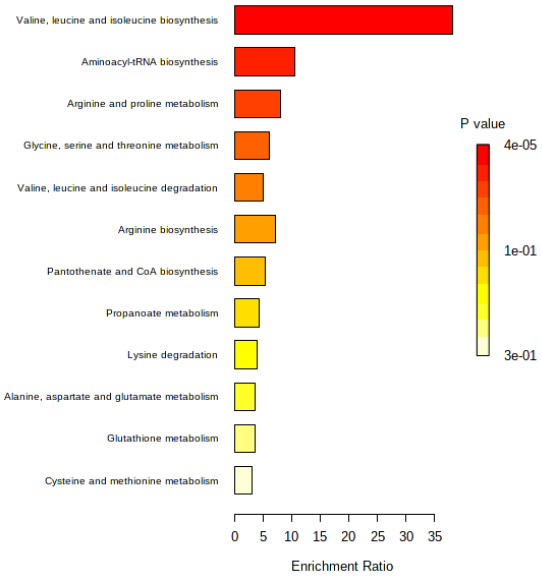


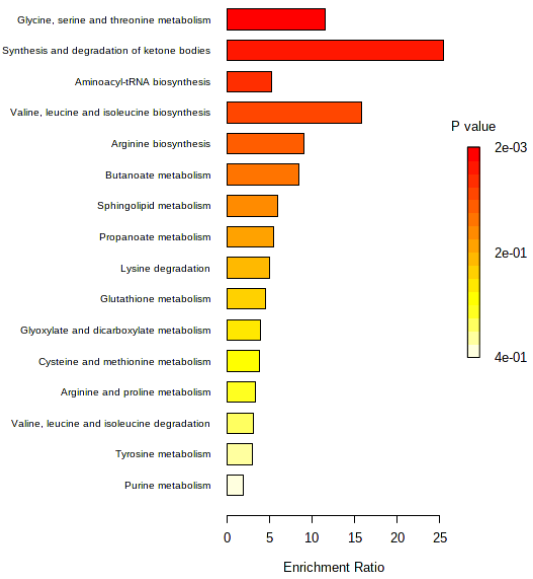
Figure 3.7. Summary of pathway enrichment analysis of short-term seaweed intake (A) heat stressed birds (B) and strain effects (c). The *p*-value of potentially affected metabolic pathways are ranked from low (red) to high (white), corresponding to the most and the least significant values, respectively.



B


Valine, leucine and isoleucine biosynthesis
Aminoacyl-tRNA biosynthesis
Arginine and proline metabolism
Glycine, serine and threonine metabolism
Valine, leucine and isoleucine degradation
Arginine biosynthesis
Pantothenate and CoA biosynthesis
Propanoate metabolism
Lysine degradation
Alanine, aspartate and glutamate metabolism
Glutathione metabolism
Cysteine and methionine metabolism
0 5 10 15 20 25 30 35
Enrichment Ratio
P value
4e-05
1e-01
3e-01

C


Glycine, serine and threonine metabolism
Synthesis and degradation of ketone bodies
Aminoacyl-tRNA biosynthesis
Valine, leucine and isoleucine biosynthesis
Arginine biosynthesis
Butanoate metabolism
Sphingolipid metabolism
Propanoate metabolism
Lysine degradation
Glutathione metabolism
Glyoxylate and dicarboxylate metabolism
Cysteine and methionine metabolism
Arginine and proline metabolism
Valine, leucine and isoleucine degradation
Tyrosine metabolism
Purine metabolism
0 5 10 15 20 25
Enrichment Ratio
P value
2e-03
2e-01
4e-01

CHAPTER 4. METABOLOMICS STUDY OF AVIAN INTESTINAL EPITHELIAL CELLS IN RESPONSE TO SHORT AND LONG-TERM SEAWEED SUPPLEMENTATION AND GENETIC STRAIN

4.1. Introduction

The gastrointestinal mucosa, present in the intestinal lumen, is a highly vascular and richly perfused organ with important functions including digestion, absorption, and protection against bacteria and toxic substances. The intestinal epithelium is a single cell layer lining the entire intestine and forming a natural, physical, and biochemical barrier to recognize and respond to macro and micronutrients, microbial components, and pathogenic insults (Zhang et al., 2015). It was previously reported that several factors, such as genetics, general state of health, diseases, dietary intake, stress, and hormones could impact epithelial cell function, growth, and proliferation (Yang et al., 2016; Ownes and Simmons, 2013). However, studies that have characterized epithelial cell changes have largely relied on cell culture outside of their natural environment (Zimmermann et al., 2007; Jiang et al., 1999; Hartmann et al., 2008). There is still much to be revealed about the cellular and molecular process or pathways potentially regulated by various factors that can impact intestinal epithelial cell metabolism.

Cell metabolomics is the study of all the metabolites present in cells that can illustrate the ultimate host responses to genetic or environmental changes by identifying specific metabolite biomarkers (Zhang et al., 2013). The application of metabolomics in cell research is relatively lagging behind and needs to be broadened and deepened. Epithelial cells have the main role in the absorption of dietary compounds, passing them from one side to the other of the epithelium, with the highest absorption occurring in the jejunum, and transporting products of digestion into the blood (Matuschek et al., 2006). Therefore,

a combination of blood and epithelial cell metabolome might be considered as the best indicator of an organism's digestive system responding to stimuli. Seaweeds are not only considered an excellent source of bioactive compounds including dietary fiber, vitamins, minerals, polyphenols, polysaccharides, polyunsaturated fatty acid, and sterols (Souza et al., 2012; Holdt et al., 2011), but also have been identified as a prebiotic source due to their high content of polysaccharides (Xiaolin et al., 2018). Prebiotics may confer certain gut health-promoting properties by favouring the growth of beneficial microbes, inhibition of pathogen colonization, and modulating the immune response (Hindu et al., 2019). Recently, the beneficial effects of seaweed as a prebiotic source were demonstrated on altering chicken performance, gut health, and immunity (Kulshreshtha et al., 2014; Carrillo et al., 2008). Therefore, prebiotics may induce direct or indirect effects on intestinal epithelial cells. Metabolomic studies may evaluate and measure the metabolic effects of seaweed prebiotic intake on the intestinal epithelium. Despite the growing appreciation of defining prebiotic impacts on metabolomic pathways, our understanding of changes to the metabolome of host cells, particularly epithelial cells, is incomplete. The gathering and analysis of previous blood data along with the intestinal epithelial cell metabolome should enhance our ability to predict different aspects of host responses to prebiotic intake, particularly the impacts on metabolic pathways.

Two strains of Lohmann LSL-Lite and Brown laying hens are among the most efficient in most markets of the world at converting feed into egg mass, high output, and good egg quality (Preisinger, 2000). Genetic strain as an indicator to impact gut health and cause physiological adaptions in the gastrointestinal tract is also expected to have a significant impact on intestinal epithelium functions.

The present study was undertaken to test the effects of seaweed supplements and genetic strain on the metabolome of epithelial cells in chicken jejunum using the NMR approach. In the present work, we aimed to provide insights into the metabolic pathways that drive the beneficial effects of seaweed and strain on production, performance, and overall health of laying hens. Our previous studies on blood metabolome, chemistry, and hematology, combined with the cell metabolome data in this study, could be a piece of the puzzle to the understanding of the intestinal mechanism of seaweed and strain actions and possible associations with chicken performance, welfare, and health traits (Borzouie et al., 2020).

4.2. Materials and Methods

4.2.1. Experimental Overview

We conducted two trials, one short-term and one long-term, at the Atlantic Poultry Research Center of the Dalhousie Agricultural Campus in Truro, Nova Scotia, Canada. The experimental methods in this study have been described in detail previously (Borzouie et al., 2020), but briefly as can be seen on Figure 4.1, the short-term experiment was a randomized trial in which 100 laying hens, 50 Lohmann LSL-Lite (White) and 50 Lohmann Brown-Lite (Brown), in 20 cages with 5 hens per cage were supplemented with 0, 3% red seaweed (*Chondrus crispus*; CC). The seaweed inclusion level and strain were the main effects, and the cage was the experimental unit (2×2 factorial trial). In this trial, we supplemented 55-week-old birds with 0 or 3% CC for 21 days prior to sampling. One bird from each cage was randomly euthanized and sampled from the middle of the jejunum, and four replicates per treatment used for NMR analysis. The long-term trial consisted of 240 hens, 120 White and 120 Brown, in 48 cages with 5 hens per cage that were

supplemented with 0, 3% red seaweed (CC) or 0.5% *Ascophyllum nodosum* (AN) (Trademark name Tasco®, Acadian Seaplants Ltd., Dartmouth, Nova Scotia, Canada), from weeks 31 to 72. This trial was designed to investigate the main effects of seaweed supplementation and laying hen strains (3×2 factorial trial). We randomly took one bird from each cage at the end of the trial at week 72 for jejunum sampling and four replicates of each treatment were taken for NMR analysis. Trials were maintained in a temperature-controlled room (25°C) with a photoperiod of 16-h of light and 8-h of darkness per day. The birds were allowed access to food and water *ad libitum* and their health was monitored daily throughout the whole experimental period. Birds were fed isocaloric and isonitrogenous diets with basal diets formulated to adequately meet the Lohmann LSL-Lite requirements as provided by Lohmann Tierzucht GmbH, Cuxhaven, Germany.

Research protocols and animal experiments were carried out at the Dalhousie University Faculty of Agriculture Animal Care & Use Committee (Protocol number 2018-031; approved 31.5.2018; Protocol number 2016-054; approved 1.9.2016) according to the guidelines of the Canadian Council of Animal Care in Science (CCAC, 2009).

4.2.2. Epithelial Cell Sample Isolation and Western Blot Analysis

At the end of the experimental period, we randomly selected one bird per cage and euthanized them by electric device, and the middle section of jejunum tissues were removed and rinsed free of intestinal content by flushing with ice-cold phosphate buffer saline (PBS).

The epithelial cell isolation was performed as previously described by Zeineldin and Neufeld (2012) with some modifications. All the isolation steps are done at 4° C (ice) to

minimize epithelial cell death and metabolite degradation. Jejunum samples were first incubated for 15 min in 15 ml of a cold solution containing 2.7 mM potassium chloride (KCl), 150 mM sodium chloride (NaCl), 1.2 mM potassium phosphate monobasic (KH_2PO_4), 680 mM disodium hydrogen orthophosphate (Na_2HPO_4), 1.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM dithiothreitol (DTT) in double distilled water (ddH_2O). The solution was then discarded, and samples were incubated in 5 ml of cold PBS, followed by quick vertexing. These steps were repeated 3 times to collect more cells. The cells were mixed and centrifuged at 300 ×*g* for 15 min at 4 °C. The cells were suspended in 5 ml of lysis buffer containing CelLytic M (Sigma-Aldrich, St. Louis, MO) and 100x protease inhibitor.

To validate the epithelial cell preparation, the Western blotting procedure was performed for the immunodetection of proteins. Subsequently, 10 µg of isolated and lysed epithelial cell protein was heated at 100°C for 15 min in a sample buffer (62.5 mM Tris-HCl, pH 8.5, 10% glycerol, 2% SDS, 0.025% bromophenol blue, 0.1 M dithiothreitol) and loaded on sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel, using the standard methods on the Bio-Rad Mini-Protein system, with 4-15% precast polyacrylamide stain free mini gel, 8.6 × 6.7 cm (Biorad, Richmond, CA) (Bio-Rad Guide, 2020). Proteins on the gel were transferred onto nitrocellulose membrane (Bio-Rad) using the Transblot-Blot SD Semi-Dry Transfer Cell (Bio-Rad, CA) at 15 volts for 15 min. The membrane was reacted with mouse monoclonal pan anti-cytokeratin antibody (Sigma Aldrich, 1:1000, 0.014 µg/µl) in 1% dry milk/TBS for 1.5 h at room temperature. It was then treated for 1 h with goat anti-mouse IgG HRP-conjugated secondary antibodies (Cell Signal, at 1:2000, 0.015 µg/µl). The membrane was incubated with SignalFire™ Elite ECL Reagent (Cell

Signaling Technology, Danvers, MA, USA). The blots were visualized by the BioRad ChemiDoc XRS System in Chemi Hi Sensitivity mode and analyzed using Image Lab software (BioRad).

The same procedure was applied using rabbit polyclonal anti-vimentin (Abcam; 1:2000, 0.014 μg/μl) as primary antibody, biotinylated anti-mouse/rabbit IgG as secondary antibody (1:200, Vector Labs, Burlingame, CA.) and the VECTASTAIN elite ABC system Kit (Vector Laboratories) for signal development, followed by imaging and visualization.

4.2.3. NMR Sample Preparation and Analyses

NMR sample collection, preparation, and storage were strictly followed according to the standard procedures (Beckonert et al., 2007; Vignloi et al., 2019) to ensure that downstream NMR analysis accurately reflects the metabolome. Following the lysis step, 570 μL of lysed cells were mixed with 70 μL deuterium water and 60 μL of a standard NMR buffer solution (11.667 mM disodium-2,2 dimethyl 2-silapentane-5-sulphonate (DSS)), 730 mM imidazole, and 0.47% sodium azide (NaN3). Samples were held in a thin walled 5 mm NMR tube (Wilmad, Buena, NJ, USA) for subsequent NMR spectral analysis.

All 1 H-NMR spectra were carried out on a 700 MHz Bruker Avance III (Bruker Biospin, Fällanden, Switzerland) at 25°C, equipped with a 5 mm TCI CryoProbe™ (Bruker Biospin) using the first transient of the NOESY pre-saturation pulse sequence. All free induction decays were zero-filled to 250 K data points. The DSS was used as an internal standard for chemical shift referencing (0 ppm), quantification, and the alignment of spectra during data processing. All 1H-NMR spectra processing, phasing, baseline correction, integration, analysis, and quantifications were carried out manually using

Chenomx NMR Suite Professional Software (version 7.7; Chenomx Inc., Edmonton, AB, Canada) (Ravanbakhsh et al., 2015).

4.2.4. NMR Statistical Analysis

Univariate statistical analyses were conducted with SAS software version 9.4 (SAS Institute, Cary, NC) using a two-way analysis of variance (ANOVA) at the 95% probability level. The general linear model (GLM) was used on individual metabolite concentrations to examine the effects of strain and seaweed supplementation and the interaction of these factors. A Tukey's multiple comparison test was used to compare the means of different treatments when significant differences existed.

Multivariate statistical analysis on NMR-derived data was performed using the MetaboAnalyst 5.0 web server. The metabolite concentrations were imported to the server according to previously published protocols to fully analyze quantitative metabolomic data (Xia & Wishart, 2016). The data was normalized and scaled through Pareto scaling, in which the response for each variable was mean-centered and divided by the square root of the standard deviation. Finally, the valid data was handled for multivariate statistical analysis using principal components (PCA) and partial least-squares discriminant analyses (PLS-DA). PCA is a non-supervised chemometric method to reduce the dimensionality of the resultant features while retaining the maximum variability present in the data. The PLS-DA is a predictive supervised method that uses class information to maximize the separation between classes and rank the importance of each variable for the classification of the metabolic profiles using the variable importance in the projection (VIP). For the metabolites significantly affected by seaweed intake or bird strain, Enrichment Analysis

and Pathway Analysis were performed using MetaboAnalyst 5.0 with reference to the KEGG Database to explore the top correlated metabolic pathways of epithelial cells impacted by genetic and environmental factors.

4.3. Results

4.3.1 Validation of the isolated epithelial cells using Western Blot Analysis

The expression of cytokeratin in mammalian cells is considered as a reliable marker to differentiate epithelial from non-epithelial cells. Thus, western blotting analysis was performed to examine the epithelial cell (EPC) existence by comparing cytokeratin protein expression in epithelial cells versus stroma cells (STC) as the control. As shown in Figure 4.2A, cytokeratin protein was expressed properly in the epithelial cells isolated from six independent samples (EPC1-EPC6; lanes 2-7). In contrast, stroma cells did not exhibit a significant expression of the cytokeratin protein (STC1 and STC2; lanes 1 and 8). Likewise, vimentin, a marker for stroma cells, was abundantly expressed in stroma cells (STC1 and STC2; lanes 1 and 8), while this pattern was almost reversed in epithelial cells (EPC1-EPC6; lanes 2-7); with no or very low vimentin expression (Figure. 4.2B). Together, these results confirmed that most isolated cells were epithelial cells. The intensities of the western blot bands were also analyzed for more precise validation of epithelial cell isolation method, using the BioRad Image Lab program (Table 4.1). The expression of anti-cytokeratin (lanes 2 to 7) was $\simeq$ 14-fold higher than those of control (lanes1 and 8) (Table 4.1), while the expression of anti-vimentin on stroma cells (lanes 1 and 8) was $\simeq$ 15-fold higher than epithelial cells (lanes 2 to 7).

4.3.2 Epithelial Cell Metabolites were significantly affected by seaweed supplementation

Seaweed supplementation significantly (P <0.05) influenced 44 and 35 epithelial cell metabolites in the short-term and long-term trials, respectively (Table 4.2). Our long-term trial results showed that among three treatments, the highest metabolite values were observed in birds supplemented with 0.5% of AN which resulted in increased levels of 27 epithelial cell metabolites (Table 4.2). By comparing 3% CC seaweed and control diets, the majority of metabolite levels were significantly higher in control birds than those supplemented with 3% CC seaweeds (41 compounds in the short-term trial and 26 compounds in the long-term trial). In both trials, 2-phosphoglycerate, xylulose, and lactose concentrations were significantly (P <0.05) increased by the seaweed intake (Table 4.2). Long-term seaweed supplementation caused a significant (P <0.05) increase in the levels of six more metabolites including 4-carboxyglutamate, fructose, ethanol, isopropanol, threonate, and xylose (Table 4.2). Most high-level metabolites were sugars, while the minority of those were amino acids.

Furthermore, there were nine compounds that were not impacted either by short- or long-term seaweed intake, including 1-propanol, acetaldehyde, acetone, formate, glycolate, isobutyrate, isocitrate, malonate, and ribose (Table 4.2). In addition, six epithelial cell metabolites including 2-hydroxyisobutyrate, ethanol, isopropanol, homocysteine, threonine, and xylose were remained unchanged in short-term seaweed intake treatment. Similarly, the following 15 metabolites were not altered by long-term seaweed intake treatment; 2-oxoglutarate, 4-aminobutyric-acid, acetate, glucuronic acid, glucose,

glutamate, glutamine, glycerol, lactate, phosphorylcholine, taurine, theophylline, uracil, xanthine, and myo-inositol (Table 4.2).

4.3.3. Genetic Strain Impacts on Epithelial Cell Metabolites

Our results indicated significant changes in epithelial cell metabolites in the two hen genetic strains. Overall, 45 and 49 metabolites were affected in the short-term and long-term trials respectively, that the vast majority of them were amino acids (see Table 4.3). Among all quantified compounds in the two strains, the most concentrated metabolites were some sugars including fructose and xylulose, as well as sugar alcohols, and alcohols including glycerol, isopropanol, and ethanol. In the short-term trial that birds were at 58 weeks of age, 43 metabolites were found significantly higher in the White strain than in the Brown. However, in birds at 72 weeks of age, 42 metabolites were found to be significantly higher in the Brown strain. As shown in Table 4.3, 40 metabolites were impacted by genetic strain in both short and long-term trials, including 2-aminobutyrate, 2-oxoglutarate, 4-carboxyglutamate, acetaldehyde, acetone, alanine, alloisoleucine, anserine, aspartate, carnitine, choline, creatine, fructose, xylulose, dimethylsulfide, glutamate, glutamine, glycerol, glycine, glycolate, guadinoacetic acid, isobutyrate, isoleucine, homocysteine, lactate, leucine, 2-oxalacetic acid, oxypurinol, phenylalanine, phosphorylcholine, proline, taurine, threonine, trimethylamine N-oxide, tryptophan, tyrosine, uracil, valine, xanthine, myo-inositol. Most of these differential metabolites were amino acids. There were five epithelial cell metabolites that were not impacted by genetic strain in any of the trials, including ethanol, formate, isocitrate, isopropanol, and malonate.

4.3.4. Multivariate Analyses of Treatment Effects

Figure 4.3 illustrated the multivariate analysis of the data to discriminate between epithelial cell metabolites of hens fed with 0% and 3% CC in the short-term trial. The unsupervised principal component analysis (PCA) and supervised partial least squares-discriminant analysis (PLS-DA) score plots were used to investigate possible epithelial cell metabolite differences between seaweed and control diets. The PCA score plot of short seaweed intake, explaining 86.6% of the variation, showed that the 3%CC seaweed-treated group was not well separated from the control group, demonstrating no distinctive clustering between the CC diet and the control (Fig. 4.3A). As shown on Figure 4.3C, the PLS-DA score plot did not completely enable group differentiation. The control diet samples in the red cluster demonstrated higher chemical variations, while the CC diet samples in the green cluster were tightly clustered, showing lower variations. The PCA score plot of the long-term seaweed supplementation (data not shown), covering 85.7 % of the observed variance in the sample set, showed that there were no significant distinctions between the control, 3% CC, and 0.5% AN groups, while the PLS-DA score plot (Fig. 4.4A) by maximizing class discrimination could illustrate better discrimination between the groups, with acceptable values of R2 and Q2 (R2 and Q2 $\geq$ 0.6) and a successful permutation test of the PLS-DA model for PC1 vs PC2 ($p < 0.05$). The PLS-DA covered 73.6% of the variance and cross-validation indicated that the PLS-DA model had modest quality (Q2 was smaller than R2). The variable importance in the projection (VIP) was constructed from the loading plots of PLS-DA (Fig. 4.4C) to rank the importance of each metabolite in discriminating seaweed supplemented birds from the control group and to select the metabolites having the highest discrimination potential (VIP $\geq$ 1) (Fig. 4.4C). Using this VIP score from PLS-

DA together with ANOVA, we found eleven compounds as group discriminators with a significant VIP score (VIP ≥ 1) and a 95% probability level ($p < 0.05$) which accounted for most of the observed separation including ethanol, fructose, homocysteine, xylulose, 2-phosphoglycerate, 2-oxalacetic acid, valine, dimethyl sulfide, and alanine. These observations suggest that the noted metabolites accounted for most of the separation between treatments in the long-term study and can be suggested as the potential biomarkers in intestinal epithelial cells of laying hens, associated with seaweed intake. The heat map on the right side of the VIP plots (Fig. 4.4B) indicates that except ethanol all the above-listed metabolites were enhanced in birds supplemented with long-term AN seaweed intake, while the levels of homocysteine, xylulose, 2-oxalacetic acid, glycerol, valine, dimethyl sulfide, and alanine were lowest by long-term CC seaweed supplementation. This can be confirmed by the PLS-DA loading plot that indicates how the responses are affected by descriptors and describes the positive or negative relationships between variables (Fig. 4.4C).

Multivariate analysis of the data to discriminate between epithelial cell metabolites of White and Brown strains is shown on Figures 4.5 (short-term trial) and 6 (long-term trial). The PCA score plot of the short- and long-term trial did not discriminate between the metabolites of two strains (data not shown). The supervised multivariate analysis with PLS-DA could separate classes into two distinct clusters in both trials, as can be seen on Figures 4.5A and 4.6A. In both trials, cross-validation indicated that the PLS-DA models had good quality (Q2 comparable to R2, and Q2>0.8, $Q^2 \sim 0.65$) with successful permutation tests ($p < 0.05$). There was a difference observed between discriminating metabolites, showing possible impacts of age, season, stage of laying on the epithelial cell metabolome. As

observed from the corresponding VIP plots and PLS-DA loading plots of two trials (Fig 4.5B, 4.5C, 4.6B, and 4.6C), alanine, fructose, xylulose, dimethyl sulfide, ethanol, glycerol, homocysteine, and oxaloacaetic acid were the strongest discriminating metabolites for separating the two strains (VIP ≥ 1 and $p < 0.05$). Moreover, glycolate and isopropanol were found as discriminating metabolites only in the short trial and phosphorylcholine only in the long-term trial.

4.3.5. Pathway Analysis

Based on our quantitative metabolomic data, we aimed to find significantly affected intestinal epithelial cell pathways. Metabolite set enrichment and pathway analyses were performed using MetaboAnalyst 5.0 in conjunction with the chicken (Gallus gallus) library from the KEGG database, to identify and interpret changes in chicken pathway-associated metabolite concentrations. The potential epithelial cell metabolites with a VIP score of greater than 1 and *p*-value smaller than 0.05 were included in the analysis to evaluate the metabolic pathways affected by short- and long-term seaweed intake, as well as the strain. The enriched pathways were ranked from low to high according to the pathway fold enrichment. The significantly affected metabolic pathways, the fold enrichment, and P-values were summarized in Figure 4.7. We revealed that there were wide-ranging effects on intestinal epithelial cell metabolic pathways as four and five metabolic pathways were found to be significantly impacted by short and long seaweed intake, respectively, as well as four and four pathways impacted by the short-term genetic strain and the long-term strain effect, respectively (Fig. 4.7).

The total number of metabolites involved in each biological pathway, the number of metabolites hit in a certain pathway, and the corresponding statistical significance are shown on Table 4.4. The statistically significant pathways were those KEGG pathways that hit two or more metabolites in each detected pathway with the pathway impact greater than 0.1 and the *p*-value less than 0.05. Enrichment analysis as a tool for visualization and biological interpretation of metabolite data suggested that short-term seaweed supplementation affected four metabolic pathways, including galactose metabolism; starch and sucrose metabolism; glycolysis/gluconeogenesis; and neomycin, kanamycin, and gentamicin biosynthesis (shown on Table 4.4). The long-term seaweed supplement trial affected five metabolic pathways, that glycolysis/gluconeogenesis was the common pathway between short and long-term trials. While four pathways including alanine, aspartate, and glutamate metabolism; glyoxylate and dicarboxylate metabolism; aminoacyl-tRNA biosynthesis; valine, leucine, and isoleucine biosynthesis were impacted only by the long seaweed supplementation in epithelial cells. Genetic strain induced a similar impact on epithelial cell metabolic pathways in short-and long-term trials by affecting galactose metabolism; starch and sucrose metabolism; and neomycin, kanamycin, and gentamicin biosynthesis. Alanine, aspartate, and glutamate metabolism was only impacted by the short-term strain trial, while phosphonate and phosphinate metabolism was impacted by the long-term strain trial.

4.4. Discussion

Despite the decent number of studies on epithelial cells, intestinal epithelial cells have been rarely considered to be an important factor in analyzing metabolomics data to date. In a

similar study, metabolites such as creatine, glycine, taurine, carnitine, choline, phosphatidylcholine, aspartate, glutamate, acetate, lactate, valine, isoleucine, and leucine were also detected in chicken enterocytes using NMR spectroscopy (Quirk et al., 1989). These findings are consistent with the metabolites identified in our own study. In the present study, 1H NMR spectroscopy on the metabolomics profiling of epithelial cells in chicken revealed significant strain variations as well as distinct metabolic responses between the prebiotic supplemented group and the control. There were four and five differential metabolic pathways impacted by short-term and long-term seaweed intake, respectively, and four pathways impacted by the strain in both short-term and long-term trials. There were more factors, including age, season, and stage of lay, that may have been expected to have metabolic effects on birds' responses to seaweed intake or genetic strain. The age, season, and stage of lay were considered consistent factors within each trial; however, they exhibited differences between the short-term and long-term trials. These variations in age, season, and stage of lay across the trials have the potential to impact the results when comparing the two trials with each other. However, it is difficult to differentiate the source of these pathway changes, but our findings indicate that the observed impacts on the metabolome were primarily attributed to genetic and dietary factors rather than age and laying cycle.

Despite a 16-week age difference between the hens before slaughter, our analysis suggests that this time gap had minimal influence on the metabolome compared to the factors we specifically investigated, namely genetics and prebiotic supplementation. Notably, our analysis of the long-term trial revealed no shared pathways between seaweed and genetic strains, indicating that the differentially expressed pathways were primarily influenced by

the treatments themselves. This finding highlight that the treatments had a significant influence on the observed differences in pathway expression. In contrast, factors like age, laying cycle, and seasonal variations had a relatively minor impact in comparison.

4.4.1. Metabolic pathways and metabolites associated with hen red and brown seaweed intake

Short-term seaweed supplementation significantly changed the epithelial cell metabolite involved in the glycolysis pathway (Table 4.2). It would thus appear that seaweed supplementation reduced intestinal glycolysis by reduction of glycolytic capacity, reducing the dependence of intestinal cells to produce ATP from glycolysis and lactate release. Glycolysis is the energy supplying pathway by converting glucose into pyruvate and pyruvate into lactate and alanine (Wang et al., 2018). Short seaweed treatment significantly decreased levels of lactate and alanine, indicating that glycolysis might be suppressed during short-term prebiotic supplementation (Table 4.2). In addition, the levels of the gluconeogenic precursors including alanine, glutamate, and lactate declined in response to short-term seaweed supplementation, suggesting that gluconeogenesis is the target of the seaweed under short-term supplementation conditions. These quantitative results were further supported by the pathway analysis data obtained from the KEGG which exhibited reduced glycolysis pathway activity after seaweed intake (Tables 4.2 and 4.4). It was previously reported that prebiotic supplements decreased glycolysis metabolism in fish and mice (Guerreiro et al., 2015; Zhang et al., 2019). Similar results were also observed in our previous study on the chicken plasma metabolome in which short-term prebiotic

supplementation reduced blood glucose by impacting the glycolysis pathway. Our long-term metabolome results on glucose were differ from the short-term, suggesting there may be some adaptation to longer-term prebiotic supplementation. Although further studies on glucose response to prebiotic intake are required to better understand the differences between the short and long-term impacts. Additionally, the short-term inclusion of red seaweed in laying hen diets reduced feed intake (Borzouie et al., 2020) probably via a glycolysis-dependent pathway. Similarly, functional enrichment analysis of Pacific white shrimp genes revealed the glycolysis pathway associated with residual feed intake (Dai et al., 2017). The present data speculated the glycolysis/gluconeogenesis pathway as the potential biological pathway related to residual feed intake.

The conversion of galactose to glucose is the major pathway in galactose metabolism (Liu et al., 2000) that was downregulated in epithelial cells, through short-term seaweed supplementation. Furthermore, it has been reported that galactose metabolism, as well as the starch and sucrose metabolism pathways were enriched by feed intake related candidate genes in ducks (Guo et al., 2022). Likewise, these pathways impacted by the short-term seaweed intake in the present experiment. Also, our previous report showed a reduction in the feed intake of laying hens with a short-term dietary supplementation of 3% CC (Borzouie et al., 2020). Therefore, it is likely that galactose, starch, and sucrose metabolisms are the key pathways associated with the feed intake of chickens. Long-term seaweed intake had no effect neither on these two pathways, nor on feed intake, confirming the association of the starch and sucrose metabolism, as well as the galactose metabolism with feed intake.

Lactose is a disaccharide of glucose and galactose that is hydrolyzed into its component monosaccharides in the brush border membrane of small intestinal enterocytes (Coelho et al., 2015). The significant increase in lactose and decrease of glucose levels following short-term seaweed intake may suggest a reduction in lactose hydrolysis in intracellular epithelial cells of the small intestine.

Intestinal epithelial cells have a high energy demand to renew every 5 to 7 days with energy substrates from both luminal and blood origins (Berger et al., 2017). Glucose, glutamate, glutamine, and aspartate are the main blood-derived fuel substrates that are consistently delivered from the blood to epithelial cells (Blachier et al., 2017). Decreased epithelial cell levels of glucose, glutamate, glutamine, and aspartate in the short-term seaweed supplemented groups might indicate that extensive anabolic activity of epithelial cells relies on these metabolites as an energy source. Interestingly, results of our previous study showed a reduction in plasma levels of glucose, glutamate, and aspartate indicating the circulatory system between blood and epithelial cells metabolites. Moreover, epithelial cells absorb short-chain fatty acids as the cell luminal energy source directly from the gut lumen, where they are produced by bacterial fermentation. Among short-chain fatty acids, acetate, formate, and isobutyrate were detected in epithelial cells, with lower levels in the short-term seaweed supplemented birds. Thus, these results support the idea that seaweed is acting as a prebiotic in chicken, affecting microbial fermentation and subsequently plasma short-chain fatty acid composition. These findings are in good agreement with our previous study on plasma.

In the long-term seaweed supplemented group, we observed downregulation in seven epithelial cell metabolites including alanine, acetate, glutamate, glutamine, valine, leucine,

and isoleucine that were involved in alanine, aspartate, and glutamate metabolism, as well as valine, leucine, and isoleucine biosynthesis pathways. Glutamine is the differential metabolite in this pathway (Huang et al., 2019), which was also found to be downregulated in epithelial cells in response to long-term seaweed intake.

In support of our results, similar pathways including alanine, aspartate, and glutamate metabolism; valine, leucine, and isoleucine biosynthesis; as well as starch and sucrose metabolism were impacted in ileal mucosa of goats that were fed for 28 days with a high-grain diet, a great source of prebiotics (Jiyao et al., 2019). Our NMR metabolic profiling and KEGG metabolic pathway analysis showed a similar expressional pattern for alanine, aspartate, and glutamate metabolism pathway (raw *p*-value = 0.01, Holm-Bonferroni p value= 0.885, FDR=0.4) and valine, leucine, and isoleucine biosynthesis pathway (raw *p*-value = 0.04, Holm-Bonferroni p value= 1, FDR=0.7), indicating that these pathways were highly activated in our study. Notably, amino acid metabolism pathways were highly expressed in the epithelial cells of seaweed supplemented birds, implying the prebiotic impact on protein metabolism. The reduction in protein synthesis suggested that prebiotic intake might inhibit poultry protein metabolism. A very similar pattern was observed in our previous plasma study indicating that amino acid metabolism pathways were highly expressed in seaweed supplemented birds.

The aminoacyl-tRNA synthesis pathway plays a central role in protein biosynthesis by providing substrates for the direct attachment of an amino acid to the corresponding tRNA (Ibba and Soll, 2001). Hence, this pathway involves a variety of amino acid metabolic pathways. There are 21 distinct pathways of aminoacyl-tRNA formation including 20 canonical amino acids plus selenocysteine (Ibba and Soll, 2001). In the present study, 13

amino acids involved in aminoacyl-tRNA biosynthesis pathway including alanine, aspartate, glutamate, glutamine, glycine, isoleucine, leucine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine were modulated by seaweed treatment, indicating that this pathway was highly seaweed-responsive. These findings indicate that seaweed intake may affect the translation process by influencing the tRNA function of these 13 amino acids. We observed depleted levels of all these amino acids in the epithelial cells of the short-term seaweed supplemented group. In the long-term CC seaweed intake group, glutamate, glutamine, and tryptophan were upregulated while other amino acid levels were reduced in intestinal epithelial cells. Interestingly, brown seaweed supplementation resulted in the upregulation of all the detected amino acids except glutamate, glutamine, and tryptophan. The altered levels of the above-mentioned amino acids in long-term seaweed supplemented birds were well correlated with the beneficial role of long-term prebiotic intake in protein synthesis and metabolism. Chen et al., reported that dietary probiotic supplementation affected the aminoacyl tRNA synthesis pathway in the mouse gut. Similarly, probiotic yeast supplementation affected aminoacyl-tRNA biosynthesis and amino acid metabolism in pigs (Alassane-Kpembi et al., 2020). This pathway was also detected in fecal samples of young mice that were fed with probiotic chow for four weeks (Vemuri et al., 2018). Therefore, we speculate that prebiotic intake could be involved in amino acid metabolism and the activation of other amino acid pathways.

It is important to highlight that the impact of seaweed on amino acid metabolism in poultry is contingent upon its nutrient composition and anti-nutrient properties. The nutrient composition of seaweed, encompassing proteins, minerals, vitamins, and essential amino

acids, plays a significant role in shaping amino acid metabolism. These nutrients contribute to the overall amino acid pool available for metabolism, thereby influencing protein synthesis and degradation. Conversely, seaweed may contain anti-nutrients that can impede amino acid metabolism. Anti-nutrients, such as phytic acid and enzyme inhibitors, can bind to amino acids or inhibit the activity of enzymes involved in amino acid metabolism, thereby altering their availability and utilization within the bird's body (Reddy et al., 1982; Hagerman et al., 1998). Furthermore, the presence of anti-nutrients in seaweed can impede the functioning of certain enzymes that play a crucial role in protein digestion and the metabolism of amino acids. This, in turn, can have a significant impact on the breakdown and utilization of dietary proteins (Dunaief et al., 2012).

Another study that analyzed biological networks and pathways associated with a probiotic supplement (*Bacillus subtilis*) in laying hens has revealed some significant biological processes including starch and sucrose metabolism, as well as galactose metabolism (Guo et al., 2018). Our epithelial cell metabolome data confirmed that seaweed could also impact the same pathways. Based on our results, it is likely that starch, sucrose, and galactose metabolism pathways play an important function in gut bacterial activity.

4.4.2. Metabolic pathways and metabolites associated with hen strain

Our study showed that genetic strain had a strong influence on the epithelial cell metabolome (Table 4.3). Zimmerman et al., (2021) revealed that genetic strain influenced the epithelial cell response to environmental factors in mice. The present data suggested that in both trials, more metabolites were observed to be impacted by strain seaweed supplementation. Interestingly, the long-term trial had a little greater influence on the

metabolic changes of the two laying hen strains in contrast to the short-term trial. Although we observed distinct separation between the White and Brown strains in the short-term group, the Brown strain metabolome was influenced more during the long-term trial. Our previous blood chemistry study has demonstrated that the White strain had higher plasma protein levels in the short-term trial that might be associated with higher amino acid metabolism, which is consistent with our epithelial cell metabolomic results here (Borzouie et al., 2020). Our quantitative NMR data here showed that the White strain had higher levels of amino acids in the short-term trial, which may result from increased protein synthesis in the specific strain. It has also been reported that developing primary follicles imparted a major impact on hen egg production that was mainly associated with amino acid synthesis (Wadood et al., 2021). Our previous study demonstrated higher egg production in the White strain (Borzouie et al., 2020). In the current short-term study, the epithelial cell metabolome of the White strain demonstrated higher levels of amino acids including alanine, aspartate, creatinine, glutamate, glutamine, glycine, isoleucine, leucine, phenylalanine, proline, threonine, tryptophan, tyrosine, and valine, suggesting that amino acid synthesis might be associated with egg production. These epithelial cell metabolome results were also in agreement with our previous plasma chemistry results in which total protein of the White strain in the short-term trial increased (Borzouie et al., 2020). Therefore, we speculate that biosynthesis of amino acids and total protein levels could be associated with egg production.

The strain-induced changes in epithelial cell metabolism pathways were in agreement with those reported in previous studies, as we observed galactose metabolism, as well as starch and sucrose metabolism pathways were most significantly affected across two hen strains

that impacted intestinal epithelial cell metabolism. Similarly, galactose metabolism was reported as one of the most activated pathways in breast, liver, and fecal samples of Arbor Acres broilers as well as the blood samples of a Chinese local chicken (Won et al., 2016; Wang et al., 2020; Ali et al., 2017, Xiao et al., 2019). Moreover, starch and sucrose metabolism, a pathway related to digestion, was found to be upregulated in chicken intestinal intraepithelial lymphocytes, the breast and leg muscles of Ross 308 broiler chickens, the breast and leg of Chinese local Wuding broilers, and the pectoralis muscle of broiler chickens (Shi et al., 2022; Zhou et al., 2020; Beauclercq et al., 2016; Zhao et al., 2019). This may imply that the above-mentioned pathways are strain dependent. Accordingly, the genetic homogeneity of the strains is expected to significantly reduce the variability of the metabolome.

4.4.3. Comparison of the Epithelial Cell and Plasma Metabolome

Differences in metabolite composition between intestinal epithelial cells and the bloodstream arise due to processes involved in nutrient metabolism and distribution (Chen et al., 2019; Shi et al., 2017). Intestinal cells produce unique metabolites that are then transported to the liver, where they undergo further changes, altering the original dietary supplement metabolites. Variations in the distribution and utilization of metabolites across different tissues contribute to distinct metabolite patterns between these compartments.

When it comes to nutrient absorption, food digestion, or microbial infections, changes in the composition of epithelial cells can occur. This is because electrolytes, nutrients, and chemical compounds directly pass from epithelial cells to blood vessels in the lamina propria, resulting in an impact on blood composition (Kong et al., 2018). Therefore,

studying the metabolome of both blood and intestinal epithelial cells is important in understanding their interaction and exploring biological functions and molecular mechanisms. In our study, we compared the results with our previous research on the plasma metabolome of chickens, and we found that 15 and 14 metabolites were commonly detected in plasma and epithelial cells in the short- and long-term seaweed supplemented groups, respectively (Fig. 4.8A). Furthermore, the strain treatment had an impact on 12 and 11 metabolites in the short- and long-term trials, which were commonly found in both plasma and epithelial cells (Fig. 4.8B). Short-term seaweed intake could affect more metabolic pathways in plasma than in epithelial cells (nine versus four). Glycolysis/gluconeogenesis was the altered pathway in both plasma and epithelial cells in the short-term seaweed supplemented birds. Alanine, aspartate, and glutamate metabolism, as well as aminoacyl-tRNA biosynthesis, were the pathways commonly impacted by plasma and epithelial cells in the long-term seaweed trial. Genetic strain impacts on plasma were found to be different than epithelial cells. There were more metabolic pathways impacted by short and long-term trials in epithelial cells than in plasma (four versus two). Glycine, serine, and threonine; as well as synthesis and degradation of ketone bodies were previously found to be impacted by plasma but not by epithelial cells (Borzouie et al., 2022; Table 4.4; Figure 4.7). The significant change in acetone and methanol levels as metabolic byproducts may indicate enhanced microbial activity and fermentation in the gut due to the prebiotic intake. Acetone is a ketone body that can be generated from the metabolism of fatty acids, while methanol can be formed through the degradation of pectin, a type of dietary fiber found in prebiotic-rich foods (Kistler et al., 2018).

4.5. Conclusions

Through the NMR analysis of the chicken epithelial cells, we found that the metabolome of laying hens was significantly impacted in response to seaweed supplementation and genetic strain treatment. We also provided insights into the pathways and mechanisms that drive the beneficial effects of seaweed and strain on production, performance, and gut health of laying hens. Given that two different stains exhibited distinct responses to the seaweed treatments, it can be concluded that the metabolite pathway modulation is strain dependent. Taken together, this work has laid a foundation for further studies revealing the association of the metabolomic pathways with nutrition, growth, and performance strategies. The results of this study can pave the way for identification of the molecular markers that can facilitate layer production improvement programs.

Table 4.1. Quantification of the cytokeratin and vimentin using the BioRad Image Lab program to analyze the intensities of the western blot bands.

Lane	Band Label	Band intensity (arbitrary unit)	Band intensity (arbitrary unit)
		Anti-cytokeratin antibody	Anti-vimentin antibody
1	STC1	1.04	1.00
2	EPC1	13.45	0.05
3	EPC2	15.24	0.04
4	EPC3	16.61	0.08
5	EPC4	15.35	0.04
6	EPC5	11.62	0.03
7	EPC6	11.23	0.13
8	STC2	1.00	0.92

Table 4.2. Concentrations (micromolar) of metabolites impacted by short-term and long-term seaweed supplementation in laying hen epithelial cells, as measured by NMR.

		Short-Term Trial [a]				Long-Term Trial				
	Metabolites	**0% CC**	**3% CC**	**SEM**	***P-value***	**0% CC**	**3% CC**	**0.5% AN**	**SEM**	***P-value***
1	1-Propanol	42.4	34.6	4.6	NS [b]	27.1	28.5	26.2	2.9	NS
2	2-Aminobutyrate	195.7	87.9	14.5	<0.01	145.4	79.6	126.2	9.4	<0.01
3	2-Hydroxyisobutyrate	22.7	12.9	4.4	NS	27.5	19.8	39.4	2.4	<0.01
4	2-Oxoglutarate	31.9	17.1	4.3	0.03	18.6	15.4	17.1	1.7	NS
5	2-Phosphoglycerate	127.7	264.3	34.6	0.02	52.7	97.5	274.6	7.1	<0.01
6	4-Aminobutyric-acid	67.8	24.8	1.8	<0.01	29.8	38.6	48.8	7.7	NS
7	4-Carboxyglutamate	98.6	51.6	7.7	<0.01	83.9	109.2	130.6	10.4	0.01
8	Acetaldehyde	206.5	174.7	17.2	NS	435.6	433.9	407.9	48.5	NS
9	Acetate	44.5	31.1	3.4	0.01	33.2	31.4	37.3	3.1	NS
10	Acetone	43.3	42.2	6.8	NS	46.6	47.5	47.1	5.1	NS
11	Alanine	613.3	331.2	133.0	0.01	549.8	370.2	656.7	50.5	<0.01
12	Alloisoleucine	112.6	63.8	6.5	<0.01	108.1	68.8	98.7	8.8	0.01
13	Anserine	40.2	26.7	2.6	<0.01	48.2	37.7	33.9	2.8	<0.01
14	Aspartate	376.6	200.7	15.8	<0.01	337.3	238.9	384.0	26.1	<0.01
15	Carnitine	65.2	28.4	4.9	<0.01	37.8	24.9	21.3	2.4	<0.01
16	Choline	114.2	43.8	20.1	0.02	80.5	36.1	83.5	7.9	<0.01
17	Creatine	47.4	29.2	4.7	<0.01	22.8	13.1	23.4	2.8	0.03
18	Creatinine	55.1	27.9	2.8	<0.01	40.4	17.9	30.6	5.4	0.04
19	D-Fructose	47777.4	1713.2	223.2	<0.01	1409.7	3469.9	4053.5	624.9	0.02
20	D-Glucuronic acid	103.6	51.2	3.9	<0.01	86.1	83.0	99.7	12.1	NS
21	D-Xylulose	675.2	1180.2	502.7	0.03	708.0	858.3	1377.7	106.1	<0.01

22	Dimethylsulfide	1148.1	452.6	98.5	<0.01	966.2	543.2	1033.7	69.4	<0.01
23	Ethanol	66219.7	74320.1	8009.3	NS	69264.6	85769.5	50676.6	3950.8	<0.01
24	Formate	34.2	29.3	2.7	NS	26.2	30.6	29.4	1.4	NS
25	Glucose	1353.1	311.2	253.1	0.01	406.4	480.4	400.8	117.2	NS
26	Glutamate	98.6	51.6	7.7	<0.01	342.1	259.2	302.0	23.9	NS
27	Glutamine	159.9	51.9	4.6	<0.01	71.1	65.2	70.9	9.2	NS
28	Glycerol	2006.4	1066.2	83.3	<0.01	1294.8	1088.1	1328.3	101.4	NS
29	Glycine	510.5	267.1	27.1	<0.01	401.8	333.0	449.6	26.8	0.02
30	Glycolate	1324.8	1434.5	74.2	NS	879.4	1276.8	1025.4	145.2	NS
31	Guadinoacetic acid	394.7	156.4	19.5	<0.01	232.7	207.1	267.3	14.9	0.04
32	Isobutyrate	22.7	12.9	4.4	NS	78.9	76.0	66.4	5.5	NS
33	Isocitrate	14.4	15.9	2.7	NS	16.1	15.5	16.5	0.8	NS
34	Isoleucine	308.4	122.5	14.6	<0.01	214.0	158.7	274.9	21.3	<0.01
35	Isopropanol	28791.9	32893.7	3288.5	NS	29070.6	37279.7	23569.0	2028.1	<0.01
36	L-Homocystine	1001.1	641.9	122.3	NS	1047.8	1173.3	1730.5	177.5	0.03
37	Lactate	264.9	140.5	19.5	<0.01	130.4	136.6	185.0	18.4	NS
38	Lactose	30.1	43.9	3.8	0.02	26.5	36.3	63.7	6.1	<0.01
39	Leucine	506.1	197.7	13.4	<0.01	274.7	204.0	326.2	20.5	<0.01
40	Malonate	466.7	427.9	37.6	NS	498.4	489.2	485.8	44.5	NS
41	Methanol	436.3	334.8	29.1	0.03	370.9	293.6	450.1	20.9	<0.01
42	Oxalacetic acid	1028.7	478.3	40.7	<0.01	900.6	581.6	1103.4	86.4	<0.01
43	Oxypurinol	31.7	14.8	3.1	<0.01	17.9	14.6	8.3	1.4	<0.01
44	Phenylalanine	205.6	58.3	9.3	<0.01	54.2	32.6	106.5	5.2	<0.01
45	Phosphorylcholine	470.6	192.7	34.5	<0.01	337.2	517.6	383.0	63.5	NS
46	Proline	285.7	126.8	30.1	<0.01	245.7	158.8	291.1	21.4	<0.01
47	Ribose	161.2	160.8	16.1	NS	142.7	194.9	149.5	15.1	NS
48	Taurine	265.5	168.4	25.2	0.02	156.8	113.9	166.9	16.5	NS
49	Theophylline	4.2	8.5	1.3	0.04	3.2	3.9	3.6	0.7	NS
50	Threonate	460.7	393.1	52.1	NS	350.5	375.2	411.0	14.8	0.03
51	Threonine	308.1	156.7	10.9	<0.01	238.6	174.9	255.4	19.1	0.02
52	Trimethylamine N-oxide	11.8	6.7	0.8	<0.01	11.2	6.8	17.3	1.4	<0.01
53	Tryptophan	48.5	23.1	2.5	<0.01	33.8	25.0	33.5	2.4	0.03
54	Tyrosine	210.4	81.1	8.3	<0.01	119.9	64.7	140.3	8.8	<0.01
55	Uracil	61.8	38.4	4.7	<0.01	69.2	51.2	79.1	9.8	NS
56	Valine	373.2	203.0	14.7	<0.01	300.3	209.7	425.7	26.4	<0.01
57	Xanthine	53.7	34.5	5.5	0.03	64.1	35.7	51.1	9.1	NS
58	Xylose	56.4	57.4	3.7	NS	44.0	72.9	59.8	6.3	0.02
59	myo-Inositol	201.3	139.0	14.8	0.01	181.5	162.7	215.2	20.9	NS

[a] Mean concentration in micromolar

[b] NS: no significant differences detected (*p-value > 0.05*).

Table 4.3. Concentrations (micromolar) of epithelial cell metabolites in two strains of laying hens as measured by NMR.

		Short-Term Trial				Long-Term Trial			
	Metabolites	**Brown strain**	**White strain**	**SEM**	***P-value***	**Brown strain**	**White strain**	**SEM**	***P-value***
1	1-Propanol	25.6	51.4	4.6	<0.01	27.8	26.7	2.5	NS
2	2-Aminobutyrate	83.7	199.9	14.5	<0.01	156.0	78.2	7.7	<0.01
3	2-Hydroxyisobutyrate	23.8	11.8	4.4	NS	39.0	18.8	1.9	<0.01
4	2-Oxoglutarate	12.3	36.7	4.3	<0.01	24.5	9.5	1.4	<0.01
5	2-Phosphoglycerate	192.1	199.9	34.6	NS	78.1	205.2	5.8	<0.01
6	4-Aminobutyric-acid	26.4	66.2	1.8	<0.01	45.1	33.1	6.1	NS
7	4-Carboxyglutamate	32.4	117.8	12.4	<0.01	134.3	81.4	8.3	<0.01
8	Acetaldehyde	144.1	237.2	16.1	<0.01	572.5	279.1	39.6	<0.01
9	Acetate	34.7	40.9	3.4	NS	38.6	29.4	2.5	0.02
10	Acetone	21.4	64.2	6.3	<0.01	64.5	29.6	4.2	<0.01
11	Alanine	239.5	705.2	76.8	<0.01	757.8	293.3	41.2	<0.01
12	Alloisoleucine	36.3	140.1	6.5	<0.01	128.8	54.9	7.2	<0.01
13	Anserine	21.7	45.2	2.6	<0.01	42.8	37.0	2.3	<0.01
14	Aspartate	144.9	432.4	15.8	<0.01	463.3	176.8	21.3	<0.01
15	Carnitine	13.1	80.7	4.9	<0.01	35.7	20.3	2.1	<0.01
16	Choline	46.9	111.1	20.1	0.03	93.8	39.6	6.5	<0.01
17	Creatine	23.6	52.9	4.6	<0.01	27.3	12.2	2.3	<0.01
18	Creatinine	30.4	52.6	2.8	<0.01	27.5	31.7	4.4	NS
19	D-Fructose	1549.9	4940.7	223.2	<0.01	4149.9	1805.5	510.3	<0.01
20	D-Glucuronic acid	68.7	86.1	3.2	<0.01	102.9	76.3	9.8	NS
21	D-Xylulose	1195.3	660.1	534.8	0.02	562.2	1400.4	86.5	<0.01
22	Dimethylsulfide	400.5	1200.2	98.5	<0.01	1226.7	468.6	56.7	<0.01
23	Ethanol	734955.6	67044.2	8009.3	NS	65810.3	71330.2	3225.8	NS
24	Formate	35.4	28.1	2.7	NS	27.8	29.6	1.2	NS
25	Glucose	170.9	1493.4	304.8	NS	648.1	210.3	93.3	<0.01
26	Glutamate	32.4	117.8	7.7	<0.01	423.0	179.2	19.5	<0.01
27	Glutamine	31.8	180.1	4.6	<0.01	95.9	42.3	7.5	<0.01
28	Glycerol	759.2	2313.4	83.3	<0.01	1599.8	874.3	82.8	<0.01
29	Glycine	264.5	513.1	27.1	<0.01	536.2	253.4	21.9	<0.01
30	Glycolate	962.8	1796.5	74.2	<0.01	880.6	1240.4	118.5	0.05
31	Guadinoacetic acid	131.9	419.1	19.5	<0.01	310.3	161.1	12.2	<0.01
32	Isobutyrate	37.8	100.7	11.6	<0.01	83.2	64.3	4.5	<0.01
33	Isocitrate	14.3	16.1	3.1	NS	16.8	15.3	0.6	NS
34	Isoleucine	86.1	344.9	14.5	<0.01	298.3	133.4	17.4	<0.01
35	Isopropanol	32369.5	29316.1	3288.5	NS	30341.1	29605.0	1561.2	NS

36	L-Homocystine	496.7	1146.4	122.3	<0.01	1671.7	962.7	144.9	<0.01
37	Lactate	103.6	301.8	19.5	<0.01	182.5	118.8	15.1	<0.01
38	Lactose	35.4	38.6	3.8	NS	28.6	55.7	4.9	<0.01
39	Leucine	160.9	542.8	11.2	<0.01	383.9	152.7	16.7	<0.01
40	Malonate	461.8	432.9	37.6	NS	469.8	512.5	36.3	NS
41	Methanol	427.4	343.8	29.1	NS	309.0	434.0	16.4	<0.01
42	Oxalacetic acid	379.3	1127.6	40.7	<0.01	1264.4	459.3	70.6	<0.01
43	Oxypurinol	11.6	35.0	3.1	<0.01	15.4	11.8	1.1	0.04
44	Phenylalanine	51.3	212.6	9.3	<0.01	76.4	52.5	3.9	<0.01
45	Phosphorylcholine	167.5	495.8	34.5	<0.01	601.2	223.9	51.8	<0.01
46	Proline	123.4	289.1	30.1	<0.01	303.9	159.9	17.5	<0.01
47	Ribose	150.9	171.0	16.1	NS	134.3	190.4	12.3	<0.01
48	Taurine	111.0	322.9	25.2	<0.01	207.1	84.7	13.5	<0.01
49	Theophylline	4.9	7.7	1.3	NS	2.1	4.9	0.5	<0.01
50	Threonate	383.9	469.9	52.1	NS	434.5	323.3	12.8	<0.01
51	Threonine	121.3	343.5	10.9	<0.01	323.5	122.4	15.6	<0.01
52	Trimethylamine N-oxide	6.4	12.1	0.8	<0.01	15.7	7.8	1.1	<0.01
53	Tryptophan	15.3	56.4	2.5	<0.01	41.0	20.6	1.9	<0.01
54	Tyrosine	78.9	212.6	8.3	<0.01	155.3	61.3	7.2	<0.01
55	Uracil	32.2	68.1	4.7	<0.01	87.7	45.3	7.8	<0.01
56	Valine	131.4	444.9	13.7	<0.01	439.5	184.3	21.6	<0.01
57	Xanthine	28.6	59.7	5.5	<0.01	67.7	32.9	7.8	<0.01
58	Xylose	81.4	32.4	3.9	<0.01	56.9	60.8	5.4	NS
59	myo-Inositol	119.8	220.5	14.8	<0.01	253.1	119.9	17.1	<0.01

Table 4.4. KEGG metabolic pathways significantly impacted hen epithelial cells by seaweed supplementation and genetic strain as revealed by Metabolic Pathway Analysis.

Treatment	Pathway	Hits [a]	*P*-value [b]
Short seaweed intake	Galactose metabolism	7/27	<0.001
	Starch and sucrose metabolism	2/18	0.004
	Glycolysis / Gluconeogenesis	2/26	0.007
	Neomycin, kanamycin and gentamicin biosynthesis	1/2	0.01
Long seaweed intake	Glycolysis / Gluconeogenesis	3/26	<0.001
	Alanine, aspartate and glutamate metabolism	2/28	0.01
	Glyoxylate and dicarboxylate metabolism	2/32	0.01
	Aminoacyl-tRNA biosynthesis	2/48	0.03
	Valine, leucine and isoleucine biosynthesis	1/8	0.04
Short-term strain effect	Galactose metabolism	3/27	<0.001
	Starch and sucrose metabolism	2/18	0.004
	Alanine, aspartate and glutamate metabolism	3/28	0.01
	Neomycin, kanamycin and gentamicin biosynthesis	1/2	0.01
Long term strain effect	Galactose metabolism	3/27	<0.001
	Starch and sucrose metabolism	2/18	0.003
	Neomycin, kanamycin and gentamicin biosynthesis	1/2	0.01
	Phosphonate and phosphinate metabolism	1/6	0.03

[a] Number of metabolites hit in the biological pathway/ total metabolites involved in each biological pathway.
[b] *p*-Value calculated from data.

Figure 4.1. Research design and experimental overview of two trials.

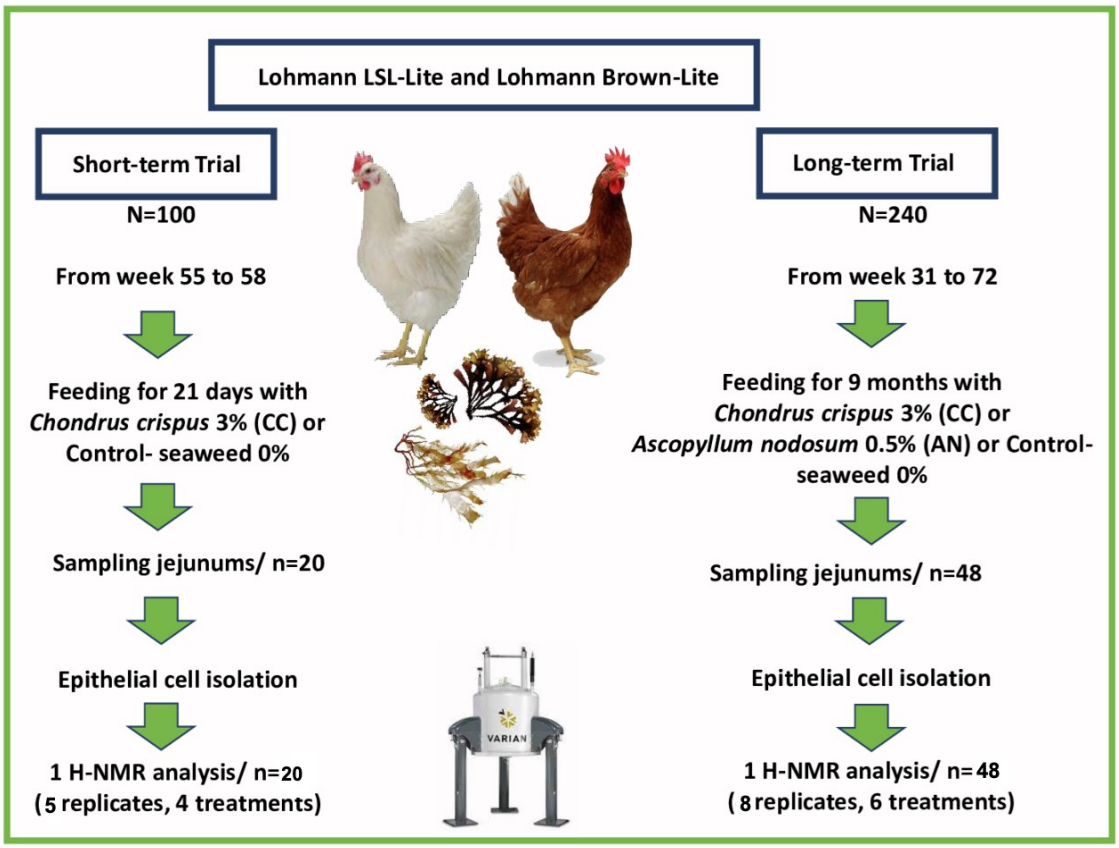


Figure 4.2. Western blotting detection method using pan anti-cytokeratin antibody **(A)** as the primary antibody and goat anti-mouse IgG HRP as the secondary antibody to compare protein expression between 10 µg of intestinal epithetical cells (EPC1-EPC6) and 10 µg of stroma cells (STC1 and STC2). Target protein (green arrow) was Cytokeratin with molecular weight of 46-58. **(B)** Rabbit polyclonal anti-vimentin was used as the primary antibody and biotinylated anti-rabbit IgG (secondary antibody) to compare protein expression between 10 µg of intestinal epithetical cells (EPC1-EPC6) and 10 µg of stroma cells (STC1 and STC2). Target protein was vimentin with molecular weight of 57.

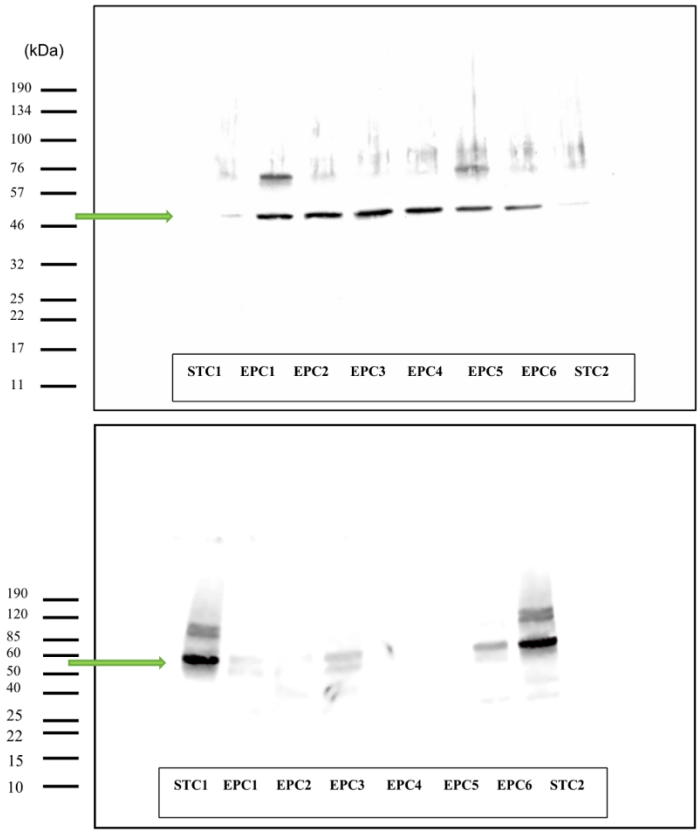


Figure 4.3. Multivariate analysis of the data to discriminate between metabolites of hen epithelial cells fed with 0% seaweed (control diet) and 3% *Chondrus crispus* (CC diet) in the short-term trial.

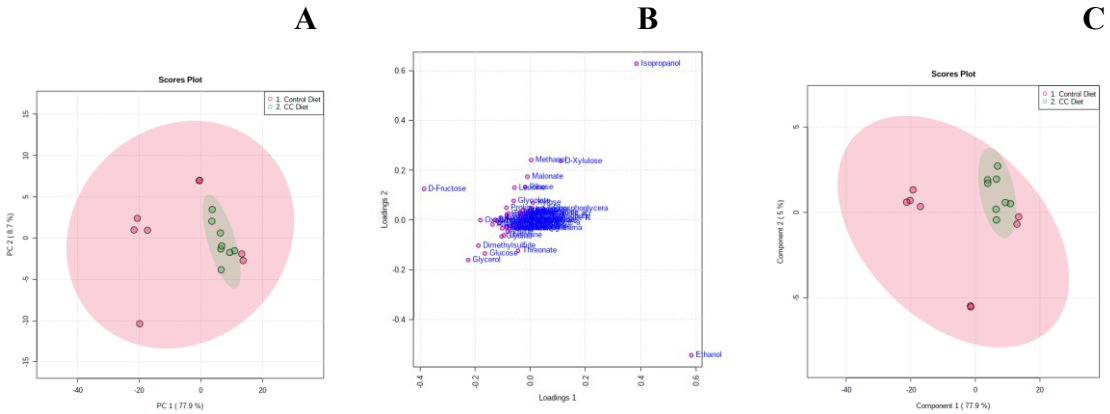


Figure 4.4. Multivariate analysis of the data to discriminate between metabolites of hen epithelial cells fed with 0% seaweed (control), 3% *Chondrus crispus* (CC) and 0.5% *Ascopyllum nodosum* (AN) in the long-term trial.

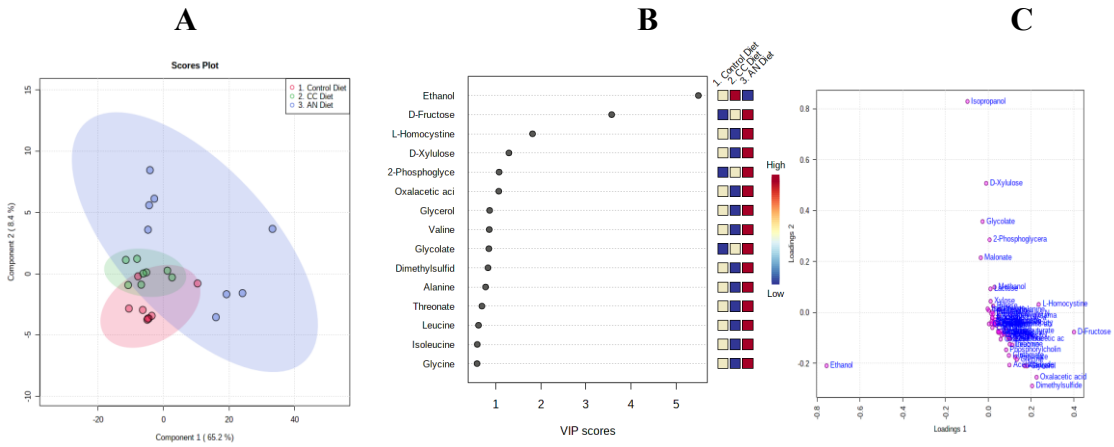


Figure 4.5. Multivariate analysis of the data to discriminate between epithelial cell metabolites of two strains, Lohmann LSL-Lite (White) and Lohmann Brown-Lite (Brown) in the short-term trial.

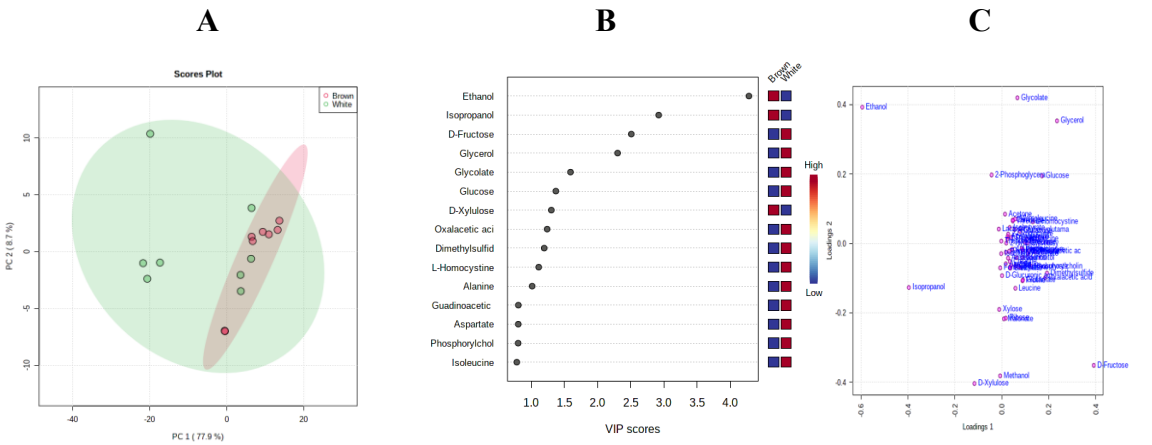


Figure 4.6. Multivariate analysis of the data to discriminate between metabolites of Lohmann LSL-Lite (White) and Lohmann Brown-Lite (Brown) in the long-term trial.

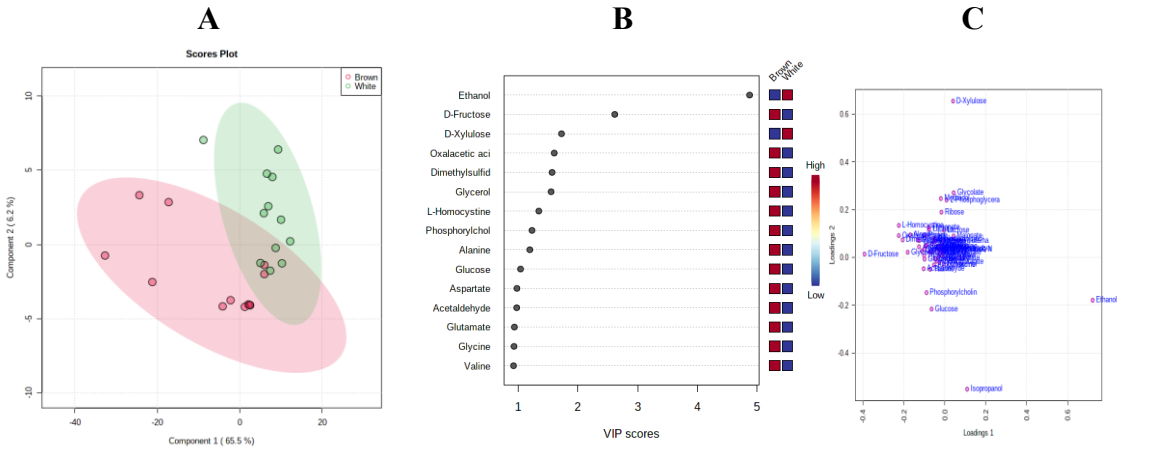


Figure 4.7. Summary of pathway enrichment analysis of the short-term seaweed supplementation (A) long-term seaweed supplementation (B), short-term strain effects (c), and long-term strain effects on epithelial cells. The *p*-value of potentially affected metabolic pathways are ranked from low (red) to high (white), corresponding to the most and the least significant values, respectively.

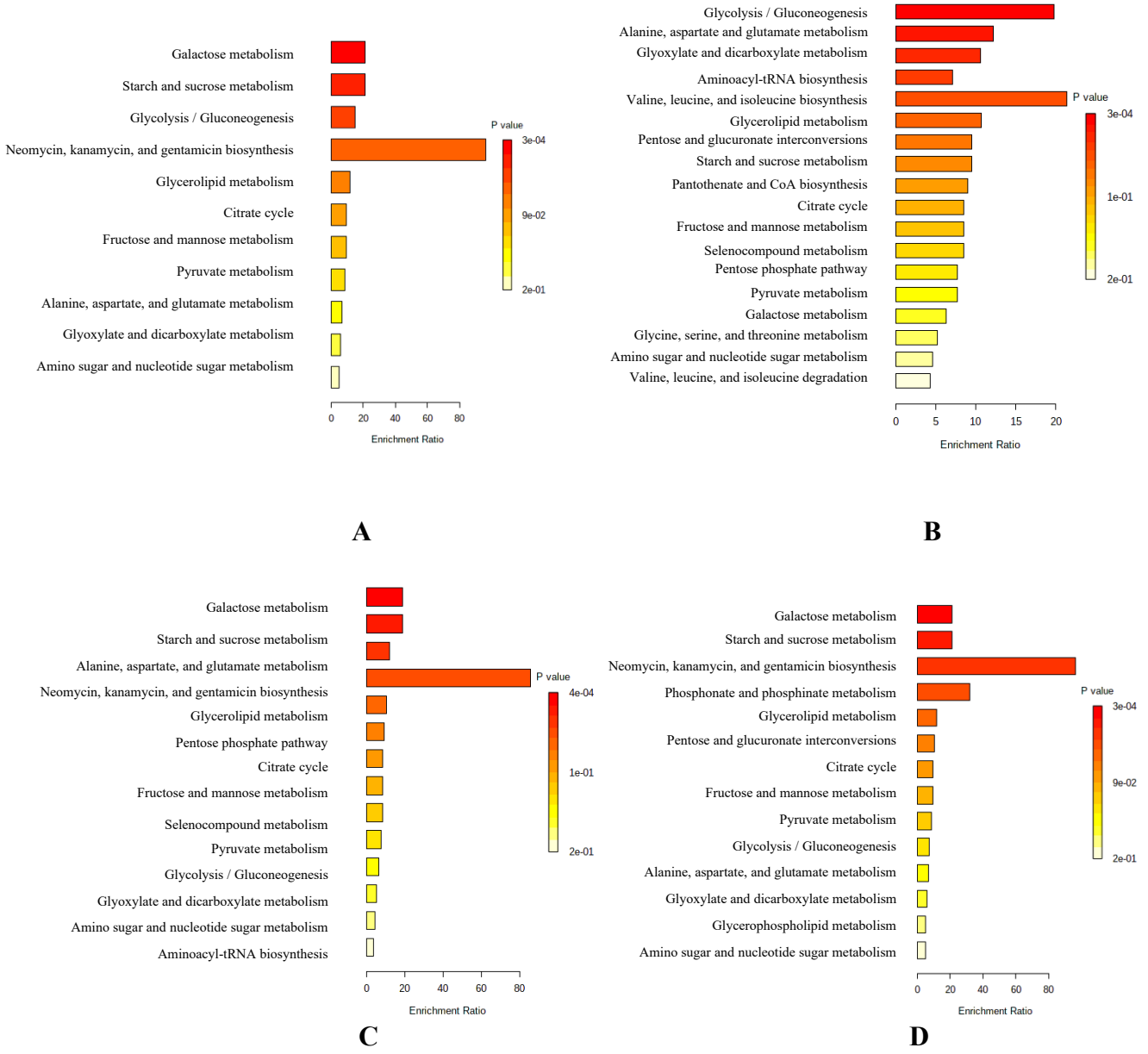
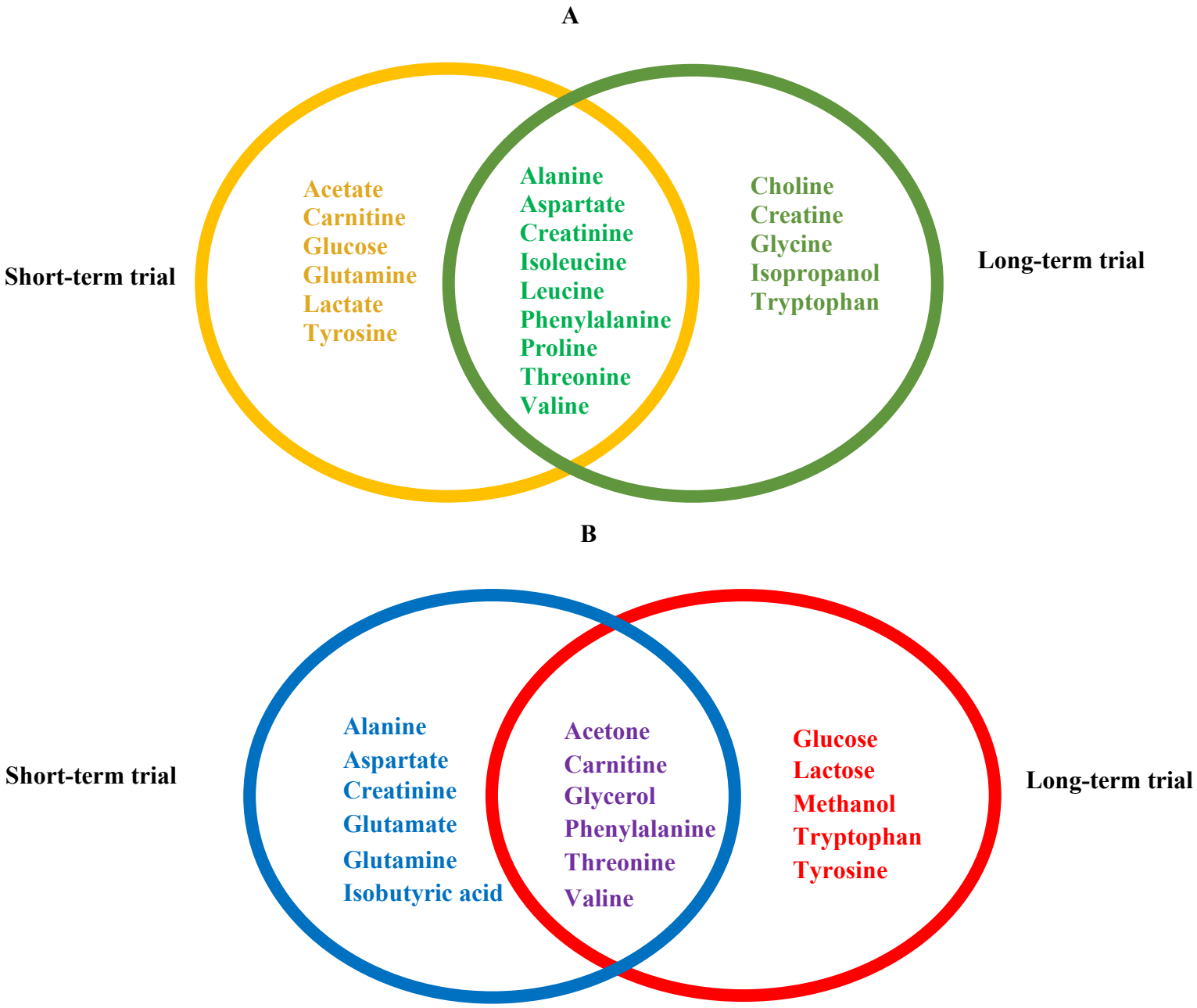


Figure 4.8. Overview of differentially expressed metabolites in plasma and intestinal epithelium. Panel A shows metabolites that were significantly affected by seaweed supplementation in both plasma and intestinal epithelium. Panel B shows metabolites that differed significantly between genetic strains in both plasma and intestinal epithelium. Short-term trial results are in left circle (yellow, blue), whereas long-term trial results are in right circle (green, red). Intersections show affected metabolites common to both short- and long-term trials.



CHAPTER 5. BROILER BLOOD METABOLOME RESPONSE TO IN-OVO AND ORAL PROBIOTIC ADMINISTRATION

5.1. Introduction

Interest in the use of probiotics as live microorganisms for poultry production is high as commitments to reduce antibiotic use have been undertaken for almost 8 decades (Kirchhelle, 2018). Probiotics have the potential to improve chicken immunity, morphology, production, host health and several other parameters. (Jin et al., 2000; Apajalahti et al., 2005; Turnbaugh et al., 2006; Wei et al., 2013; Afsharmanesh and Sadaghi, 2014; Zhang and Kim, 2014). Spore-forming *Bacillus* species, as one of the most widely used probiotic bacteria in commercially farmed poultry are resistant to physical stress, heat, chemical treatments, and any harsh conditions in the gastric environment (Hooge, 2003; Jha et al., 2020).

In the poultry industry, it is crucial to consider the route of probiotic delivery as an important determinant of the intervention, which may affect the efficacy and output of the probiotic treatment. There are several probiotic administration routes that exist, including in-ovo, feed, water, oral gavage, and litter. Probiotic provision in the form of feed additives is the simplest and most conventional method used in organic poultry production, but often involves large volumes of probiotic waste. Probiotic administration in drinking water is used to a limited extent due to the need for precision watering devices and the possibility of infection related to water quality (Olnood et al., 2015; Oladokun et al., 2021). The in-ovo probiotic administration technique can help to significantly reduce the difficulties associated with traditional methods and be more economical by using fewer probiotic products (Bednarczyk et al., 2016). The in-ovo technique is defined as the injection of

probiotic bacteria into developing chick embryos prior to hatching, which can result in superior long-term effects (Oladokun and Adewole, 2020). In the modern poultry production system, establishing gut microflora through in-ovo inoculation can be a key factor in chick health as chicks are susceptible to disease pathogens immediately post-hatch and prior to consuming the first feed.

Despite the significant advancements in applying metabolomics to chicken research in recent years, there is still a shortage of research and a noticeable lag in progress. The majority of studies primarily concentrate on investigating biomarker compounds associated with environmental exposures, nutrition, health, production, as well as their implications for chicken egg and meat products (Fotakis et al., 2017; Tomonaga et al., 2018; Zampiga et al., 2018; Hofmann et al., 2019; Brugaletta et al., 2020; Chen et al., 2021). However, the application of metabolomics to probiotic intervention in chickens and differentiating supplementation methods are still lagging, with few published studies on probiotic-mediated effects (Nothaft et al., 2017; Ito et al., 2022; Park et al., 2020).

The aim of this study was to evaluate the effects of the probiotic *Bacillus subtilis* delivery route on the plasma metabolome and metabolic pathways using quantitative NMR analysis.

5.2. Materials and Methods

5.2.1 Birds, diet, and experimental procedures

A total of 300 fertile eggs with an average weight of 64 ± 0.2 g were collected from a commercial Cobb 500 breeder flock in Nova Scotia, Canada. Eggs were assigned to six replicate trays that were incubated for 21 days in a ChickMaster single-stage incubator (ChickMaster G09, Cresskill, NJ, USA) at 37.5 °C and 55% relative humidity (75 eggs/per

tray). Eggs were candled individually on day 17 of incubation to discard the unfertilized eggs. On day 21 of incubation, unhatched eggs were opened to determine the reason for embryo mortality. The handling of the diets, injection of probiotics, and data collection have been previously reported in detail (Oladokun et al., 2021). Briefly, hatchlings were allocated to 24 cages (six replicate cages of four treatments) with seven birds per cage. Hatchlings were allocated into four treatment groups including the control, in-ovo probiotic, in-water probiotic, and in-feed probiotic groups. The control group consisted of 42 chicks fed a basal diet containing corn-soybean meal-wheat-based diet. The in-water probiotic group included 42 chicks that were fed the control diet plus *B. subtilis* fermentation extract at a concentration of 2.5 g/L of drinking water (containing 2.5 x 10^8 cfu of *B. subtilis*/L of drinking water); while 42 chicks as the in-feed probiotic group were fed a control diet plus 0.005% *B. subtilis* fermentation extract, premixed with preground corn (containing 5 x 10^8 cfu/kg of feed); The in-ovo probiotic treatment included 66 eggs that were injected with 200 µl of *B. subtilis* fermentation extract (each egg received 10 x 10^6 cfu of the bacterium) and 42 of these hatched chicks were fed with a basal diet. The egg injection protocol was borrowed from what was described by Tako et al., (2004) with some modifications. Briefly, eggs were disinfected by wiping with 70% ethanol and followed by making a small hole into the shell at the center of the air cell using an 18-gauge size tuberculin needle. Injections were performed manually using a self-refilling injector (Socorex ultra-1810.2.05005, Ecublens, Switzerland) equipped with a 22-gauge needle at a 45-degree angle into the amnion of each egg. The injection site was sealed with sterile paraffin wax immediately.

Basal diets (Table 5.1) and environmental conditions were experimentally monitored based on the Cobb Broiler Management Guide (Cobb-Vantress, 2020). Broilers were housed for 28 days in cages with dimensions of 0.93 m x 2.14 m with ad libitum access to food and water, all equipped with feed hoppers and water troughs. Birds were fed mash diets during the starter phase and pellets during the grower phase. The room temperature was gradually reduced from 31 ◦C on day 0 to 23 ◦C on day 28, in the relative humidity range of 45-55%. The Bacillus subtilis fermentation extract probiotic product utilized in this study was obtained from a commercial source (Probiotech International, St. Hyacinth, QC, Canada) at a concentration of 10 x 10^9 cfu/g. We prepared the probiotic solution for 100 eggs, by dissolving 0.1 g of the B. subtilis product into 20 ml of 0.9% saline.

Animal experiments were carried out according to the guidelines of the Canadian Council of Animal Care in Science (CCAC, 2009) at the Dalhousie University Faculty of Agriculture Animal Care & Use Committee (Protocol number 2018-031; approved 31.5.2018; Protocol number 2016-054; approved 1.9.2016).

5.2.2 Sampling and preparation

At the end of the trial on day 28, one bird from each cage was randomly selected (4 treatments with 6 replicates), and euthanized via electrical stunning, and blood samples were collected for analysis in vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ, USA) containing sodium heparin. The plasma was separated by centrifugation of 2 mL of blood sample at 2000× g for 10 minutes at 4 ◦C. Samples were then subjected to deproteinization using ultrafiltration to remove plasma proteins (Amicon centrifugal filter devices 10 kDa cut-off, Millipore, Billerica, USA), followed by quenching in liquid

nitrogen and storing at -80 ◦C until analysis. Then 570 µL of plasma samples were mixed with 50 µL of a standard buffer solution (54% D2O:46% 1.75 mM KH2PO4 pH 7.0 v/v containing 5.84 mM DSS (2,2-dimethyl-2-silcepentane-5-sulphonate), and 0.1% NaN3 in H2O) (Sigma-Aldrich, Mississauga, ON). Samples were immediately transferred to NMR tubes (Wilmad, Buena, NJ, USA) for subsequent NMR spectral analysis.

5.2.3 NMR Spectroscopy

Chicken plasma samples were analyzed using nuclear magnetic resonance (NMR) spectroscopy.

A total sample volume of 250 µL was transferred to 5mm SampleJet NMR tube for spectral analysis, using a 700 MHz Avance III (Bruker, USA) spectrometer at 25 °C. The spectrometer was equipped with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) cryoprobe using the first transient of the NOESY pre-saturation pulse sequence (noesy1dpr). All free induction decays were zero-filled to 250 K data points. The internal standard used for quantification was the singlet peak produced by sodium trimethylsilylpropanesulfonate (DSS) methyl for chemical shift reference and set to 0.00 ppm. all 1H-NMR spectra were then processed, quantified, and analyzed using a modified version of the Bayesil automated analysis software package (Ravanbakhsh et al., 2015).

5.2.4 Statistical analyses

The SAS program version 9.4 was used to perform an analysis of variance (ANOVA) using Tukey's method for multiple comparisons with the general linear model (GLM) approach to determine mean differences prior to testing the normality of residuals (using the

Shapiro-Wilk test; $p < 0.05$). Normalization, filtering, and Pareto scaling transformation (using the square root of the standard deviation of the data) were performed prior to analyses, using standard methods available via MetaboAnalyst 5.0 (Chong et al., 2019). Multivariate statistical analyses were then performed according to previously published MetaboAnalyst protocols to analyze quantitative metabolomic data using principal components (PCA) and partial least-squares discriminant analyses (PLS-DA) with statistical significance set to $P < 0.05$ (Xia et al., 2009; Xia and Wishart, 2016). PCA is a non-supervised pattern recognition method to obtain a preliminary overview of the data. The PLS-DA performs supervised data reduction and classification to rank the importance of each variable and identify potential biomarkers using the variable importance in the projection (VIP). Metabolites with VIP values of more than one can be distinguished as important for optimal PLS-DA model performance and considered the potential candidate biomarkers for discriminatory significance. The PLS-DA models were validated using cross-validation and the *P*-value, obtained from permutation testing. The multivariate statistical analysis was complemented by pairwise analysis between the three treatments and the control group to screen for potential metabolic differences between each probiotic group and the control. To interpret the metabolomic data, multiple metabolites were selected following the statistically significant ANOVA test ($P < 0.05$) and significant permutation test ($n = 1{,}000$; $P < 0.05$). Selected metabolites as the potential biomarkers were then imported to MetaboAnalyst 5.0 for Enrichment Analysis and Pathway Analysis using KEGG pathway libraries targeting the *Gallus gallus* database to explore the top correlated metabolic pathways impacted by probiotic treatments. Statistical significance

pathways were defined by a *p*-value less than 0.05 and the pathway impact of more than 0.1 for those KEGG pathways that hit two or more metabolites in each pathway.

5.3. Results

5.3.1 Probiotic Impacts on Broiler Plasma Metabolome

A total of 52 metabolites were detected and quantified from the plasma of broilers. Probiotic supplementation significantly ($P < 0.05$) affected 17 plasma metabolites (Table 5.2). These metabolites could be classified into their chemical classes using the ClassyFire ontology that allows for compounds to be grouped by structure (Djoumbou Feunang et al., 2016). These chemical classes included 10 amino acids and derivatives and one metabolite from each of the following classes: alpha-hydroxy acids and derivatives; alpha-keto acids and derivatives; carboxylic acids; carnitines; hypoxanthines; as well as indolyl carboxylic acids and derivatives. Of the 17 changed metabolites, nine metabolites were upregulated in all three types of probiotic groups compared to the control group. These upregulated metabolites included acetic acid, glycine, hypoxanthine, proline, lysine, pyruvic acid, methionine, valine, and tryptophan. Betaine levels decreased responsively among all probiotic treatment groups. The majority of metabolites were expressed at the highest levels after in-ovo probiotic supplementation (carnitine, glycine, hypoxanthine, proline, lysine, lactic acid, pyruvic acid, and valine), while the minority of concentrated metabolites were found among the in-feed probiotic supplemented group. Threonine was the only metabolite with increased levels after in-feed probiotic supplementation. The in-water probiotic treatment increased the levels of six compounds including acetic acid, proline, aspartate, methionine, tryptophan, and propylene glycol. There was a reduction in the

aspartate levels after in-water probiotic intake. Based on our data, the metabolite concentration ranges detected in our NMR analysis of broiler plasma varied from 1.3 μM (2-hydroxyisovalerate in response to in-feed probiotic) to 848.3 μM (acetic acid due to in-water probiotic treatment). Amino acids were the main metabolite classes that were measured by NMR in the plasma of broilers supplemented with probiotics. Among the short-chain fatty acids, acetic acid and isobutyric acid were detected in the plasma of each group, but only acetic acid was significantly ($P < 0.05$) increased in the three groups.

5.3.2 Univariate and Multivariate Analyses of Treatment Effects

Analysis of variance identified 17 metabolites which were significantly different between the three treatment groups (Table 5.2). The results of Tukey's multiple comparisons test is summarized in Table 5.2.

The PCA score plot of probiotic supplementation showed that the three types of probiotic supplementation were not well separated from one another, demonstrating no distinctive clustering between the probiotic groups and the control (data not shown here). Similarly, PCA pairwise analysis revealed no separation of probiotic groups, with no clear clustering linked to the treatments (Fig. 5.1A, 5.1B, and 5.1C). However, the PLS-DA score plots of groupwise showed better discrimination by maximizing the separation between classes and minimizing the distance between intragroup clustering by covering 64.9 % of the observed variance in the sample set (data not shown). The pairwise PLS-DA models could successfully discriminate each probiotic group from the control group (Fig. 5.1D, 5.1E, and 5.1F). The successful permutation tests showed that the models were reliable with no overfitting ($p < 0.05$) and the results of the cross-validation were acceptable (accuracy, R2,

and Q2). The VIP scores obtained for the PLS-DA models are shown in Figure 5.2, which ranked the metabolites based on their importance in discriminating probiotic groups from the control group. The plot highlighted the top 15 metabolic changes with the highest VIP score (VIP score ≥ 1) that contributed significantly to the categorization of the probiotic groups and accounted for groupwise separation (Fig 5.2D). As such, the most powerful group discriminators included glucose, lactic acid, proline, betaine, methanol, citric acid, acetic acid, serine, threonine, pyruvic acid, valine, aspartate, arginine, glycerol, and methionine. The heat map on the right side of the plot indicated the respective increased or decreased concentrations of the metabolites (Fig. 5.2). As shown in Figure 5.2, ten metabolites were more abundant in the in-water probiotic supplemented group, while three were more abundant in the in-feed probiotic group (threonine, pyruvic acid, and valine). There was only one metabolite that was increased relative to the in-ovo probiotic intake (lactic acid), and one that was increased in the control group (glucose). Ten metabolites, including methionine, aspartate, valine, pyruvic acid, threonine, acetic acid, betaine, proline, and lactic acid, overlapped with the metabolites identified as significantly different between the three probiotic groups according to our univariate analysis ($p < 0.05$). These metabolites were selected as potential discriminatory compounds for broiler plasma samples supplemented with our probiotic treatments. These results were complemented by pairwise analysis to screen for potential discriminatory metabolites in each specific treatment. The VIP score of the key metabolites and their relative levels that were downregulated and upregulated by probiotic intake were shown in Figures 5.2A, 5.2B, and 6.2C. In the in-ovo, in-water, and in-feed probiotic supplemented groups, 14, 12, and 13 group-discriminating metabolites were detected, respectively. The Venn diagrams in

Figure 5.3 is showing the pairwise metabolites overlap between the three groups. The results indicated that there were three overlapping significantly differential metabolites between the three groups, including citric acid, glucose, and proline. Five metabolites including acetic acid, choline, lactic acid, lysine, and methionine were overlapped between the in-ovo and in-water probiotic supplementation groups, while there were four overlapped differential metabolites including 2-hydroxybutyrate, betaine, pyruvic acid, and valine between the in-ovo and in-feed probiotic supplementation groups. Serine was the only overlapped metabolite between in-water and in-feed probiotic supplemented birds.

5.3.3 Pathway Analysis

We performed Enrichment and Pathway Analyses using the chicken (*Gallus gallus*) library from the KEGG database of the metabolites identified as affected by our treatments. The key significantly differential metabolites with a *p*-value less than 0.05 and VIP score higher than one were imported to the server for groupwise and pairwise analysis (Table 5.3). Figure 5.4 summarizes the differentiating metabolic pathways along with the fold enrichment and *P*- values for individual and groupwise treatments. We identified 9, 3, and 4 metabolic pathways that distinguished in the in-ovo, in-water, and in-feed probiotic supplementation groups, respectively. The in-ovo probiotic treatment with the highest metabolic impact was involved in nine metabolic pathways including, aminoacyl-tRNA biosynthesis; glyoxylate and dicarboxylate metabolism; pyruvate metabolism; glycolysis/gluconeogenesis; cysteine and methionine metabolism; glycine, serine, and threonine metabolism; arginine and proline metabolism; valine, leucine, and isoleucine biosynthesis; and biotin metabolism (Table 5.3). Aminoacyl-tRNA biosynthesis was

overlapped between in-water and in-feed probiotic treatments. The in-water probiotic delivery was associated with biotin metabolism and pyruvate metabolism, while the expression of glycine, serine, and threonine metabolism; arginine and proline metabolism; as well as valine, leucine and isoleucine biosynthesis pathway were observed in the in-feed probiotic treatment.

5.4. Discussion

In this work, we studied the effects of microbiome modulation on chicken metabolites and metabolic pathways and compared the metabolome of probiotic *B. subtilis* delivery routes (in-ovo, in-water, and in-feed). Furthermore, we predicted chicken response to probiotic supplementation by finding the association between the detected pathways here and known literature reported responses to health, performance, and production.

In total, we identified and quantified 51 plasma metabolites in broilers supplemented with probiotic intake. A previous study by our collaborator Oladokun et al., (2021), on the same trial reported the enhancement of intestinal morphology by in-ovo probiotic supplementation. Interestingly, we observed that the in-ovo delivery of the probiotic product by changing the embryonic milieu could affect more plasma metabolic pathways than in-feed and in-water treatments. This might be associated with improved morphology of the jejunum in response to in-ovo delivery of B. subtilis, suggesting that early inoculation could enhance the intestinal absorptive function and increase the expression levels of more metabolites and metabolic pathways.

Metabolic pathways are the key functional units in living organisms. Glycolysis is the energy supplying pathway that is activated to compensate for a lack of ATP by converting

glucose into pyruvate and pyruvate into lactate (Suissa et al., 2021). During glycolysis, 2 ATP molecules are used to generate 4 ATP, 2 NADH, and 2 molecules of pyruvate per glucose molecule (Chaudhry and Varacallo, 2022). Our results illustrated the fine relationship between probiotic supplementation and glycolysis metabolism in broilers. As shown in Table 5.2, higher levels of glucose, pyruvate, lactate, acetate, and ethanol, the key metabolites in the glycolysis pathway, were detected in the plasma of broilers supplemented with in-ovo probiotics. These quantitative results demonstrate that the pathway might be upregulated in response to probiotic supplementation. This interpretation was further supported by the KEGG pathway analysis data, which indicated the glycolysis pathway activity in response to in-ovo probiotic supplementation. Likewise, the enhancement of the glycolysis pathway and expression of glycolysis enzymes were observed in sheep supplemented with the probiotic *Bacillus amyloliquefaciens* (Schofield, 2017). Similarly in a separate study, probiotic exposure to *Lactobacillus paracasei* and *Lactobacillus rhamnosus* stimulated the glycolysis pathway in mice (Martin et al., 2008). In recent years, more studies have reported that probiotic supplements could impact the glycolysis metabolism using whole-genome sequencing and amino acid sequencing of the proteins encoded by the glycolysis genes (Francois-Pierre et al., 2008; Brandt and Barrangou, 2016; Wu et al., 2020; Illina et al., 2022). These results might imply that gut bacteria and probiotics may utilize the glycolytic pathway for the catabolism of carbohydrates to produce energy in the form of ATP.

Moreover, it has been reported that the glycolysis pathway continues producing glycogen after slaughter, so the pH would not significantly change, which can improve chicken meat quality by altering the tenderness and color (Suryadi et al., 2019). Bai et al., (2016) also

reported that the dietary probiotic *Bacillus subtilis* fmbj in broiler diets could improve meat quality by protecting the breast meat against storage-induced oxidative stress and suppressing oxidative damage. The present data suggest that probiotic intake, particularly in-ovo probiotic treatment, could improve chicken meat quality by alleviating the pH decline through the expression of the glycolysis pathway.

We discovered that levels of lactate, the main gluconeogenic precursor (Gerich et al., 2001), were significantly higher in the in-ovo probiotic treatment in broilers, implying that the gluconeogenesis pathway might be responding to the in-ovo probiotic supplemented birds. Similarly, the transcriptomic analysis of the probiotic *Lacticaseibacillus casei* revealed that the gluconeogenesis metabolism was promoted in soybean meal-wheat bran medium (Wu et al., 2020).

The pyruvate metabolism pathway in the chicken liver (Kumar et al., 2019) plays a critical role in converting pyruvate, which is formed during glycolysis, to lactate and synthesizing glucose from lactate. Our results here showed that the metabolites including pyruvate, acetate, and lactate associated with pyruvate metabolism in response to probiotic intake in broilers were differentially expressed (Tables 5.2 and 5.3). The pyruvate metabolism pathway was upregulated in the in-ovo and in-water probiotic supplementation groups, and the participated metabolites were enhanced in both groups. Similarly, a metagenomic approach revealed that pyruvate metabolism was enriched in blood samples of 6-week rats supplemented with the probiotic *Lactobacillus plantarum* for 28 days (Yuyu et al., 2017). Likewise, the transcriptomic analysis of *Lacticaseibacillus casei* produced from soybean meal-wheat bran fermentation indicated that pyruvate metabolism was widely up-regulated to generate lactate, acetate, ethanol, and succinate (Pengyu et al., 2020). In addition, a

previous study on broiler chickens fed with the dietary probiotic *Enterococcus faecium* determined that differentially expressed proteins in pectoral muscles were involved in pyruvate metabolism with a central role in meat quality (Zheng et al., 2015). As previously shown by Tang and colleagues (2021), the meat quality and carcass traits of broilers were improved in response to the intake of the probiotic strain *B. subtilis* (Tang et al., 2021). Thus, the present study suggests that the upregulation of the pyruvate metabolism pathway might be linked to the meat quality of broilers supplemented with *B. subtilis,* particularly in the in-ovo and in-water probiotic supplemented birds.

The aminoacyl-tRNA synthesis pathway plays a key role in protein biosynthesis and amino acid signaling pathways by supplying substrates for the ribosomal translation of messenger RNA during protein synthesis (Yu et al., 2021). The pathway is involved in protein biosynthesis translation by pairing mRNA codons with aminoacyl-tRNA on the ribosome (Corti et al., 2003). In the present work, the aminoacyl-tRNA synthesis pathway was highly expressed in all three types of probiotic supplements. We observed that this pathway was composed of seven interactive metabolites that were upregulated in probiotic supplemented birds, including glycine, phenylalanine, proline, threonine, methionine, valine, and tryptophan. These amino acid levels were all significantly elevated in all probiotic supplemented groups. Moreover, our results here demonstrated that the aminoacyl-tRNA biosynthesis pathway was significantly upregulated in all broiler groups. These findings suggest that probiotic intake may affect the translation process by influencing the tRNA function of the above-mentioned amino acids. Similarly, aminoacyl-tRNA biosynthesis upregulation was observed by gene sequencing in young adult mice, supplemented for 12 weeks with the probiotic *Lactobacillus casei* Shirota (Chen et al., 2021). The impact of

probiotic intake on aminoacyl-tRNA synthesis and amino acid metabolism was also identified in a study on the plasma of male piglets supplemented with probiotic yeast *Saccharomyces cerevisiae* boulardii (Alassane-Kpembi et al., 2020). Likewise, the metabolomic profiles of yogurt by Wang et al., (2021) showed that this pathway was significantly upregulated due to the presence of probiotic bacteria including *Lactobacillus casei* and *Bifidobacterium animalis*. Thus, we could speculate that the upregulated metabolites that were involved in the aminoacyl-tRNA biosynthesis pathway, might be associated with the probiotic supplementation of broilers.

Our quantitative NMR data showed that 18 amino acids were detected in plasma broiler samples (Table 5.2). Among these amino acids, glycine, phenylalanine, proline, threonine, lysine, aspartate, methionine, valine, and tryptophan were significantly impacted by probiotic supplementation. As can be seen in Table 5.2, probiotic supplementation resulted in the upregulation of all the noted amino acids except threonine and aspartate in the in-ovo probiotic treatment and tryptophan in the in-feed probiotic treatments. Our KEGG metabolic pathway analysis showed a similar expressional pattern for amino acid metabolism pathways as glycine, serine, and threonine metabolism; arginine and proline metabolism; and valine, leucine, and isoleucine biosynthesis were expressed in both the in-ovo and in-feed probiotic supplemented groups, while cysteine and methionine metabolism was only expressed in the in-ovo group (Figure 5.4). The current data suggests that in-ovo and in-feed probiotic supplementations may contribute to protein synthesis and energy metabolism. The results indicated no protein pathway expression in the in-feed group. This might be attributed to the fact that in-ovo and in-water techniques could provide more sufficient nutrients than in-feed for chickens (Das et al., 2021).

The enhanced protein synthesis might be associated with microorganisms as Suryadi et al., (2019) reported that a local microorganism-based probiotic supplement containing *Lactobacillus spp*., *Bifidobacterium spp*., *Streptococcus spp*., and *Bacillus spp.*, resulted in an increase in the chicken meat protein content. In another study, upregulation of amino acid metabolism was observed in broilers fed with the probiotic *Bacillus amyloliquefaciens* (Cao et al., 2018). Similarly, Liu et al., (2022) reported upregulated amino acids in the plasma and stool of newborn mice in response to feeding with the probiotic *Lactobacillus reuteri.* Several studies exhibited the increased capacity of protein synthesis in response to probiotic supplementation (Rhoads and Wu, 2009; Harding et al., 2008; Tian et al., 2021). The possible reason for this enhancement might be attributed to supplying a carbon and energy source for microorganisms to satisfy the increasing energy demand of the gut bacteria. It also might be possible that the enhancement of amino acid metabolism could accelerate the metabolism of nitrogenous substances in poultry manure composting, particularly ammonia synthesis, supplying nitrogen for microbial growth and production (Li et al., 2021).

The valine, leucine, and isoleucine biosynthesis pathway play an essential function in the breast muscle development of chickens and serve as important precursors for the synthesis of low-molecular-weight substances, hormones, and hormone-like substances (Wu. 2009; Shi et al., 2022). Leucine is not only an inhibitor of protein degradation, but also stimulates muscle protein synthesis and regulates multiple cell-signaling pathways of protein formation (Wu, 2009; Shi et al., 2022). Previous studies have reported that chicken muscle growth consumed more branched-chain amino acids, particularly valine and leucine (Shi et al., 2022; Tomonaga 2022). In the present study, we observed the upregulation of

leucine, isoleucine, and valine in response to the in-ovo and in-feed probiotic intake but not in the in-water probiotic treatment. These results suggest that in-ovo and in-feed probiotic supplementation might be positively associated with meat quality, particularly breast muscle development and growth in broilers.

Proline and arginine as energy substrates in animals and bacteria are used by mitochondria to fuel energy metabolism (Du et al., 2021). In the present work, the differentially expressed metabolites, including pyruvate, arginine, and proline, that were involved in the arginine and proline metabolism pathway were also upregulated by all the probiotic treatments that might be further used by probiotic bacteria as the energy substrates. The KEGG analysis illustrated the upregulation of the arginine and proline metabolism pathway only in in-feed probiotic supplemented birds (Table 5.3). Raza et al., (2020) showed that the gut microbiota of *Bactrocera dorsalis* promoted the arginine and proline metabolism pathway that was along with host resistance to low-temperature stress by influencing mitochondrial functionality. Thus, it might be suggested that in the presented study, in-feed probiotic supplemented birds have better host resistance to low-temperature stress although further validation is required to confirm these findings.

The pathway and set enrichment analyses showed that the glycine, serine, and threonine metabolism pathway was enriched in the in-ovo and in-feed probiotic treatments. The results indicated that glycine, betaine, threonine, and pyruvate metabolites were involved in the pathway. Except for threonine, other metabolite levels were significantly increased in response to probiotic supplementation. There were more studies reporting the expression of glycine, serine, and threonine metabolism pathway in blood and fecal samples of probiotic supplemented pigs (Yang et al., 2017; Cui et al., 2020). Yang et al., (2017)

showed that glycine, serine, and threonine metabolism was positively associated with porcine feed efficiency. These results suggest that there might be a potential association between glycine, serine, and threonine metabolism pathway and feed efficiency in broilers, but the confirmation is beyond the scope of this study and will require further verification with larger trial with larger sample size.

5.5. Conclusions

In this study, the quantitative NMR analysis demonstrated significant differences in metabolic profile of broilers plasma supplemented with various delivery routes of *B. subtilis* probiotic strain. The differentially expressed metabolites involved in each probiotic treatment were successfully linked to the significantly impacted metabolic pathways, concluded that the biochemical pathways could be modulated by the delivery route. Taken together, these results can establish a foundation for further investigation of potential molecular markers involved in chicken meat quality traits such as the taste, tenderness, odor, color, and overall acceptability. While these initial results are promising, further validation using a larger sample size and a greater diversity of probiotic supplements is required to confirm the association between the altered metabolic pathways and broiler chicken traits.

Table 5.1. Diet formulation (g/kg) and calculated composition (as fed basis) of broiler phases.

	Starter		Grower	
	Control Diet	**Probiotic Diet**	**Control Diet**	**Probiotic Diet**
Corn	51.08	51.08	44.32	44.31
Soybean Meal-46.5	41.44	41.44	36.48	36.48
Animal/Vegetable Fat	2.93	2.93	4.59	4.60
Wheat	-	-	10.00	10.00
Limestone	1.80	1.80	1.65	1.65
Dicalcium Phosphate	1.24	1.24	1.06	1.06
DL-Methionine premix[1]	0.59	0.59	0.53	0.53
Lysine HCl	0.01	0.01	0.00	0.00
Vitamin/Mineral Premix[2]	0.50	0.50	0.50	0.50
Salt	0.41	0.41	0.37	0.37
Pellet Binding Agent[3]	-	-	0.50	0.50
BMD 110G[4]	-	-	-	-
Bacillus subtilis	-	0.005	-	0.005
Total	100	100	100	100
Calculated Composition				
ME (Kcal/kg)	3,000	3,000	3,100	3,100
CP	23.00	23.00	21.50	21.50
Calcium	0.96	0.96	0.87	0.87
Available Phosphorus	0.48	0.48	0.44	0.44
Sodium	0.19	0.19	0.18	0.18
Digestible Lysine	1.28	1.28	1.16	1.16
Digestible Methionine + Cysteine	0.95	0.95	0.87	0.87
Analyzed Composition				
DM	89.23	90.85	87.10	86.83
CP	22.77	24.16	21.72	21.87
Crude Fat	5.06	5.17	6.77	6.35
Calcium	1.13	1.04	0.89	0.89
Total Phosphorus	0.65	0.62	0.55	0.57
Sodium (%)	0.19	0.33	0.15	0.21

[1]Supplied/kg premix: DL-Methionine, 0.5 kg; wheat middlings, 0.5 kg.
[2]Starter vitamin–mineral premix contained the following per kg of diet: 9,750 IU vitamin A; 2,000 IU vitamin D3; 25 IU vitamin E; 2.97-mg vitamin K; 7.6-mg riboflavin; 13.5-mg Dl Ca-pantothenate; 0.012-mg vitamin B12; 29.7-mg niacin; 1.0-mg folic acid, 801-mg choline; 0.3-mg biotin; 4.9-mg pyridoxine; 2.9-mg thiamine; 70.2-mg manganese; 80.0-mg zinc; 25-mg copper; 0.15-mg selenium; 50-mg ethoxyquin; 1543-mgwheat middlings; 500-mg ground limestone. Grower vitamin–mineral premix contained the following per kg of diet: 9,750 IU vitamin A; 2,000 IU vitamin D3; 25 IU vitamin E; 2.97-mg vitamin K; 7.6-mg riboflavin; 13.5-mg Dl Ca-pantothenate; 0.012-mg vitamin B12; 29.7-mg niacin; 1.0-mg folic acid, 801-mg choline; 0.3-mg biotin; 4.9-mg pyridoxine; 2.9-mg thiamine; 70.2-mg manganese; 80.0-mg zinc; 25-mg copper; 0.15-mg selenium; 50-mg ethoxyquin; 1,543-mg wheat middlings; 500-mg ground limestone.
[3]Pel-stik: Uniscope, Inc., Johnstown, CO.
[4]Bacitracin methylene disalicylate (providing 55 mg/kg mixed feed); Alpharma, Inc., Fort Lee, NJ.

Table 5.2. Concentrations (micromolar) of broiler plasma metabolites impacted by *Bacillus subtilis* fermentation extract delivery route, as measured by NMR.

	Metabolites	Control	In-ovo probiotic	In-water probiotic	In-feed probiotic	SEM	*P*-value
1	2-Hydroxybutyrate	26.4	23.8	25.6	19.2	3.04	NS
2	Acetic acid	59.3^{b}	212.6^{b}	848.3^{b}	63.7^{b}	83.7	<0.0001
3	Betaine	678.3^{a}	642.9^{ab}	643.8^{ab}	347.2^{b}	76.7	0.02
4	Acetoacetate	10.2	9.6	8.1	10.4	1.4	NS
5	L-Carnitine	5.6^{ab}	9.6^{a}	4.6^{b}	5.2^{b}	1.1	0.02
6	Creatine	68.8	71.5	57.1	62.1	8.4	NS
7	Dimethylglycine	154.4	148.9	134.6	99.1	15.8	NS
8	Citric acid	359.2	721.7	813.7	480.8	151.3	NS
9	Choline	27.2	206.5	325.3	52.9	91.4	NS
10	Ethanol	6.4	8.3	22.1	8.1	4.8	NS
11	D-Glucose	18334.8	19296.8	18306.8	15566.1	924.6	NS
12	Glycine	618.6^{b}	845.8^{a}	703.9^{ab}	750.9^{ab}	48.6	0.03
13	Glycerol	136.2	203.9	391.7	163.1	9.0	NS
14	Formate	90.7	93.1	98.3	83.1	6.8	NS
15	L-Glutamic acid	289.1	303.3	249.7	210.2	34.3	NS
16	Hypoxanthine	12.4^{b}	23.3^{a}	15.1^{ab}	12.4^{b}	2.1	0.02
17	Tyrosine	344.8	334.7	314.5	307.2	28.4	NS
18	L-Phenylalanine	174.6^{ab}	207.3^{a}	171.1^{b}	165.5^{b}	28.7	0.01
19	L-Alanine	1179.5	1404.6	1283.7	1082.6	106.9	NS
20	L-Proline	531.9^{b}	746.2^{a}	788.9^{a}	715.4^{a}	42.3	0.002
21	L-Threonine	618.3^{ab}	610.7^{ab}	438.4^{b}	708.2^{a}	63.2	0.04
22	L-Asparagine	219.3	280.1	182.9	191.4	72.4	NS
23	Isoleucine	119.2	151.4	127.2	143.9	12.2	NS
24	L-Lysine	208.6^{c}	388.8^{a}	296.2^{b}	280.9^{b}	16.2	<0.0001
25	L-Serine	612.9	779.1	729.2	663.9	51.7	NS
26	L-Lactic acid	4686.1^{ab}	6120.5^{a}	4178.4^{b}	4227.5^{b}	371.3	0.007
27	L-Aspartate	181.4^{ab}	168.9^{b}	257.8^{a}	256.7^{a}	24.9	0.04
28	L-Acetylcarnitine	11.2	14.1	13.2	10.1	2.1	NS
29	Oxoglutarate	40.6	52.7	44.8	43.8	6.3	NS
30	L-Ornithine	55.4	60.5	76.6	57.9	11.7	NS
31	Pyruvic acid	140.5^{c}	238.9^{a}	189.5^{b}	209.2^{ab}	10.2	<0.0001
32	Succinate	26.3	66.2	174.6	34.7	46.9	NS
33	Sarcosine	47.3	39.7	27.6	28.1	7.7	NS
34	3-Hydroxybutyric acid	744.2	642.9	582.5	575.7	75.9	NS
35	2-hydroxyisovalerate	1.6	1.9	2.2	1.3	0.4	NS
36	L-Alpha-aminobutyric acid	8.2	8.6	7.8	6.9	3.9	NS

37	3-Methyl-2-oxovaleric acid	12.3	13.7	12.4	13.1	1.1	NS
38	L-arginine	577.1	632.5	644.5	600.6	46.9	NS
39	Creatinine	8.1	7.6	4.9	8.3	1.5	NS
40	L-Glutamine	884.6	642.8	858.1	1358.9	177.7	NS
41	L-Leucine	233.2	285.5	243.5	262.1	22.1	NS
42	Malonate	15.7	21.6	37.3	21.7	7.1	NS
43	Ketoleucine	16.9	18.1	17.9	17.4	1.5	NS
44	Methionine	219.1^{c}	610.5^{b}	1185.1^{a}	231.3^{c}	45.7	<0.0001
45	Valine	230.4^{c}	348.3^{a}	245.4^{bc}	305.4^{ab}	18.1	0.0005
46	Tryptophan	31.1^{ab}	32.1^{ab}	41.6^{a}	28.1^{b}	2.9	0.02
47	Acetone	20.7	17.5	17.6	21.6	20.7	NS
48	Isobutyric acid	16.2	16.1	14.2	16.3	1.9	NS
49	Methanol	463.8	473.5	479.1	476.8	5.5	NS
50	Propylene glycol	44.5^{ab}	39.8^{ab}	83.1^{a}	29.5^{b}	13.1	0.04
51	Dimethyl sulfone	33.8	48.6	40.4	32.5	10.2	NS

Table 5.3. KEGG metabolic pathways of broiler plasma significantly impacted by *Bacillus subtilis* fermentation extract delivery route.

Treatments	Pathways	Hits [a]	*P*-value [b]
Groupwise[c]	Aminoacyl-tRNA biosynthesis	5/48	<0.001
	Pyruvate metabolism	3/22	<0.001
	Glycolysis / Gluconeogenesis	3/26	<0.001
	Glycine, serine and threonine metabolism	3/34	<0.001
	Valine, leucine and isoleucine biosynthesis	2/8	<0.001
	Pantothenate and CoA biosynthesis	2/19	0.005
	Alanine, aspartate and glutamate metabolism	2/28	0.01
	Glyoxylate and dicarboxylate metabolism	2/32	0.01
	Cysteine and methionine metabolism	2/33	0.02
	Arginine and proline metabolism	2/38	0.02
In-ovo probiotic	Aminoacyl-tRNA biosynthesis	5/48	<0.001
	Glyoxylate and dicarboxylate metabolism	3/32	<0.001
	Pyruvate metabolism	2/22	0.004
	Glycolysis / Gluconeogenesis	2/26	0.006
	Cysteine and methionine metabolism	2/33	0.01
	Glycine, serine and threonine metabolism	2/34	0.01
	Arginine and proline metabolism	2/38	0.01
	Valine, leucine and isoleucine biosynthesis	1/8	0.04
	Biotin metabolism	1/10	0.04
In-water probiotic	Aminoacyl-tRNA biosynthesis	3/48	<0.001
	Biotin metabolism	1/10	0.03
	Pyruvate metabolism	1/22	0.05
In-feed probiotic	Glycine, serine and threonine metabolism	2/34	0.003
	Arginine and proline metabolism	2/38	0.004
	Aminoacyl-tRNA biosynthesis	2/48	0.006
	Valine, leucine and isoleucine biosynthesis	1/8	0.02

[a] Number of metabolites hit in the biological pathway/ total metabolites involved in each biological pathway.
[b] *p*-Value calculated from data.
[c] Running 3 treatment data through statistical analyses as one treatment.

Figure 5.1. Multivariate analysis of the data to discriminate between the plasma metabolome of broilers supplemented with probiotic *Bacillus subtilis* fermentation extract. The PCA loading plots allow for the separation of the variation between the control and in-ovo (A), in-water (B), and in-feed (C) probiotic supplementation groups. The PLS-DA score plots distinguish the metabolic profiles of plasma in broilers supplemented with in-ovo (D), in-water (E), and in-feed (F) probiotic treatments.

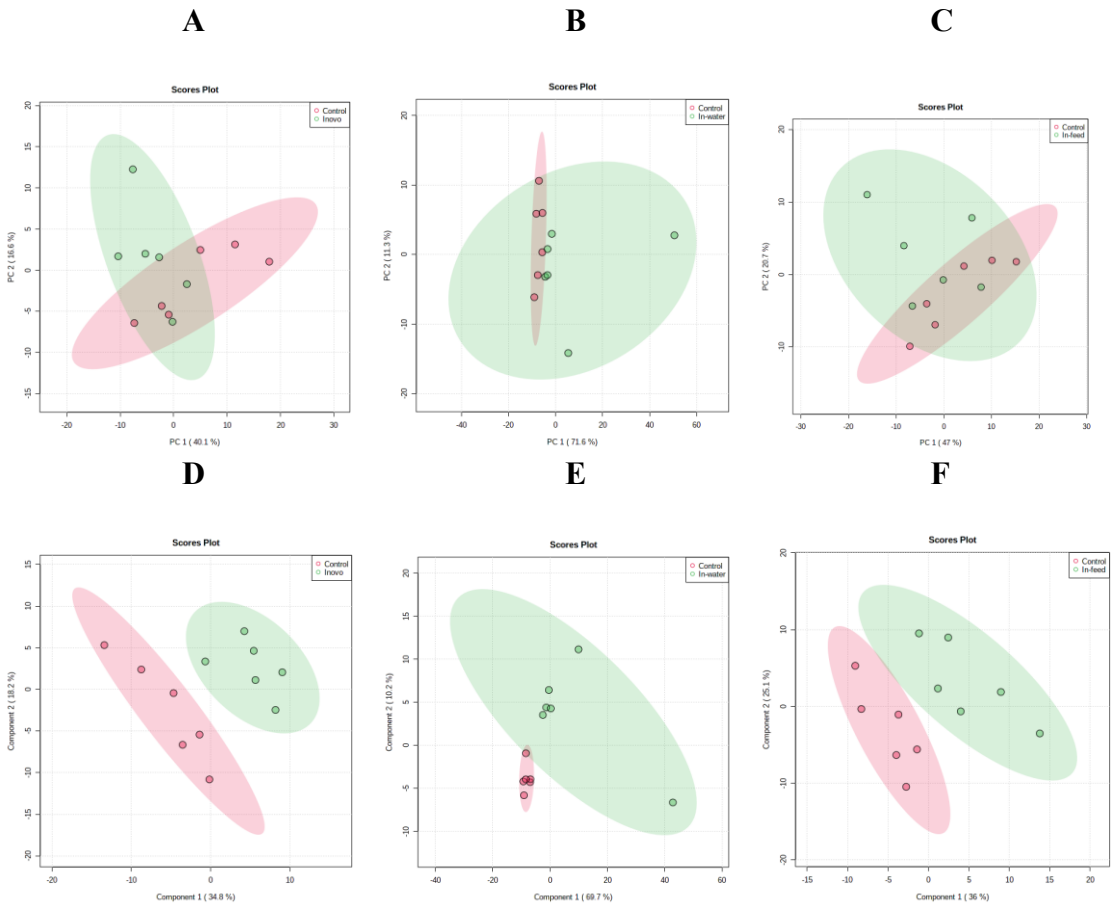


Figure 5.2. The variable importance in projection plot (VIP) ranking markers for their contribution to separation of in-ovo (A), in-water (B), and in-feed (C) probiotic groups, as well as the groupwise separation (D).

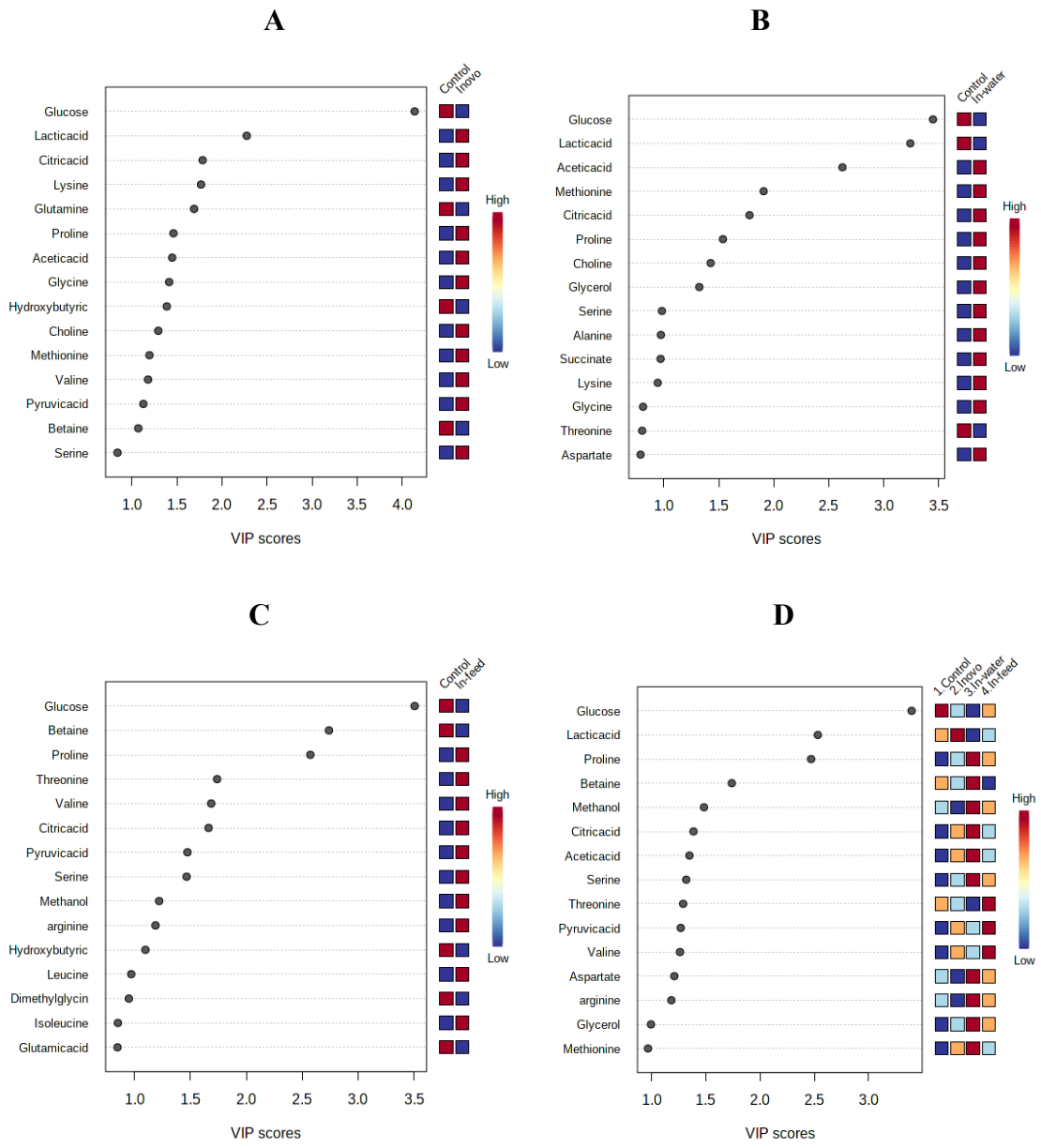


Figure 5.3. Visualization of the significantly differential metabolites overlap between the three groups.

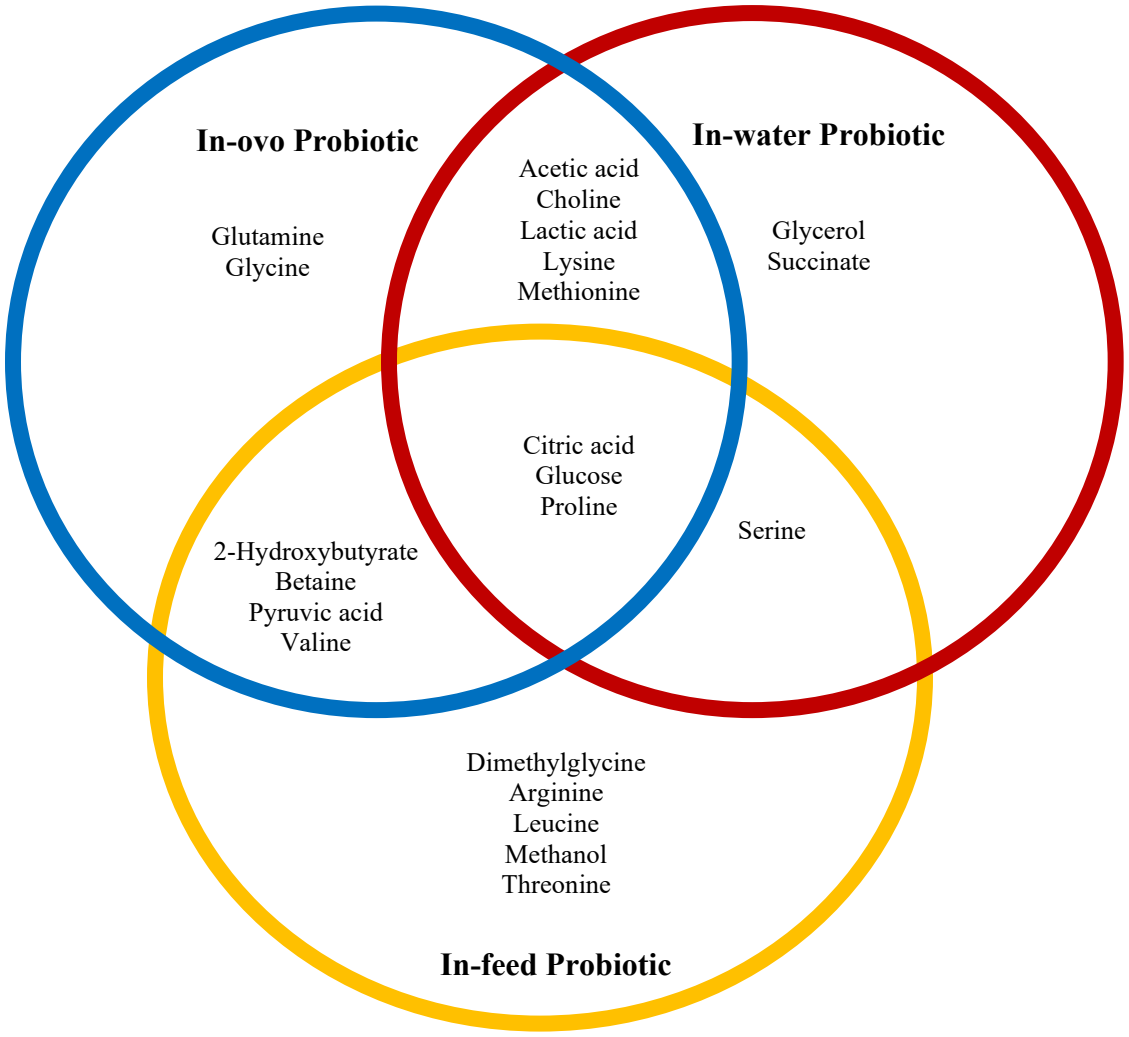
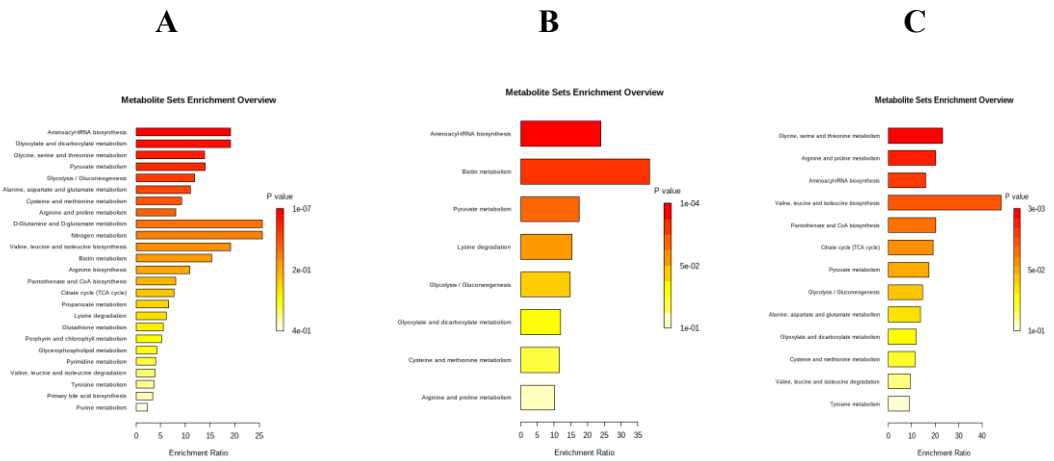


Figure 5.4. Summary of the pathway enrichment analysis of broilers supplemented with the in-ovo (A), in-water (B), and in-feed(C) probiotic *Bacillus subtilis* fermentation extract. The *p*-value of potentially affected metabolic pathways are ranked from low (red) to high (white), corresponding to the most and the least significant values, respectively.



CHAPTER 6. DISCUSSION

6.1. General Discussion

Through the application of 1H NMR spectroscopy, we demonstrate the utility of metabolomics in uncovering the metabolic impacts of pre- and probiotics, heat stress, and genetic strain in chickens. Differentially expressed metabolites were associated with corresponding metabolic pathways using the predictive KEGG software, potentially linked to bird health and production. These findings suggest that the plasma and epithelial cell metabolome can serve as a platform for novel biomarker discovery.

The observed disparity between short- and long-term trials was interesting. More specifically, in this study, we observed the short-term inclusion of seaweed improved feed efficiency, while long-term supplementation affected plasma protein and enzyme profiles. The White strain showed superior production, feed efficiency, and heat stress resistance compared to the Brown strain. In our short-term trial, we noticed higher levels of blood amino acids in the White strain, which could be associated with increased protein synthesis and egg production. The findings emphasize the importance of considering genetic factors and the duration of supplementation in optimizing the health and performance of layer hens. Notably, among the treatments examined, seaweed consumption had the most significant impact on blood metabolite levels, while genetic variation had the least effect on metabolism.

The long-term supplementation of seaweed and exposure to heat stress in birds did not yield significant performance or production responses that may demonstrate the remarkable adaptability of birds to maintain satisfactory levels of performance despite

environmental challenges. Notably, we found that the metabolic changes observed in different genetic strains were associated with significant performance variations. These outcomes suggest that genetic strain could play a pivotal role in determining the birds' ability to adapt and respond to environmental challenges, underscoring its significance in their overall resilience.

Seaweed supplementation affected nine different metabolic pathways primarily related to amino acid metabolism, while heat stress caused changes in pathways involved in the production of valine, leucine, and isoleucine, as well as the synthesis of aminoacyl-tRNA and other relevant pathways. Genetic strain differences potentially included two pathways associated with the production of ketone bodies and the metabolism of glycine, serine, and threonine. Seaweed supplementation demonstrated a significant impact on epithelial cell metabolites, resulting in 29 overlapping metabolites observed in both short and long-term trials, and associations with blood metabolome responses. This supplementation also affected key metabolic pathways, such as glycine, serine, and threonine metabolism, as well as aminoacyl-tRNA biosynthesis, suggesting its potential influence on avian health in the context of genetic or environmental changes.

Seaweed-fed layers exhibited reduced levels of various amino acids in their blood, such as glycine, alanine, proline, glutamic acid, aspartic acid, and serine, which are typically abundant in red seaweed. Several factors including inefficient metabolism of seaweed-derived amino acids, digestion-related degradation, dietary interference, as well as genetic factors, gut microbiota composition, and physiological differences may contribute to the observed variations in amino acid processing and utilization in chickens.

As earlier discussed, the purpose of conducting short- and long-term trials was to assess the effects of seaweed intake duration and determine whether the length of supplementation could lead to differences in the metabolome profiles. When comparing the results of the short- and long-term seaweed trials, the observed metabolome changes in the supplemented birds could be influenced by the duration of seaweed supplementation as well as other environmental factors such as age, season, and stage of laying. It is important to consider that fluctuations in nutrient requirements, utilization, and the physiological stress associated with egg production can impact metabolic pathways and specific metabolite levels (Gloux et al., 2019; Brown et al., 2022). While age, season, and stage of lay were consistent within both the short- and long-term trial, they did differ between the trials and may have contributed to the differences seen. That was out of the scope of this study to determine the homeostasis impacts on the metabolome, but the long-term trial could also evaluate the birds' response to homeostasis and adaptations to long-term environmental changes. Our results suggest that the effects on the metabolome were mainly driven by genetic and dietary factors, rather than age and laying cycle, as the 16-week age difference had minimal influence compared to our targeted factors. This highlights the significant impact of the treatments on pathway expression, while age, laying cycle, and seasonal variations had relatively minor effects.

Our results showed a reduction in the feed intake and weight gain of laying hens due to the short-term dietary supplementation of 3% CC. However, the long-term inclusion of red seaweed in laying hen diets had no effect on production traits. There are some scenarios that could explain this reduction. First, the taste of the seaweed could be a deterrent that causes a reduction in feed intake and body weight in the short term for birds. However,

after a long-term administration of seaweed, birds might get used to the taste and flavour, resulting in no significant impact on production traits during long-term seaweed inclusion in their diets. It is also possible that seaweed causes a reduction in the metabolic energy requirement of chicken. A reduction in metabolic energy requirement might ultimately result in reduced bird activity and metabolic heat production, which requires further evaluation. Another possibility might be that seaweed-supplemented birds are more efficient in the absorption and utilization of nutrients, so seaweed might impact their nutritional requirements and feed intake. Moreover, weight loss in birds might result from muscle catabolism and muscle breakdown. However, our metabolomics data on the plasma amino acids (lower levels in the control than seaweed) and our metabolic pathway analysis (overexpression of amino acid metabolism pathways in seaweed supplemented birds) could not support this hypothesis. These conflicting perspectives suggest that further research is needed to understand the biology behind these results.

Seaweed may have a potential impact on amino acid metabolism in poultry, as it can vary based on its nutrient composition and anti-nutrient properties. The nutrient composition of seaweed, which includes proteins, minerals, vitamins, and essential amino acids, can influence amino acid metabolism. These nutrients contribute to the overall pool of amino acids available for metabolism, thereby modulating protein synthesis and degradation. In contrast to what was anticipated, layers fed with seaweed exhibited significantly lower levels of amino acids in their blood, including glycine, alanine, proline, glutamic acid, aspartic acid, and serine, which are typically found in higher concentrations in red seaweed. Possible explanations for this unexpected finding could be inefficient metabolism or utilization of seaweed-derived amino acids, degradation or modification during digestion,

interference from other dietary components or factors, as well as potential influences from genetic factors, gut microbiota composition, or physiological differences that affect amino acid processing and utilization in chickens. Anti-nutrients, such as phytic acid and enzyme inhibitors, possess the capability to bind to amino acids or inhibit the activity of enzymes involved in amino acid metabolism, thereby perturbing their availability and utilization within the avian organism (Reddy et al., 1982; Hagerman et al., 1998). Additionally, the presence of anti-nutrients in seaweed can hinder the functionality of specific enzymes that play a pivotal role in protein digestion and amino acid metabolism. Consequently, this can have a substantial impact on the breakdown and utilization of dietary proteins (Dunaief et al., 2012). Further research is needed to fully understand the specific mechanisms by which seaweed nutrient composition and anti-nutrients interact with amino acid metabolism in different contexts.

In Chapter 5 of our study, we specifically selected broilers as the subject to explore the influence of genetic background and environmental changes on metabolic responses. Our primary focus was to investigate the impact of different probiotic delivery routes on the metabolome, emphasizing their significance. Interestingly, despite significant changes in metabolites and metabolic pathways observed in both layers and broilers, no effects were observed on physiological and performance traits. These findings highlight the accuracy and sensitivity of metabolomics techniques. Furthermore, organisms possess compensatory mechanisms that maintain stability and homeostasis, mitigating the effects of these changes and keeping the traits unaffected. The interconnectedness and redundancy of metabolic pathways provide flexibility and robustness, allowing adaptation without significant impact on traits. It's also possible that the observed changes did not surpass threshold levels

to trigger physiological and performance responses, and a longer study period might be necessary to observe effects.

We could detect 51 plasma metabolites in broilers supplemented with probiotics and found that in-ovo delivery had a greater impact on plasma metabolic pathways compared to in-feed and in-water treatments. This may be due to improved jejunum morphology and enhanced intestinal absorptive function resulting from in-ovo delivery, leading to increased expression levels of metabolites and pathways. However, due to the significant genetic distinctions and contrasting experimental paradigms between broilers and layers, the comparison was challenging but we found six metabolic pathways to be shared between layers supplemented with a 21-day prebiotic source in study 3, and broilers supplemented with a 28-day intake of *B. subtilis* probiotic in study 5. These shared pathways include aminoacyl-tRNA biosynthesis, glycolysis/gluconeogenesis, alanine, aspartate, and glutamate metabolism, glyoxylate and dicarboxylate metabolism, cysteine and methionine metabolism, and glycine, serine, and threonine metabolism.

We focused on the glycolysis pathway, which showed higher levels of key metabolites in broilers supplemented with in-ovo probiotics, suggesting upregulation of glycolysis in response to probiotic supplementation. Aminoacyl-tRNA synthesis, involved in protein biosynthesis and amino acid signaling, was highly expressed in all probiotic supplements, with seven interactive metabolites upregulated, indicating the influence of probiotics on translation and tRNA function. Our findings indicate that probiotics can impact protein synthesis and energy metabolism, with notable upregulation observed in in-feed probiotic supplementation.

In both broilers and layers, we observed significant changes in the metabolome when subjected to long-term pre-/pro-biotic supplementation. However, these changes did not have any impact on bird performance and production. These findings highlight both the high sensitivity of metabolomics techniques and the remarkable adaptability of birds to maintain satisfactory levels of performance and production despite environmental challenges. Metabolomics is highly sensitive, which makes it a valuable tool for the analysis of minor stimuli. The sensitivity of metabolomics makes it a valuable tool for the analysis of minor stimuli which has both advantages as well as disadvantages. Its inherent sensitivity makes metabolomics very important for studies that are targeting novel biomarkers as a predictor of treatment. On the negative side, metabolomics studies require high and specific experimental considerations, such as identifying and limiting sources of all the variations. These factors could cause not only physiological differences between study groups but also influence the metabolome.

Comprehensive literature and reference databases play a crucial role in assessing the feasibility of our findings. Although we propose potential linkages between the detected metabolic pathways and chicken performance and health traits, further references on chicken metabolic responses to environmental changes are necessary to ensure the accuracy of our biological interpretations. As the field of metabolomics advances, poultry metabolomics is expected to become a routine tool with broad scientific implications for future studies.

However, given the limited exploration of the chicken metabolome, practical implications of the metabolic pathways in chicken remain limited. To address this complexity, the establishment of a centralized poultry metabolomics database containing detailed

information on chicken metabolites is essential. Such a database would facilitate knowledge sharing among researchers and aid in the interpretation of experimental results. Existing open-access platforms like the livestock metabolome database and bovine metabolome database serve as solid foundations, enabling easier performance, analysis, and comparison of livestock metabolomic studies. A dedicated poultry metabolomics database would greatly benefit metabolomics researchers, chicken health professionals, scientists, nutritionists, and consumers.

Metabolites exhibit interconnections and are subject to influence from diverse factors such as the microbiome, environment, circulating metabolites, and metabolic pathways. More specifically in this study, we encountered challenges in comprehending the complex relationship between blood and intestinal epithelial cell metabolites, as well as investigating the impact of internal and external factors on protein metabolism in layers and broilers. By elucidating these intricate interactions, we can enhance the interpretation of metabolic results, eliminating potential confounding factors and facilitating comprehension of the impact of different stimuli on metabolism.

Differences in metabolite composition between intestinal epithelial cells and the bloodstream arise from complex processes of nutrient absorption, metabolism, and systemic distribution (Chen et al., 2019; Shi et al., 2017). Intestinal cells metabolize dietary compounds through specific transporters and enzymes, generating unique metabolites. After absorption, metabolites undergo further transformations in the liver, modifying the original dietary supplement metabolites. Variations in metabolite distribution and utilization across tissues contribute to distinct patterns between intestinal cells and the bloodstream. The bloodstream acts as a dynamic medium, efficiently transporting and

distributing a diverse range of metabolites derived from nutrients and other sources, leading to inherent dissimilarities in metabolite composition (Chen et al., 2019; Shi et al., 2017). The relationship between pre- and probiotic intake and metabolites are not straightforward nor fully understood. The plasma and intestinal epithelial cell metabolome profiles are the accumulated results of both microbial and chicken metabolism. Thus, it is difficult to differentiate which of the two factors (chicken or microbiota) caused the metabolome change. The fecal metabolome can be used not only to reveal the gut microbial metabolome but also provide more insight into the relationship between the gut microbiome and the environment as well as its interaction and effect on chicken physiology. The full characterization of the gut microbiota metabolome and comparing it with the plasma and intestinal epithelial cell metabolome profiles may illustrate this point.

We determined that protein pathways and amino acid metabolism were impacted by most of the treatments in chicken plasma and epithelial cells (short- and long-term seaweed intake, genetic strain, heat stress, in-ovo and in-feed *B. subtilis* probiotic supplementation in the plasma metabolome, as well as the long-term intake of seaweed and short-term strain effect on laying hens' epithelial cells). Protein metabolism is complex and involves up to 20 essential and nonessential amino acids and a large number of metabolites. Protein synthesis is highly interconnected with other pathways and intimately networked with many feedback loops. The amino acid metabolism pathways are also affected by many factors such as age, light, or any environmental stressors that may regulate cell functions at different levels. Additionally, there are other pathways, such as gluconeogenesis, proteolysis, and the tricarboxylic acid cycle, that contribute to amino acid metabolism. The

interaction between several factors indicates that control of amino acid metabolism is complex and probably involves several regulatory molecules.

Gaining insights into chicken metabolic pathways through the analysis of blood and intestinal cells contributes to the advancement of poultry science. This knowledge can impact nutrition, health, and production efficiency. With an understanding of chicken metabolic pathways, researchers can optimize dietary strategies by tailoring feed formulations to meet the specific nutritional needs of chickens at different growth stages, thus improving overall growth, health, and well-being. Additionally, studying these pathways enables the identification of potential biomarkers for disease diagnosis, monitoring dietary interventions, and developing targeted strategies for enhancing poultry health and managing environmental stressors. By focusing on pre- and probiotic supplementation, researchers can have valuable knowledge about how these factors influence chicken metabolism, enabling targeted interventions to enhance gut health, promote beneficial microbial populations, and improve nutrient utilization. Ultimately, a deeper understanding of chicken metabolism leads to more sustainable and efficient poultry production systems, benefiting various aspects of poultry science and the industry.

Metabolomics data obtained from blood and intestinal cells can be integrated with other types of data, such as genomics, transcriptomics, and proteomics. This integration allows for the identification of significant molecular pathways and networks, leading to the construction of comprehensive models of chicken metabolism. By doing so, we can gain insights into how genetic background, environmental factors, and stressors influence the metabolism and health of poultry.

6.2. Future Directions

While metabolome analysis has grown tremendously over the last decade, metabolomics is not without its challenges and difficulties. Each biological sample can contain a vast array of metabolites, often numbering in the thousands. Consequently, the comprehensive characterization of these metabolites requires the utilization of multiple analytical platforms, each specialized in detecting specific chemical groups. In contrast to genomics, where thousands of genes, RNA, and proteins can be detected through a single experiment, metabolomics platforms have inherent limitations in their ability to detect diverse chemical groups simultaneously. Hence, a multi-platform approach becomes necessary to achieve a more comprehensive and accurate metabolic profiling of biological samples. Assigning every single peak that corresponds to metabolites requires very extensive databases of compound structures, different reference spectral libraries, and available reference standards. Likewise, carefully designed experiments are required to provide unambiguous tests of hypotheses and to control all the variables, including but not limited to diet, age, sex, species, sampling time, sample selection, sample extraction, and designing optimized, roboticized protocols. Certainly, significant effort and costs must be incurred for any type of metabolomics study to overcome these challenges and difficulties.

As seen in chapters 3, 4, and 5, we could confidently infer the existence of up to 60 metabolites in the chicken plasma using NMR analysis. Clearly, more work is needed in the poultry field to expand the number of measured chemicals and the repertoire of quantitative assays. Moreover, different metabolomics platforms are required to detect different classes of metabolites. Indeed, a combination of NMR spectroscopy, gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass

spectrometry (LC–MS), Inductive Coupled Plasma Mass Spectrometry (ICP-MS), lipidomics, and several targeted high-performance liquid chromatography-based (HPLC) assays would help detect several hundred thousand different metabolites, spanning dozens of chemical classes.

The NMR, as an ideal tool for biomarker discovery, is particularly well suited to analyze untargeted metabolites dissolved in liquids but is biased towards highly abundant compounds. Therefore, in the future, the application of more extensive targeted metabolomics techniques, including liquid chromatography (LC), gas chromatography (GC), and mass spectrometry (MS), could be used to detect more chemical compounds from complex matrices and complementarily expand the metabolite coverage of chicken.

Our metabolomics data illustrated specific pathways activated by the experimental factors that need to be tested for linkage to some production traits to be validated for their biological significance and putative functions in future poultry studies. In the current study, we showed that probiotic intake significantly impacted the arginine and proline metabolism pathway. We speculated the association between probiotic supplementation and chicken resistance to low-temperature stress as this pathway was previously found to be linked with host resistance to low-temperature (Raza et al., 2020). The upregulation of the arginine and proline metabolism pathway and significantly elevated levels of pyruvate, arginine, and proline suggested enhanced resistance to low-temperature stress in chickens supplemented with the in-feed probiotic. Further research is needed to reveal more details regarding the association between **s**tress resistance and the arginine-proline metabolism pathway.

Our plasma metabolomic results illustrated the fine relationship between probiotic supplementation and glycolysis metabolism in broilers supplemented with in-ovo

probiotic. We observed enhancement of the glycolysis pathway and higher levels of glucose, pyruvate, lactate, acetate, and ethanol, the key metabolites in the glycolysis pathway. These results might imply that gut bacteria and probiotics utilize the glycolytic pathway for the catabolism of carbohydrates to produce energy in the form of ATP. Therefore, further investigation is needed to illustrate the energy metabolism of gut bacteria through the glycolysis pathway.

Previously, it was reported that the glycolysis pathway and pyruvate metabolism pathway had a central role in meat quality (Zheng et al., 2015; Suryadi et al., 2019). We revealed that both pathways were significantly impacted by probiotic intake. Consequently, we suggested the association between probiotic treatment and chicken meat quality by impacting the glycolysis pathway and the pyruvate metabolism pathway, which warrants further investigation.

As discussed earlier, the present data speculated the linkage between three pathways, including the glycolysis/gluconeogenesis pathway, galactose metabolism, as well as starch and sucrose metabolism, associated with the feed intake of chickens. In addition, we suggested that there might be a potential association between the glycine, serine, and threonine metabolism pathway and feed efficiency in broilers. Further research is needed to reveal all these associations between the mentioned metabolic pathways and chicken physiological performance traits. Additional studies with larger cohorts, covering the influence of chicken lines, and different pre- and probiotic products would help to confirm the validity of our suggested results.

Overall, with regards to these four chapters, I believe the establishment of plasma and epithelial cell metabolomes in response to pre- and probiotic supplementations, genetic

strains, and heat stress will advance poultry research in many ways, including the following: 1) improvement of reproductive efficiency and heat stress resistance of White laying hens; 2) improvement of feed efficiency and reduction of feed intake in short-term seaweed-supplemented layers; 3) revealing changes in several blood chemistry values (albumin, ALT, and GGT) with no negative effect on plasma health indicators after long-term seaweed supplementation of laying hens; 4) demonstrating that white laying hens are less susceptible to the adverse effects of heat stress; 5) establishing a better understanding of the blood metabolic composition of the laying hens supplemented with seaweed; 6) enhanced support for the health benefits of prebiotics and probiotics by more completely determining their metabolomes and metabolic pathways; 7) speculating on associations between metabolic pathways and feed intake, protein metabolism, microbial fermentation, and energy metabolism in seaweed supplemented layers; 8) expanding the repertoire of quantitative assays available for chicken blood and epithelial cell composition analysis to define more biological functions and molecular mechanisms; 9) providing a convenient, centralized resource to study the mechanisms of prebiotics, probiotics, heat stress, and genetic strain on the metabolic profile of chicken; 10) identifying potential key metabolic biomarkers and pathways possibly linked to chicken health, performance, production, meat, and egg quality traits; 11) evaluating the blood metabolome response to in-ovo and oral probiotic administration in broilers; 12) Speculating on the blood metabolic pathways possibly linked with broiler meat quality, stress resistance, feed efficiency, and energy metabolism, associated with the probiotic supplementation delivery routes.

6.3. Summary

As discussed in the first chapter, my thesis investigated three different hypotheses. These hypotheses were described and tested in chapters 2–5 of this thesis. Hypothesis testing and the associated results can be summarized in the three steps below.

In chapter 2, seaweed supplementation, heat stress, and genetic strain could change the blood parameters and overall performance of laying hens. The results presented in this chapter showed that short-term seaweed intake had little impact on blood characteristics, while long-term seaweed supplementation impacted several blood chemistry parameters. The hen production performance data indicated that the short-term seaweed diet reduced feed intake, weight gain, and feed/egg ratio, while there was no impact on long-term performance. These findings provided more evidence of seaweed suitability for use in layer hens. Our investigations on the effects of genetic strain confirmed that the White strain had better production, feed efficiency, and heat stress resistance, with significant liver enzyme changes. These performance and blood parameter results could further help us to interpret metabolomics data related to glucose and protein metabolism, in chapters 3 and 4.

In chapters 3 and 4, NMR spectroscopy could reveal the effects of seaweed supplementation, genetic strain, and heat stress on modulating the chicken metabolome. In testing our hypothesis, we employed quantitative NMR spectroscopy on laying hen plasma and intestinal epithelial cells in response to the noted treatments. In chapter 3, we provided quantitative data on 57 plasma metabolites that was aimed to help advance the fields of quantitative metabolomics in studying biological pathways, functions, and molecular mechanisms. The greatest plasma metabolic change was observed in seaweed supplemented birds, while the genetic strain induced the least metabolic changes. We

paved the way for the identification of potential biomarkers by detecting some differentially expressed metabolites that might serve as potential prebiotic biomarkers. We identified 13 differentially expressed metabolites in plasma (L-carnitine, isopropanol, L-alpha-aminobutyric acid, oxoglutarate, dimethyl sulfone, L-asparagine, L-aspartate, malonate, acetic acid, acetone, hypoxanthine and propylene glycol) that could be suggested as potential biomarkers associated with seaweed intake in laying hens. Additionally, we suggested ten potential biomarkers for heat stress in laying hens, mainly involved in protein metabolism pathways (valine, leucine and isoleucine biosynthesis; aminoacyl-tRNA biosynthesis; arginine and proline metabolism; and glycine, serine, and threonine metabolism). This was indicating that heat stress modulated chicken metabolism and might cause the diversion of energy to muscle catabolism, leading to muscle breakdown during periods of heat stress.

In chapter 4, the research was followed up with a study that utilised the epithelial cell metabolome to test the effects of seaweed supplements and genetic strain in chicken jejunum. Nine differentially expressed intestinal epithelial cell metabolites were detected as the group discriminators of prebiotic supplemented birds (ethanol, fructose, homocysteine, xylulose, 2-phosphoglycerate, 2-oxalacetic acid, valine, dimethyl sulfide, and alanine), which consequently impacted some metabolic pathways, leading to changes in host systematic responses. In chapters 3 and 4, we found the connection and the circulatory system between blood and epithelial cells metabolites and compared the impacted metabolic pathways. However, we observed no overlap between the suggested seaweed potential biomarkers in the plasma and epithelial cell metabolome, showing that blood and intestinal epithelial cells had unique metabolites associated with seaweed intake.

Our results also showed that seaweed could impact some signalling molecules involved in the regulation of physiological processes, such as amino acids, short-chain fatty acids, and fuel metabolites. These findings could help us understand the underlying metabolic responses of laying hens.

Furthermore, set enrichment and pathway analyses in chapters 3 and 4 indicated that the short-term seaweed diet was involved in more plasma metabolic pathways, than the long-term trial. Genetic strain impacted the least number of blood metabolic pathways. These results suggested that metabolomics could be used for biomarker discovery, to address the challenge of identifying pathways and mechanisms, and to explain the health effects of genetic and environmental factors.

To focus on the impact of probiotic supplementation on the chicken metabolome, my subsequent study was performed in Chapter 5. The purpose of these studies was to gain a better understanding of the biology underlying the effects of pre- and probiotic supplementation. In this chapter, we hypothesized that the delivery route of probiotic supplementation could impact the plasma metabolome and metabolic pathways of broilers. The goal of this study was to evaluate the effectiveness of using untargeted metabolomics to differentiate the delivery route of probiotic supplementation in broilers by identifying key metabolic changes and pathways. The in-ovo probiotic treatment with the highest metabolic impact was involved in nine metabolic pathways, suggesting that early inoculation could enhance intestinal absorptive function and increase the expression levels of more metabolites and metabolic pathways. This study showed that probiotic delivery routes could modulate the plasma metabolome of broilers and impact host physiological responses.

6.4. General Conclusion

My thesis research has evaluated the utility of metabolomics in understanding chicken physiological responses to environmental changes and genetic background. We showed that chicken plasma and epithelial cell metabolome were sensitive to respond to internal and external stimuli, including pre- and probiotic supplementation, heat stress, and genetic strain. These findings suggested that metabolomics is a useful tool to address the challenge of identifying pathways and mechanisms linked to chicken productivity and health traits. The maximum gain from metabolic pathways may be obtained by growing poultry databases in reported metabolites.

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