WHAT'S IN A CHICKEN: USING NMR METABOLOMICS TO INVESTIGATE STRESS IN BROILER CHICKENS

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DEDICATION

To the chicken producers, without whom there would be no thesis

And to my family, without whom there would also be no thesis

ABSTRACT

Stress has the potential to adversely affect the health of chickens. Using Nuclear Magnetic Resonance (NMR) spectroscopy-based metabolomics objectives were to: ascertain the effects of physiological stress on the metabolomes of chicken liver, kidney, and breast muscle; compare sample preparation techniques for fecal metabolomics; and determine if NMR spectra can be obtained from feather pulp, and the effects of age and stress on the pulp metabolome. Physiological stress modulated chicken metabolism in all tissues examined, and caused the diversion of energy to muscle catabolism and gluconeogenesis. Ultrafiltration was the most versatile, reproducible, and efficient fecal metabolite extraction method; however, Bligh-Dyer extraction and no extraction with baseline correction produced comparable results. High quality NMR spectra were obtained from chicken feather pulp, and both age and stress significantly altered the pulp metabolome. The results obtained are foundational to the identification of novel biomarkers of stress for use in chicken production settings.

CONTRIBUTIONS OF AUTHORS

Catherine Brown is the primary author of all five chapters within this thesis, with edits and revisions from Dr. Douglas Inglis and Tony Montina. All shared content in this thesis is included with the permission of each co-author. I wish to acknowledge the contributions of authors in the following manuscript-based thesis:

Chapter 2. Dr. Sarah Zaytsoff and Dr. Inglis conceptualized and designed the experiments. Dr. Inglis obtained animal care and biosafety approvals, and provided animal and laboratory infrastructure, and personnel support. Dr. Zaytsoff performed the experiments with chickens. Tony Montina provided the NMR infrastructure. Tony Montina and Catherine Brown conducted NMR metabolomics, analyzed the metabolome data, and generated figures. Catherine Brown and Tony Montina completed statistical analyses of metabolomics data. Catherine Brown interpreted the results of experiments.

Chapter 3. Tony Montina, Dr. Gerlinde Metz, and Dr. Inglis conceptualized and designed the experiments. Tony Montina provided the NMR infrastructure. Catherine Brown, Hannah Scott, and Crystal Mulik carried out sample preparation, data collection and data analysis. Tony Montina, Mike Opyr, and Amy Freund implemented and tested the baseline correction algorithm used in the study.

Chapter 4. Catherine Brown, Tony Montina, and Dr. Inglis conceptualized and designed the experiments. Dr. Inglis obtained animal ethics approvals, provided the animal infrastructure, and personnel support. Maximo Lange arranged for and assisted with the collection of feather pulp samples from broiler breeders. Tony Montina provided the NMR infrastructure. Catherine Brown conducted NMR metabolomics and interpreted

the results of experiments. Catherine Brown and Tony Montina analyzed the metabolome data, completed statistical analyses, and generated figures.

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PREFACE

The research contained in chapters 2, 3, and 4 were carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee, and the University of Lethbridge Animal Welfare Committee before commencement of the research (LeRDC Animal Use Protocol Review #1526, #1903, and #2010; University of Lethbridge Animal Use Protocol #1715).

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LIST OF ABBREVIATIONS

^{1}H-NMR	Proton Nuclear Magnetic Resonance
AAFC	Agriculture and Agri-Food Canada
ACTH	Adrenocorticotropic Hormone
AGP	Antibacterial Growth Promoters
ASD	Autism Spectrum Disorder
AVT	Arginine Vasotocin
BCAA	Branched chain amino acids (valine, leucine and isoleucine)
BD	Bligh-Dyer
CORT	Corticosterone
CPMG	Carr Purcell Meiboom Gill pulse sequence
CRF	Corticotropin Releasing Factor
D_2O	Deuterium oxide
DNA	Deoxyribonucleic acid
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
FFE	Feather Follicle Epithelium or Feather Pulp
GC	Gas Chromatography
H:L	Heterophil to Lymphocyte ratio
HFMDB	Human Fecal Metabolome Database
HPA	Hypothalamic-Pituitary-Adrenal
HSP70	Heat Shock Protein 70
Hz	Hertz
IBD	Inflammatory Bowel Disease
IBS	Irritable Bowel Syndrome
IVC	Individually Ventilated Cage
kDa	Kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	Liquid Chromatography
LeRDC	Lethbridge Research and Development Centre
M-BD	modified version of Bligh-Dyer
MDD	Major Depressive Disorder
MetPA	Metabolomics Pathway Analysis
MND	Motor Neuron Disease
mRNA	Mitochondrial Ribonucleic acid
MS	Mass Spectrometry
MW	Mann-Whitney U test
NMDA	N-methyl-D-aspartate
NOESY	Nuclear Overhauser Effect Spectroscopy
OPLS-DA	Orthogonal Projection of Latent Structures Discriminant Analysis
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis

PLS-DA	Partial Least Squares Discriminant Analysis
ppm	Parts Per Million
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristics
rRNA	Ribosomal Ribonucleic acid
TMS	Tetramethylsilane
TSP	Trimethylsilylpropionic acid
UDP	Uridine Diphosphate
UF	Ultrafiltration
VIAVC	Variable Importance Analysis based on random Variable Combination

1 INTRODUCTION

1.1 The domestic chicken

The modern-day chicken (*Gallus Gallus domesticus)* is believed to have evolved from Red Jungle Fowl (*Gallus gallus gallus*) [1,2]. It is not known exactly how long-ago chickens were domesticated, but current archeological evidence indicates that domesticated chickens existed 8,000 years ago [3-5], and phylogenetic studies suggest genetic divergence from *G. gallus gallus* occurred between 74,000 – 42,000 years ago [6]. Chickens are now one of the most populous birds on Earth, and they are one of the most important domesticated animals [7].

There are two main purposes for which chickens are commercially reared. These are laying eggs and producing meat. Different strains of chickens have been selectively bred for different characteristics, and they are roughly divided into layer and broiler breeds [8]. Layers have been bred for high feed conversion efficiency and high egg yield, and modern breeds of layers can produce over 300 eggs per year [2,8]. In contrast, broiler breeds have been selected for enhanced growth rate and meat yield. To illustrate the impact of breeding programs, the average carcass mass of broiler chickens was 1.3 kg in 1961, as compared to 1.7 kg in 2013, representing a 30% increase in mass [9,10]. Moreover, modern breeds of broiler chickens reach market weight in about a third the amount of time it took in the 1950s. The decrease in time to market weight is largely attributed to genetics, although nutrition and management practices have changed over the years and have also contributed to the improved growth rates [11].

1.2 The chicken industry

Worldwide there were approximately 25 billion chickens in 2019 [12], and over 100 million metric tonnes of chicken meat was produced in 2020 [12]. Although production of chicken meat occurs in the majority of countries, relative rates of production vary from country to country (**Figure 1-1**). The poultry (domesticated birds including chickens, turkeys, guinea fowl, and quail) population has increased about five-fold over the last 50 years, along with the consumption of poultry meat [9]. In 2011, the world poultry meat consumption was 14.4 kg/person/year with people living in Oceania and the Americas consuming higher than the world average of meat, at 42.1 and 38.6 kg/person/year, respectively [10,13]. It is predicted that in 2021 the world will consume 132.4 metric kilotonnes of poultry meat [12]. Poultry meat production was estimated to be 98.4 million tonnes in 2014, with chicken meat representing about 88% of the global poultry meat production [14], and approximately 96 million tons of meat [15]. Poultry is one of the fastest growing agricultural sub-sectors [9], and between 1970 and 2005, poultry meat production increased faster than both that of beef and pork [16]. Poultry meat production will soon be the largest contributor to the meat industry [15]. With greenhouse gas emissions from livestock production under increasing scrutiny, broilers possess one of the most efficient feed conversion rates of all meat production animals. In this regard, broilers require 26.4 kg of dry matter feed to produce 1 kg of protein, while ruminants need an average of 133.4 kg per kg of protein [9]. Chicken feed efficiency is 4.5 kg feed/kg edible weight, which is much better than beef (20.0 kg feed/kg edible weight) and pork (7.3 kg feed/kg edible weight) (**Table 1-1**). The only commercially farmed animals with better feed efficiency than chicken are fish such as carp (2.3 kg feed/kg edible weight) and salmon (1.4 kg feed/kg edible weight) [17].

Broiler chickens consume 1.6 to 2.0 times as much water as feed and overall daily water consumption steadily increases as birds grow [18]. However, the water footprint of meat from beef cattle is on average 15,400 L/kg globally, which is much greater than the footprints for sheep (10,400 L/kg), pigs (6,000 L/kg), goats (5,500 L/kg), and chickens (4,300 L/kg) [19-21]. In summary, poultry production accounts for only 11% of the total water and approximately 11% of the total greenhouse gas emissions from livestock production, making it the smallest contributor to the global livestock sector's emissions [9]. More specifically, poultry meat production generates only 40 kg CO_2 equivalent/kg protein [9], compared to beef cattle production that generates approximately 300 CO_2 equivalent/kg protein.

Globally, economic contributions of the poultry industry are significant, generating billions of dollars annually with the global poultry market valued at $310.7 billion in 2020 and expected to grow to $322.6 billion in 2021 [22]. The largest exporter of broiler meat in 2021 is Brazil, with the United States a close second [12]. The largest importer of broiler meat is Japan [12]. How a country manages production influences both rates of exportation and importation. For example, Canada has implemented a supply management system [23], in which production rates are managed by producer quotas to achieve the following: avoid over supply; facilitate access to locally produced chicken products; ensure a sustainable and consistent local food supply for consumers; achieve optimal food safety and animal care standards; and to reduce the requirement for subsidy inputs from Government [24]. The volume of broiler meat imported into Canada is limited by tariff rate quotas, under which very high tariffs are applied on imports above a specific level [25]. Although Canada is not a leading poultry producing country, in 2019, 1.3 million metric tonnes of chicken meat were produced [10,12]. Even though the

majority of chicken meat produced in Canada is consumed domestically, in 2018, 0.127 million tonnes of broiler meat was exported, primarily to the USA, Philippines, Taiwan, Jamaica, and Cuba [26]. In Canada, the chicken industry is a multibillion-dollar industry, consisting of 2,803 farmers, 191 processors, and contributing 87,000 total jobs, $6.8 billion dollars to the GDP, and $2.2 billion in taxes [24]. The province of Alberta alone generated 8,031 total jobs, $623 million to GDP, and $203 million in taxes [24], and produced more than 165 million live kg of chicken meat in 2020 [27].

Broiler production in Canada is vertically integrated, which has contributed to economically efficient production of poultry meat. Vertical integrated production involves the control of the production continuum by a limited number of companies. Thus, companies own or control broiler egg production, hatching of eggs, broiler production, and processing (**Figure 1-2**). Moreover, broiler breeds are developed by a limited number of companies (e.g. Aviagen, Inc. and Cobb North America), who work in coordination with the production and processing companies. Although broiler hatching egg producers and broiler producers are independently owned and operated, producers operate under contract to the integrated chicken production and processing companies, and under their assigned quota, which is controlled by national and provincial marketing boards [24].

The poultry industry in Canada is currently undergoing some major changes. In 2014, the Canadian chicken sector decided to stop the use of Category I antibiotics for disease prevention [28]. By 2018, the use of Category II antibiotics for the prophylactic-administration of antimicrobials was also eliminated, and by the end of 2020, the preventive use of Category III antibiotics was stopped [28]. Category I, II and III antibiotics are still permitted in broiler production as a therapeutic treatment, but they

must be prescribed by a licensed veterinarian [29,30]. The purpose of reducing the use of antibiotics in livestock production is to reduce the emergence of antibiotic resistance in zoonotic bacterial pathogens [31], such as *Salmonella enterica* and *Campylobacter jejuni*. However, the loss of antibiotics for growth promotion has necessitated that the industry identifies suitable non-antibiotic replacements [32,33].

There are three main uses of antibiotics in poultry rearing: (1) prophylactic / disease prevention; (2) treatment of an outbreak of disease (i.e. therapeutic administration); and (3) as antibacterial growth promoters (AGPs). For poultry, antibiotics used for therapeutic reasons are usually administered through water, whereas AGPs are usually administered at non-therapeutic doses for prolonged periods via feed [34]. Moore *et al.* [35] was the first to publish about the positive effects of antibiotics on chicken growth. Followed shortly by Stokstad and Jukes in 1950 and 1956, who showed the benefits of antibiotics on growth of livestock in general [36,37]. Although the exact mode of action by which AGPs function are unknown even today, antibiotics have been used globally to increase weight gain and feed efficiency in livestock species [32,38]. The mechanisms by which AGPs function may be by modulating the microbiota and/or the immune systems of livestock [38].

Although a definitive link between the use of antibiotics in food animals and the increase in antibiotic resistant bacterial pathogens in people has not been definitively established [32], many governments have adopted a precautionary approach due to the risk posed by resistant bacterial pathogens to human health [9,34,39,40]. Sweden was the first country to ban all AGPs in 1986, followed by Denmark in 2000, the EU in 2006 [38], Canada in 2018 [41,42], and most recently China in 2020 [39]. China is the largest producer and consumer of antibiotics in the world, using almost 30,000 tons of antibiotics

for animal husbandry in 2018, 53% of which were administered as AGPs [39]. Some studies have indicated that, in poultry, the benefits of AGP are not worth the cost of the antibiotics [43]. It is currently unknown what the effects of these bans will have on disease and losses from disease in livestock production, but it is expected that rates of disease will increase [39]. Thus, rationale-based alternatives to antibiotics will be needed to mitigate bird morbidity and mortality in poultry production. It is also important to fully identify indicators of disease risk.

1.3 Stress in poultry

Due to the changes to the Canadian poultry industry, producers have started looking at other ways to maintain the health of their flocks, and it is becoming increasingly apparent that stress plays an important role [44-47]. The definition of stress used herein is a state when the Hypothalamo-Pituitary-Adrenal (HPA) axis is activated with increased secretion of glucocorticoid hormones in response to a stressor [48]. When a chicken experiences a stressor, the hypothalamus releases both Arginine Vasotocin (AVT) and Corticotropin Releasing Factor (CRF), and these hormones travel to the anterior pituitary, which produces adrenocorticotropic hormone (ACTH), which acts on the adrenal gland. In birds, ACTH causes the adrenocortical hormone, corticosterone (CORT) to be secreted from the cortex of the adrenal gland, which is located near the kidney (**Figure 1-3**) [48-50]. The HPA axis can respond to a stressor in less than three minutes [51].

The most commonly measured biomarkers, or biological characteristics that can be objectively measured as indicators of physiological stress in chickens are circulating concentrations of CORT, and the heterophil: lymphocyte (H:L) ratio in blood [48]. However, the stress response in chickens is complex, resulting in a systemic response,

which requires the examination of multiple parameters of stress [48]. An extra level of complication in quantifying stress is that two identical chickens, when faced with the same stimulus, may not both perceive the stimulus to be a stressor; therefore, variation in the population, usually referred to the effect of the "personality" of the chickens, occurs [50].

In addition to a physiological response to stress, an organism also responds to stress on a cellular level. Cells respond to stress in a multitude of ways. This includes activation of a various pathways with outcomes that range from the promotion of cell survival to eliciting programmed cell death (e.g. apoptosis) and autophagy [52]. When a tissue is in homeostatic state, an equilibrium exists between the net growth and death rates of the cells making up the tissue [53]. Upon exposure to cellular stress, homeostasis is disrupted, which can precipitate a number of cellular processes. Examples of stressors that disrupt cell homeostasis include exposure to heavy metals, ethanol, oxidative stress, or other toxic substances [54]. The overarching goal of cellular stress responses is to return the cell to a homeostatic state, and failing this, cells may commit to a death pathway [52]. For the model of physiological stress presented in this thesis, the goal was to stimulate a physiological stress response at an organismal level. However, as CORT administration is known to cause oxidative stress responses in birds [53,55,56], it is important to recognize that the foundation of the stress response measured is at a cellular level.

1.4 Impacts of stress on bird health

Stress has been shown to be associated with decreased production [48,57] as evidenced in previous studies by a decrease in growth rate and an increase in abdominal fat, which is undesirable in broiler chickens [58], and can also lead to fatty liver disease

[59]. Stress in general, but specifically heat stress, can result in a significant reduction in feed intake, which can adversely affect the growth performance of chickens [60,61], and substantially increase the feed conversion ratio (lower values are more cost effective) [62,63]. Stress leads to reductions in growth rate because energy is diverted from growth (building new protein for muscle and skeletal development) to processes that provide energy to tissues such as glycogenolysis [64], which converts stored glycogen into glucose. Heat stress in particular has been shown to increase oxidative stress in skeletal muscle [63,65]. Oxidative stress occurs when the balance of oxidants and antioxidants are in favour of the oxidants. Maintaining this redox homeostasis is important for good broiler health [55]. Birds have more robust antioxidant systems than mammals [66], but they can still be overwhelmed by oxidative stress conditions which can be brought on by a physiological stress condition. Stress leads to the formation of free radicals, the beginnings of oxidative stress, which can lead to muscular membranes losing their integrity [55]. The most common responses to oxidative stress are increased lipid peroxidation and creatine kinase activity. Although both combat oxidative stress, they can also cause tissue damage that can lead to the inhibition of growth [67]. Lipid peroxidation has been shown to increase in the liver and muscle of broiler chickens administered CORT [67]. Stress is clearly something to be avoided if possible as it has clear deleterious effects on production metrics. Another effect of stress is that it can predispose broilers to disease [68] as a result of immune system suppression [69,70], although the exact mechanisms are not fully understood. Stress also puts flocks at risk of vaccine failure and increased morbidity and mortality [71,72]. As a result of the profound negative effects that stress can impart on chicken well-being and production health, it would be beneficial to both chicken producers and bird researchers in general to have a biomarker of stress that

is correlated with bird health and is predictive of subsequent adverse health outcomes (e.g. disease manifestation). Moreover, a biomarker that can be measured in a non-destructively obtained sample from birds would be highly beneficial.

1.5 Biomarkers of stress in chickens

As a result of the transitory presence of hormones following exposure to a stressor, coupled with the logistical challenges of obtaining representative blood samples from birds in a production flock, the ability to measure relevant biomarkers in samples such as feces and feathers, which can be obtained with little to no handling of the birds, is of considerable interest. Although the stress hormone, CORT, cannot be directly measured in feces, metabolites of CORT can be measured in chicken feces as an indicator of stress [73-75]. However, there has been little research into other biomarkers of stress that can be measured in fecal samples. Feces also allows for analyses that blood does not permit, such as characterization of the fecal bacterial microbiome and its functional output (metabolome). For example, the fecal metabolome reflects host-microbiome interactions, with up to 68% of its variance arising from the gut microbiota, not the host [76]. As the relationship between the gut microbiome and the host's health becomes more evident, there is increasing interest in using fecal metabolomics as an indirect and non-invasive diagnostic indicator of host health.

Feathers are much like hair in that they are produced and supported by follicles in the skin [77]. A feather consists of a cylindrical shaft, called a calamus, which starts in the follicle and a long portion called a rachis [78-80]. The rachis is highly vascularized and creates a dermal core, referred to as feather pulp or feather follicle epithelium (FFE), which is surrounded by feather tissue and an outer sheath (**Figure 1-4**) [81].

Although there are similarities with hair, feathers differ in that they have nerves and their own muscles [80]. Feather collection can be less invasive than blood, particularly if molted feathers can be used [82]. Furthermore, feathers are often used for detecting pathogens that incite important diseases such as avian leukosis virus [83], West Nile virus [84], and Marek's disease virus [85]. It is currently unclear how CORT is deposited in feathers and how well CORT in feather reflects stress levels [86], but it is known that the concentration of CORT in feathers fluctuates [49] and is only representative of the time while the feather was actively growing [49]. Thus, feathers incorporate both the duration and amplitude of glucocorticoid secretion [87], and as such, are best suited to study the activity of the HPA axis over relatively long time frames. This contrasts with blood, which provides a snapshot of the activity of the HPA (e.g. at the time of blood sampling), because blood CORT levels change quickly and frequently. There are disadvantages to using CORT in feathers as a biomarker of stress. In this regard, CORT levels fluctuate in feathers depending on the season (with a particularly large drop during breeding season) resulting in a need for control matched samples [49]. There has been some research into other biomarkers of stress that can be measured in feathers, such as heat shock protein 70 (HSP70), although this technique has been patented meaning it may be difficult and costly to adapt to different needs [88]. Feather pulp is highly vascularized during growth and it is a fairly easy sample to obtain as the calamus of a feather can be squeezed between a sample tube and its lid to collect the sample [83]. The disadvantage of targeting feather pulp is that feathers in rest phase, in which the feather is preparing to molt, have much less pulp due to the shrinking of the size of the follicle, and the older the bird, the larger the number of feathers in rest phase [89]. Thus, in older birds there may be insufficient quantities of feather pulp to obtain a sufficient sample quantity. There are

many potential advantages to using feather pulp as a sample, and the identification of a quantifiable biomarker of stress response and disease susceptibility in feather pulp would be of significant value to chicken producers and wild bird researchers alike.

1.6 Metabolomics

One possible way to evaluate the biological effects of stress in a sensitive and comprehensive manner is through metabolomics, which is the study of small molecular weight compounds, referred to as metabolites. Studying the metabolites present in a tissue or biofluid allows for the analysis of the metabolic responses of living systems to external stimuli, such as stressors. This is because metabolites are the end products of metabolism, and as such, represent the functional responses of a cell [90]. The collective characterization of metabolites can provide valuable insight into the biochemical pathways that are potentially dysregulated due to physiological stress. Importantly, this can identify novel and diagnostic biomarkers. For example, recent research has explored the use of metabolomics to examine chicken tissues for both model development [91] and to identify biomarkers of disease [59,92-94]. Another key advantage of looking at metabolites (metabolomics), instead of genes (genomics) or proteins (proteomics) is logistical. For example, there are approximately 10^4-10^5 genes and 10^7 proteins, but there are only 10^3-10^4 metabolites, which simplifies the identification of relevant biomarkers [95].

There are two main methods of studying metabolomics: Mass Spectrometry (MS) and Nuclear Magnetic Resonance (NMR) spectroscopy [96-99]. MS provides a higher degree of sensitivity and is often used for targeted or selective analyses; however, this technique requires a large amount of demanding sample preparation, often involving

chemical derivatization, and offers only moderate reproducibility. NMR offers quantitative measurement, high reproducibility, and requires relatively minimal sample preparation [97,99-101], but has a lower level of sensitivity and specificity. However, as technology improves, the sensitivity of NMR is expected to increase through the development of larger magnetic fields, signal enhancement techniques, and more accessible cryogenic probe technology [102]; Also, if quantification of the metabolites is required, concentrations determined by NMR are more reliable [103]. Research presented in this thesis will focus on the use of NMR based metabolomics.

Samples selected for metabolomics analysis can be solids, such as tissues, or biofluids, such as saliva, blood (plasma or serum), cerebrospinal fluid, amniotic fluid, or urine [97]. Regardless of the sample chosen, the first step is to extract the metabolites from the sample. In the case of NMR-based metabolomics, it is typically the water-soluble and non-volatile metabolites that are investigated as the samples are dissolved in a buffer containing a mixture of deuterated and non-deuterated water, although it is also possible to investigate the lipid-soluble metabolites using alternative NMR solvents. There are many different extraction methods that can be utilized to obtain water soluble metabolites from biofluids and tissues [90,104,105], but common to all is the requirement to remove large macromolecules (ex. DNA and RNA), proteins, and fats from the sample. This is because all three of these biomolecules produce too many overlapping NMR peaks and broad NMR signals, both of which are undesirable for carrying out accurate NMR-based metabolomic measurements. In addition, proteins can bind to the chemical shift indicator, sodium 3-trimethylsilylpropanoate-2,2,3,3-d4 (TSP), and this leads to the acquisition of lower quality NMR spectra. The three extraction methods used in this thesis are: (1) ultrafiltration using a 3 kDa cut-off filter; (2) a methanol-chloroform-based

fat and protein extraction (Bligh & Dyer, 1959; Wu et al., 2008); and (3) no extraction in which the sample is added to buffer, vortexed/homogenized, and centrifuged.

1.7 ^{1}H-NMR

Introduction to spin and angular momentum

Proton NMR (^{1}H-NMR) is becoming one of the most popular ways to characterize the metabolome. All atoms are made up of three fundamental particles, which are electrons, protons, and neutrons, with the exception of ^{1}H. The nucleus of an atom is made up of protons and neutrons (called nucleons), and it is the nucleus of atoms that are important in NMR. Every nucleus possesses mass, charge, and spin. Spin (I) can be a positive integer (0, 1, 2, …, n) or half-integer (^{1}⁄$_{2}$, ^{3}⁄$_{2}$, …, ^{n}⁄$_{2}$), and isotopes of the same element may have different quantum numbers (e.g. ^{12}C has a spin quantum number of 0, whereas ^{13}C has a spin quantum number of ½). Nuclei with non-zero spin possess spin angular momentum (**I**), a vector, where both the magnitude and the direction are quantized. This gives 2I + 1 possible projections of the vector onto an axis, z, which is called the magnetic quantum number, m. For example, a nucleus with spin of ½ has two possible spin angular momentum values, which are m = ½ and -½, while a nucleus with spin of 1 has three possible values, which are m = -1, 0, and 1.

Usually, the energies from the different spin angular momentum values are equivalent (degenerate), and therefore cannot be used spectroscopically (the 2I+1 energy levels of a spin -1 nucleus have the same energy); however, when an external magnetic field is applied it creates a difference in the energies that can be used to perform NMR spectroscopy. When a nucleus is placed in an external magnetic field its magnetic moment (**μ**) interacts with the magnetic field ($\mathbf{B}_0$) and experiences a torque (**τ**). The torque causes the nucleus to precess (rotate) along the direction of the applied magnetic

field at an angular frequency of ω (**Figure 1-5**). The angular frequency is proportional to the strength of the applied magnetic field and the gyromagnetic ratio (also called magnetogyric ratio), which is a scaling factor derived from the mass ratio of each nucleus. The gyromagnetic ratio is unique to each nucleus and by Equation 1 the angular frequency is also unique to each nucleus and is known as the Larmor frequency.

$$\omega = -\gamma B_0 \qquad \textbf{Equation 1}$$

ω is the angular frequency / Lamor frequency (unique to each nucleus)

γ is the gyromagnetic ratio of the nucleus ($T^{-1}s^{-1}$)

B_0 is the strength of the magnetic field (T)

The energy of a magnetic moment in a magnetic field associated with this angular motion is then given by Equation 2.

$$E = -m\hbar\gamma B_0 \qquad \textbf{Equation 2}$$

Where E is the energy of a magnetic moment in a magnetic field

ħ is a constant called the reduced Planck's constant (1.055×10^{-34} J·s).

γ is the gyromagnetic ratio of the nucleus ($T^{-1}s^{-1}$)

B_0 is the strength of the magnetic field (T)

In the absence of a strong magnetic field, nuclear spins exist in all directions, and are not aligned to anything (**Figure 1-6A**). However, in the presence of a strong magnetic field, individual spins will align either with or against the field (**Figure 1-6B**). Parallel orientations correspond to the lower energy state (m = 1/2, spin up), and anti-parallel orientations correspond to a slightly higher energy state (m = -1/2, spin down). Due to the preference of being in a lower energy state, there will always be slightly more spins in the

spin up orientation creating a net magnetization parallel to the static magnetic field (**Figure 1-6C**).

The NMR experiment

There are three main components needed for an NMR experiment: (1) a strong magnetic field to polarize the spins; (2) radiofrequency (RF) radiation to excite the nuclear spins; and (3) equipment to detect the resulting NMR signal. The magnetic field is most often provided by running a constant current through a solenoid made of a metal alloy, such as niobium and tin or niobium and titanium, that superconducts (i.e. is resistance free) at extremely low temperatures (< 8 K) and can support a persistent current (**Figure 1-7D**). In order to achieve the required extreme low temperatures, the solenoid of the NMR magnet is submersed in a bath of liquid helium (T = 4.2 K, **Figure 1-7H**), which is then further surrounded by a vacuum jacket (**Figure 1-7G**), a secondary bath of liquid nitrogen (T = 77 K, **Figure 1-7F**) to reduce helium boil, and a final vacuum jacket (**Figure 1-7E**) to isolate the entire cryogenic system from atmospheric temperatures. This design, along with frequent fills of both liquid helium (once every 3 to 4 months) and liquid nitrogen (once every 1 to 2 weeks) ensures the magnet retains the current in the solenoid and continues to produce a strong and persistent magnetic field.

The NMR signal is detected as a change in the magnetization through time. Although the net nuclear magnetization is aligned with the z-axis, no change in the magnetization (signal) can be detected as the nuclear spins are rotating around the z-axis in a rapid (nanosecond time scale) and symmetric fashion. In this case, the change in the signal over time in the x- and y-axes averages to zero, as the modern electronics can only detect motion on the microsecond time scale, and the z component does not change. In order to detect the NMR signal, the magnetization from the nuclear spins must be put into the

transverse (x, y) plane. This is accomplished using a radio frequency (RF) pulse that is produced by a coil placed along the x-y plane (**Figure 1-7K**). The RF pulse must be applied at a frequency that is on-resonance, or the same frequency, as the nuclear spins being observed (the Larmor frequency) and be applied for the right amount of time to result in a 90-degree rotation of the spins into the transverse plane. Once the magnetization is in the transverse plane the RF pulse is turned off and the magnetization will precess back to where it normally aligns in a magnetic field (equilibrium), which is along the z-axis, through a process called relaxation (**Figure 1-8A**). During this relaxation process the precession in the x-y plane does not average to zero (there is a change to detect) and produces an oscillating magnetic field, which in turn induces a current in the same coil that was used to produce the RF pulse. The change over time in the induced current (signal) is referred to as the free induction delay (FID) (**Figure 1-8B**). The FID then undergoes Fourier Transformation to produce a spectrum (signal as a function of frequency) (**Figure 1-8C**).

Chemical shift and J-coupling

All hydrogen nuclei in a sample are placed in the same strength of static magnetic field ($\mathbf{B}_0$) and based on equation 1, it would be expected that all the protons in the sample would precess at the same frequency ω. However, this is not the case. The magnetic field experienced by each proton is not just $\mathbf{B}_0$ but the sum of $\mathbf{B}_0$ plus the tiny magnetic fields generated by the circulating electrons surrounding each nucleus. In other words, nuclear spins experience a local magnetic field (B_{Local}) which is the total of the static magnetic field and an induced magnetic field (Equation 3).

$$\omega = \gamma B_{Local} = \gamma(B_0 - B_{electrons}) \qquad \textbf{Equation 3}$$

Where ω is the angular frequency of the precession of the nucleus

γ is the gyromagnetic ratio of the nucleus ($T^{-1}s^{-1}$)

B_{Local} is the magnetic field experienced by the nucleus (T)

B_0 is the strength of the magnetic field (T)

$B_{electrons}$ is the strength of the magnetic field created by the electrons near the nucleus (T)

The induced magnetic field is small, especially when compared to the static magnetic field, but produces a small shift in the angular frequency known as the chemical shift (δ). Electrons around a proton nucleus tend to create a small magnetic field in the opposing direction of $\mathbf{B}_0$, which effectively decreases the magnitude of the external magnetic field experienced by the proton. The more electrons near a proton, the lower the frequency (shielding), and the fewer electrons near a proton, the higher the frequency (deshielding). In addition, there is a high degree of consistency in the amount of shielding or deshielding that is caused by different functional groups, and this knowledge has been applied to help identify structural elements of molecules using NMR. This sensitivity of the nuclear frequency to its immediate and surrounding chemical environment is what leads to each metabolite having a unique fingerprint.

Frequencies of chemical shifts in an NMR spectrum are given in parts per million (ppm), which makes the chemical shift independent of the strength of the static magnetic field (B_o). The reference frequency is usually the proton shifts from a standard molecule that are assigned to be zero ppm. In the case of non-water-soluble compounds, the zero ppm frequency is usually assigned to the resonance frequency of protons in tetramethylsilane (TMS). However, in metabolomics the solvent used is water and TMS

is not water soluble. In this case, the water soluble 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) or trimethylsilylpropionic acid (TSP) are most often used as chemical shift references. The methyl protons of TSP and DSS have almost identical chemical shifts to TMS and are used as the zero ppm chemical shift reference for metabolomics.

In solution-state NMR, spins are not isolated from one another, and neighbouring spins that are not chemically equivalent affect the chemical shift in a process called J-coupling. J-coupling results in multiplets in the spectrum based on the 2NI + 1 rule, where I is the spin of the nucleus and n is the number of inequivalent spin neighbours within three bonds. For example, in the case of the protons (I = ½) in a terminal ethyl group (R-CH_2CH_3), where R is not a methyl (CH_3), methylene (CH_2), or methine (CH), the methyl group is adjacent to two inequivalent hydrogens spins (N = 2), and this results in the chemical shift of the methyl group being split into three lines, or a triplet (2 x 2 x ½ +1 = 3) (**Figure 1-9**). Inversely, the hydrogens in the CH_2 group are adjacent to three (N = 3) inequivalent hydrogen spins and the chemical shift of the CH_2 is split into four lines or a quartet (2 x 3 x ½ +1 = 4) (**Figure 1-9**). Ultimately, the multiplet structure and chemical shift of a proton signal provide a wealth of information about the connectivity of functional groups (bonding network) of the molecule.

1.8 Thesis scope

There is currently a lack of reliable and quantifiable biomarkers of stress for chickens, which will become more important in an increasingly antibiotic-free world. It is known that stress predisposes birds to disease and has adverse effects on the production and health of chickens. However, the identification of biomarkers in samples that require minimal or no handling of chickens, such as feathers and feces, correlated with stress

would be very beneficial in managing disease and optimizing production. In that regard, this thesis has three main hypotheses and objectives:

Hypothesis 1: CORT administered in water will incite a systemic metabolic cascade including the stimulation of catabolic processes in liver, kidney, and breast muscle.

Objective 1: Investigate the effects of CORT administered in water on the metabolome of liver, kidney, and breast muscle of chickens.

Discussed in chapter 2

Hypothesis 2: *In silico* baseline correction following solvent extraction of feces will provide comparable ^{1}H-NMR metabolomics profiles to those obtained with the ultrafiltration method, but at a lower cost.

Objective 2: Compare and contrast water-soluble metabolite extraction protocols for ^{1}H-NMR metabolomics analysis of fecal samples, including the application of *in silico* spectral baseline correction, to determine the most reproducible and cost-effective method.

Discussed in chapter 3

Hypothesis 3: The vascularized feather pulp will generate a ^{1}H-NMR spectrum containing quantifiable information from numerous metabolites, similar to the metabolites found in serum, and the feather pulp metabolome will be sensitive to biological factors such as age and CORT administered to chickens.

Objective 3: Determine if ^{1}H-NMR spectra can be produced from chicken feather pulp and the best processing method for metabolomics studies, as well as the effects of age and stress on the feather pulp metabolome.

Discussed in chapter 4

Table 1-1: Animal food production efficiencies [17]

	Chicken	**Pork**	**Beef**	**Carp**	**Salmon**
Feed Efficiency (kg feed / kg live weight)	2.5	4.0	8.0	1.5	0.9
Edible portion of live weight (%)	55.0	55.0	40.0	65.0	65.0
Feed Efficiency (kg feed / kg edible weight)	4.5	7.3	20.0	2.3	1.4
Protein content (% of edible weight)	20.0	14.0	15.0	18.0	22.0

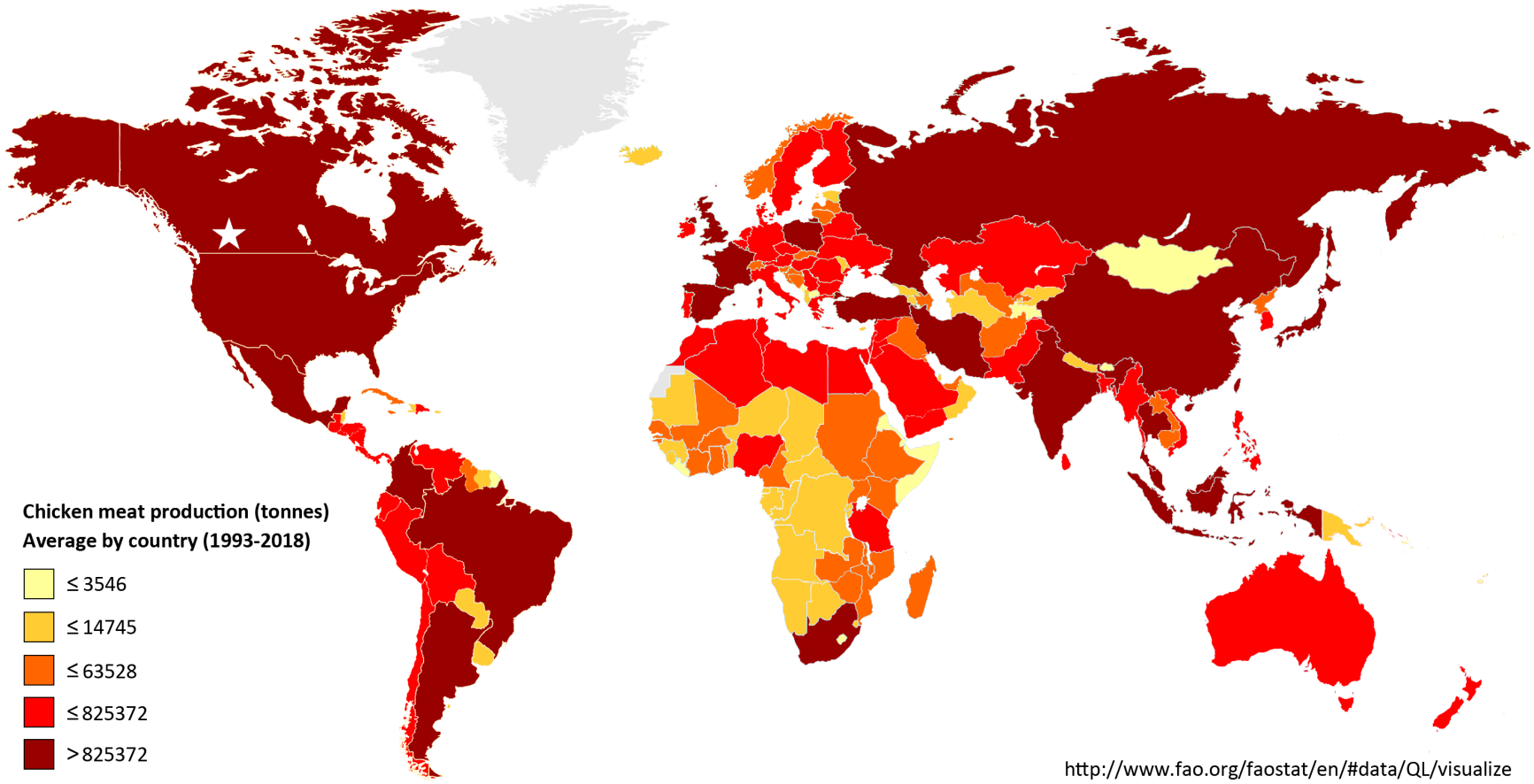

Figure 1-1: Average global production of chicken meat. The star shows southwestern Alberta. The figure was generated from data obtained from the Food and Agriculture Organization of the United Nations [10] (Figure provided by Douglas Inglis)

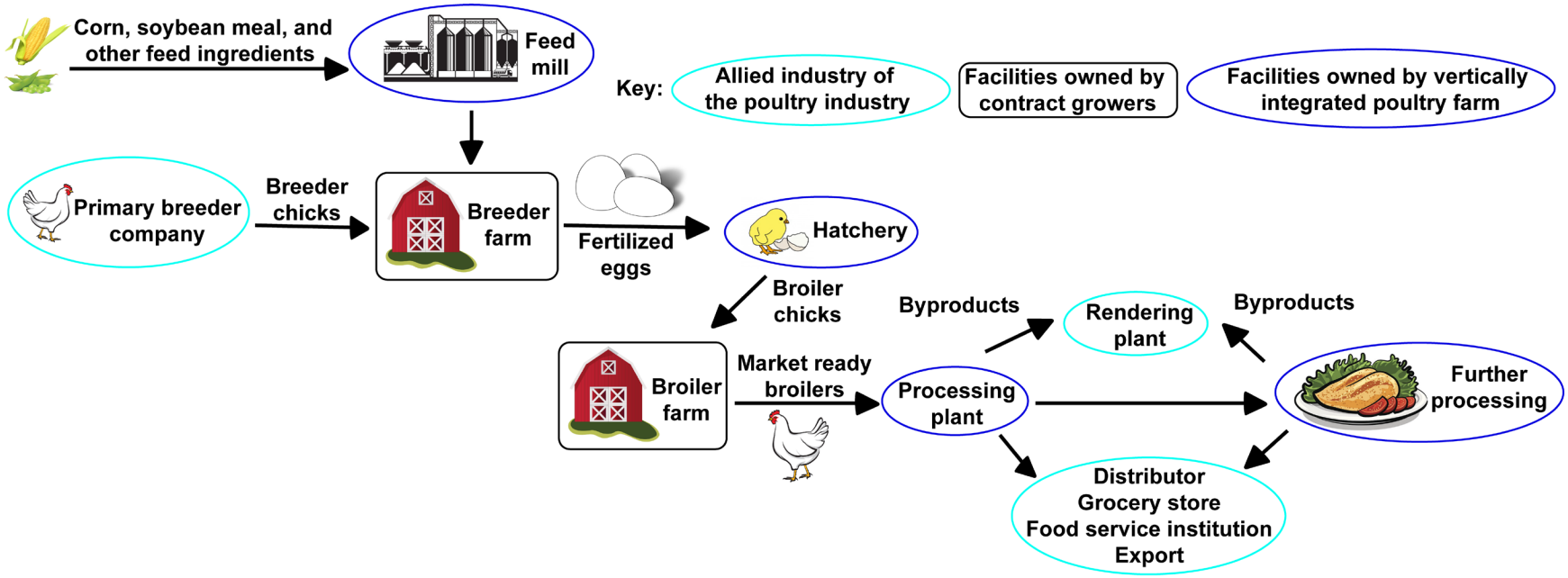

Figure 1-2: Typical operation of a vertically integrated broiler chicken farm

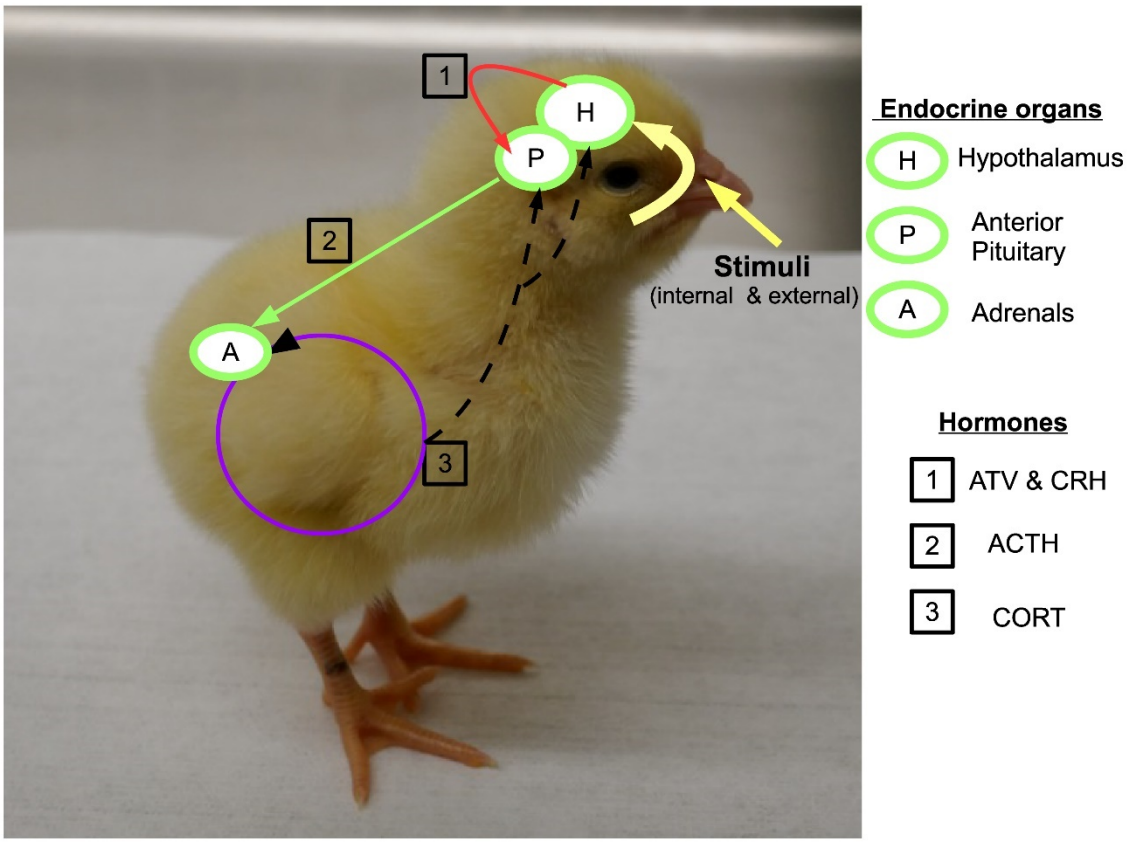

Figure 1-3: The Hypothalamo-Pituitary-Adrenal (HPA) axis

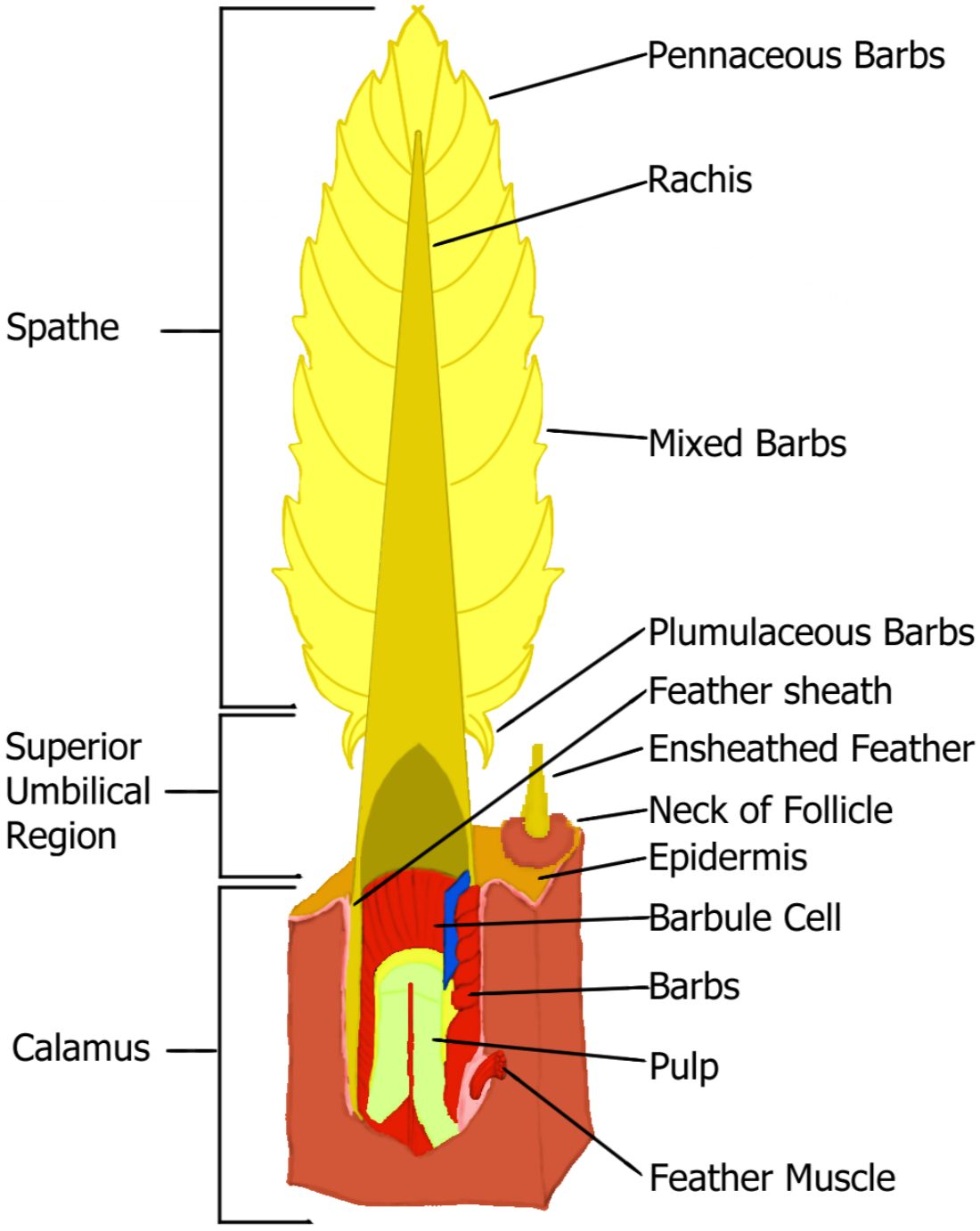

Figure 1-4: Diagram of a feather (Figure provided by Matthew Hutchinson)

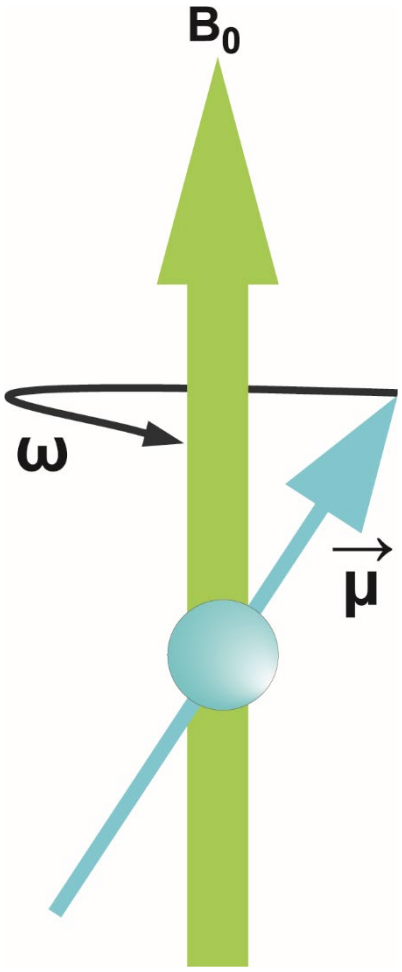

Figure 1-5: Precession of the magnetic moment of a nucleus in a static magnetic field

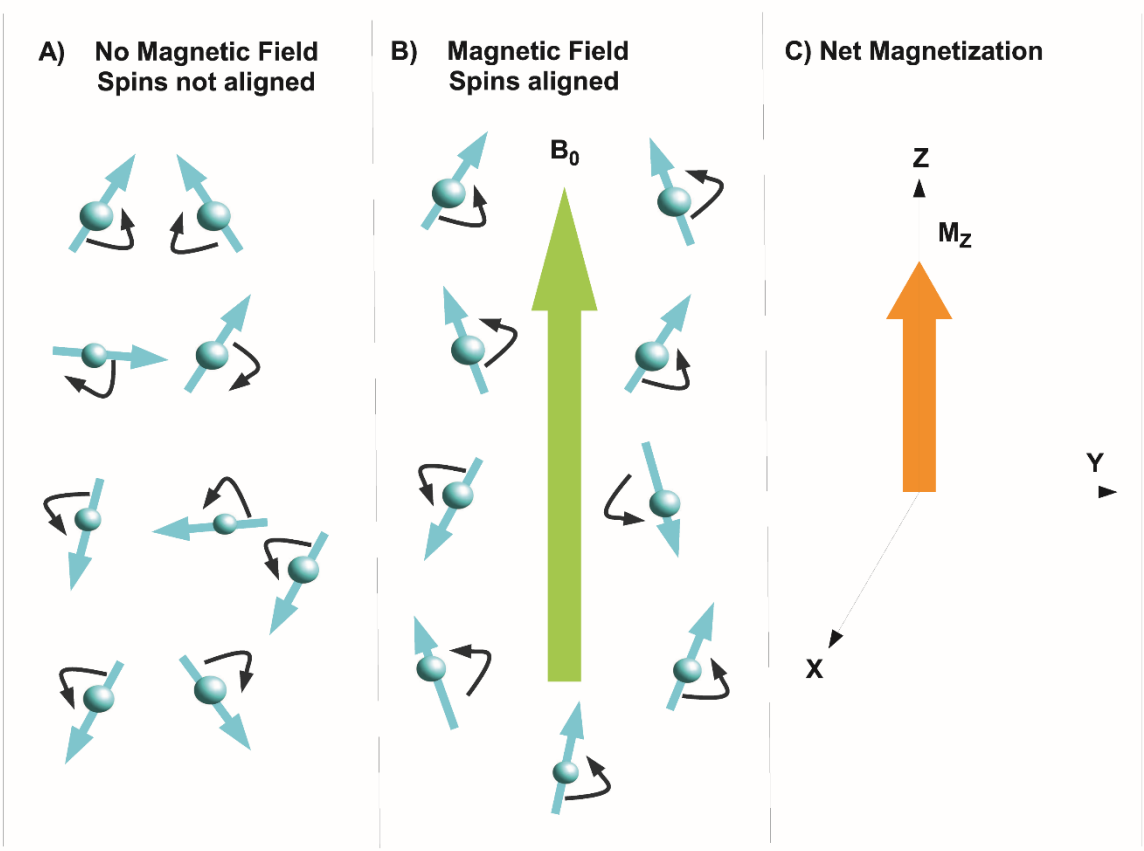

Figure 1-6: Spin. A) the spins of nuclei not in a magnetic field; B) the spins aligned in a static magnetic field; C) the net magnetization caused by the spins aligning in a static magnetic field

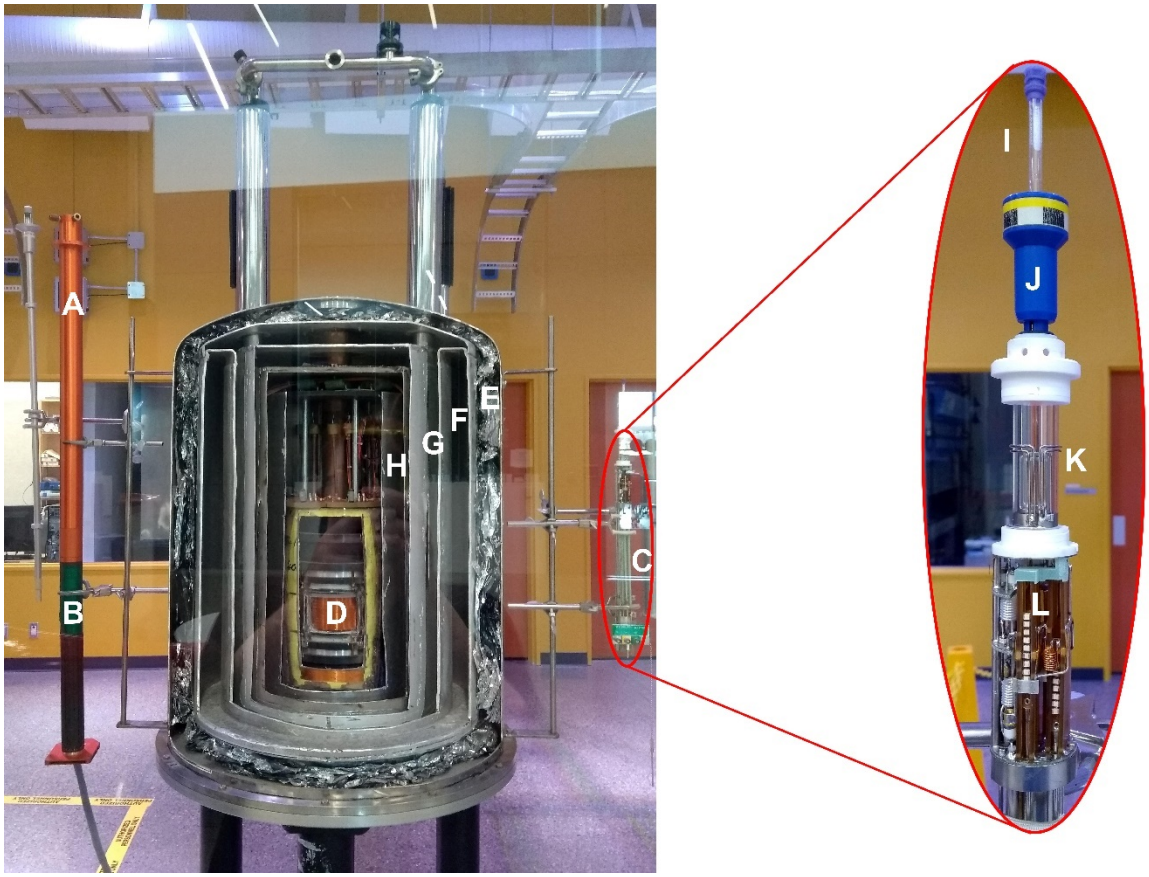

Figure 1-7: Inside an NMR magnet and probe. A) Sample transport tube; B) Room temperature shim sleeve; C) Probe; D) Superconducting solenoid; E) Vacuum jacket with mylar insulation; F) Liquid nitrogen cooling jacket; G) Second vacuum jacket; H) Liquid helium bath; I) Glass NMR sample tube; J) Spinner; K) Radiofrequency (RF); coils L) RF circuit tuning components

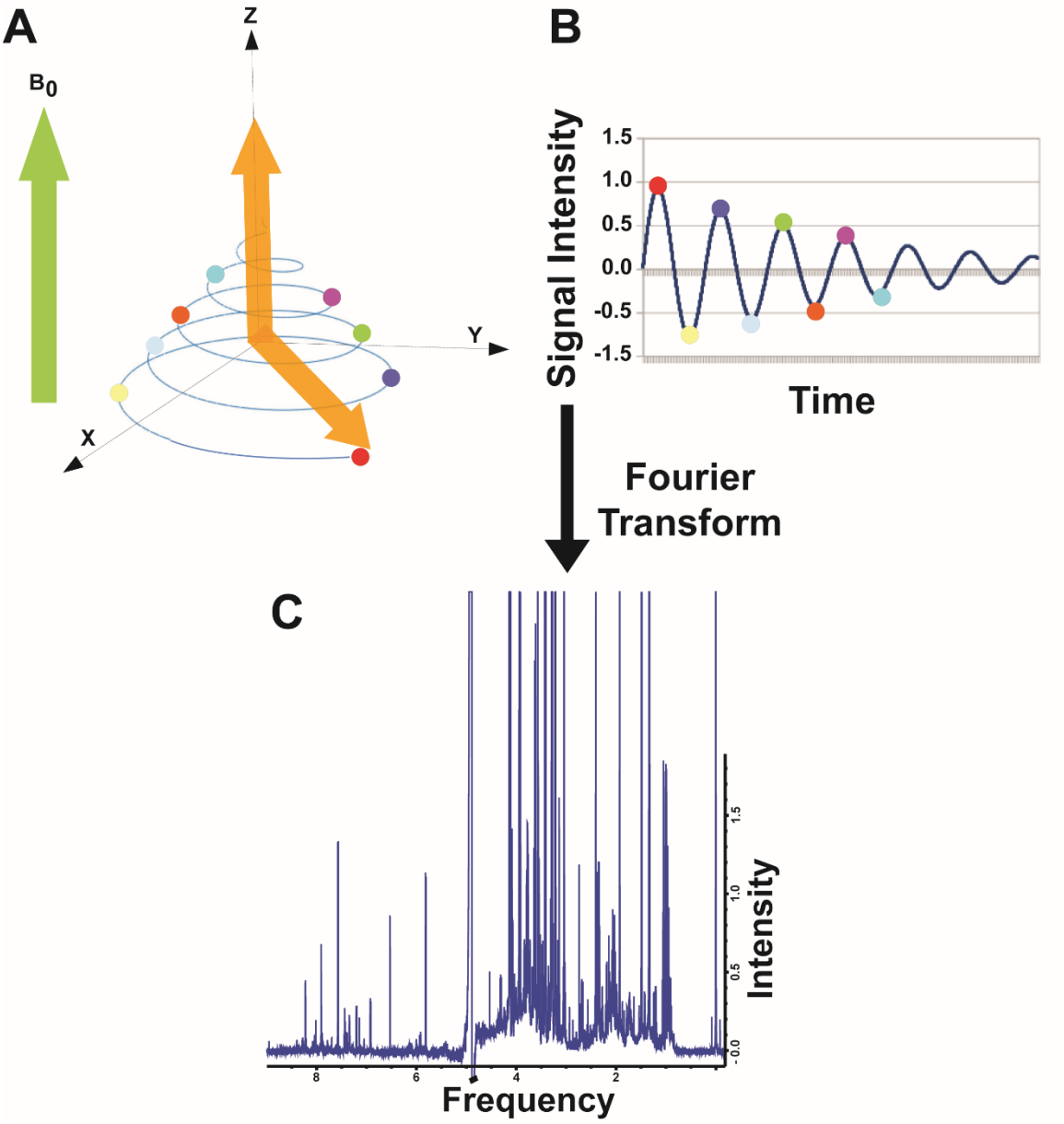

Figure 1-8: Relaxation, free induction decay (FID), and ^{1}H-NMR spectrum. A) conical trajectory of the transverse magnetization during relaxation; B) Resulting FID; C) ^{1}H-NMR spectrum resulting from Fourier transformation

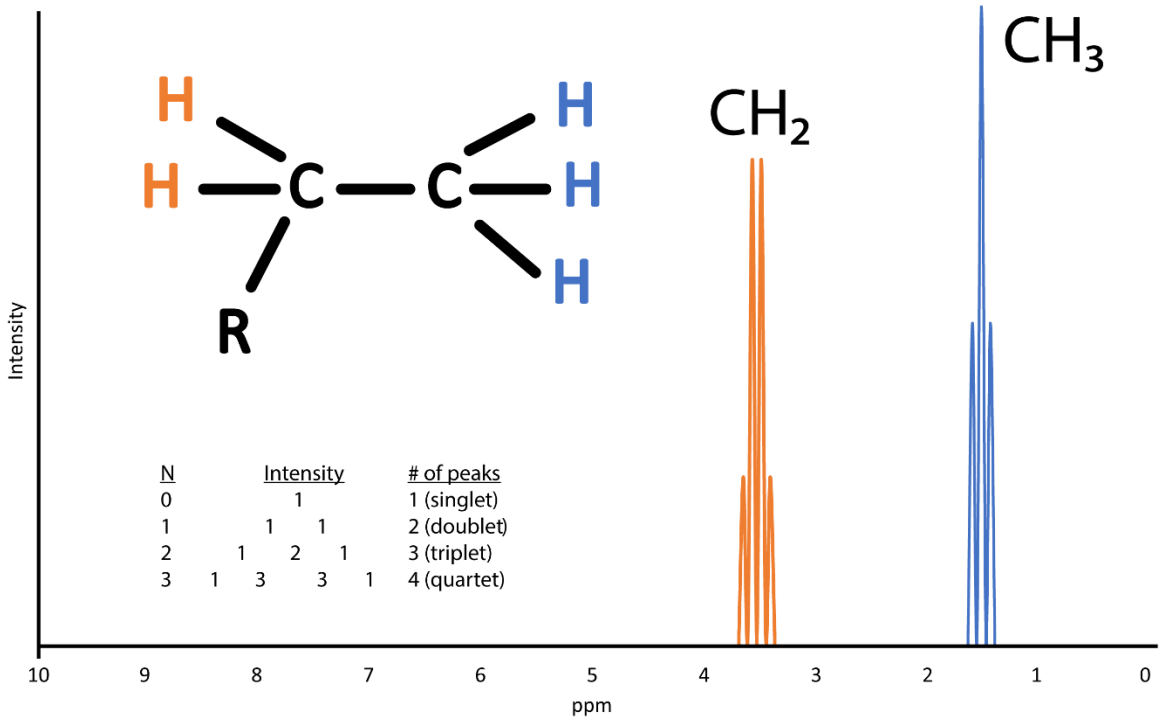

Figure 1-9: Proton NMR spectrum for the protons in a terminal ethyl group (R-CH_2CH_3), where R is not a methyl (CH_3), methylene (CH_2), or methine (CH). The Inset (left) shows Pascal's triangle giving the splitting pattern produced according to the 2NI + 1 rule for spin-spin coupling and the theoretical ratio of line intensities for different numbers of adjacent protons (N)

1.9 References

1. Fumihito, A.; Miyake, T.; Sumi, S.; Takada, M.; Ohno, S.; Kondo, N. One subspecies of the red junglefowl (*gallus gallus gallus*) suffices as the matriarchic ancestor of all domestic breeds. *Proc. Natl. Acad. Sci. U. S. A.* **1994**, *91*, 12505-12509, doi:10.1073/pnas.91.26.12505.

2. Appleby, M.C.; Mench, J.A.; Hughes, B.O. *Poultry behaviour and welfare*; CABI Pub.: Cambridge, MA, USA; Wallingford, Oxfordshire, UK, 2004.

3. West, B.; Zhou, B.-X. Did chickens go north? New evidence for domestication. *Worlds Poult. Sci. J.* **1989**, *45*, 205-218, doi:10.1079/WPS19890012.

4. Fumihito, A.; Miyake, T.; Takada, M.; Shingu, R.; Endo, T.; Gojobori, T.; Kondo, N.; Ohno, S. Monophyletic origin and unique dispersal patterns of domestic fowls. *Proc. Natl. Acad. Sci. U. S. A.* **1996**, *93*, 6792-6795, doi:10.1073/pnas.93.13.6792.

5. Nätt, D.; Rubin, C.-J.; Wright, D.; Johnsson, M.; Beltéky, J.; Andersson, L.; Jensen, P. Heritable genome-wide variation of gene expression and promoter methylation between wild and domesticated chickens. *BMC Genomics* **2012**, *13*, 59, doi:10.1186/1471-2164-13-59.

6. Sawai, H.; Kim, H.L.; Kuno, K.; Suzuki, S.; Gotoh, H.; Takada, M.; Takahata, N.; Satta, Y.; Akishinonomiya, F. The origin and genetic variation of domestic chickens with special reference to jungle fowls *gallus g. Gallus* and *g. Varius*. *PLoS One* **2010**, *5*, doi:10.1371/journal.pone.0010639.

7. Garnham, L.; Løvlie, H. Sophisticated fowl: The complex behaviour and cognitive skills of chickens and red jungle fowl. *Behav. Sci.* **2018**, *8*, doi:10.3390/bs8010013.

8. Druyan, S. The effects of genetic line (broilers vs. Layers) on embryo development. *Poult. Sci.* **2010**, *89*, 1457-1467, doi:10.3382/ps.2009-00304.

9. Mottet, A.; Tempio, G. Global poultry production: Current state and future outlook and challenges. *Worlds Poult. Sci. J.* **2017**, *73*, 245-256, doi:10.1017/s0043933917000071.

10. Food and Agriculture Organization of the United Nations. Fao statistical database. **2021**.

11. Havenstein, G.B.; Ferket, P.R.; Qureshi, M.A. Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poult. Sci.* **2003**, *82*, 1500-1508, doi:10.1093/ps/82.10.1500.

12. Bedford, E. *Poultry industry in canada*; did-66177-1; Statista: Sept 10 2020 2020.

13. Linden, J. Global poultry trends 2014: Growth in chicken consumption in americas slows. Available online: https://www.thepoultrysite.com/articles/global-poultry-trends-2014-growth-in-chicken-consumption-in-americas-slows (accessed on 16 June 2021).

14. Linden, J. Global poultry trends 2014: Poultry set to become no.1 meat in asia. Available online: https://www.thepoultrysite.com/articles/global-poultry-trends-2014-poultry-set-to-become-no1-meat-in-asia (accessed on 16 June 2021).

15. Muaz, K.; Riaz, M.; Akhtar, S.; Park, S.; Ismail, A. Antibiotic residues in chicken meat: Global prevalence, threats, and decontamination strategies: A review. *J. Food Prot.* **2018**, *81*, 619-627, doi:10.4315/0362-028X.JFP-17-086.

16. Windhorst, H.-W. Changes in poultry production and trade worldwide. *Worlds Poult. Sci. J.* **2006**, *62*, 585, doi:10.1017/s0043933906001140.

17. Smil, V. Chapter 5: Rationalizing animal food production. In *Feeding the world: A challenge for the twenty-first century*; MIT: 2000; pp. 141-180.

18. Fairchild, B.D.; Ritz, C.W. *Poultry drinking water primer*; University of Georgia: 2015.

19. Gerbens-Leenes, P.W.; Mekonnen, M.M.; Hoekstra, A.Y. The water footprint of poultry, pork and beef: A comparative study in different countries and production systems. *Water Resources and Industry* **2013**, *1-2*, 25-36, doi:10.1016/j.wri.2013.03.001.

20. Freelancer. How much water does it take to produce meat? Available online: https://www.thepoultrysite.com/news/2016/04/how-much-water-does-it-take-to-produce-meat (accessed on 2021 09 14).

21. Mekonnen, M.M.; Hoekstra, A.Y. A global assessment of the water footprint of farm animal products. *Ecosystems* **2012**, *15*, 401-415, doi:10.1007/s10021-011-9517-8.

22. The Business Research Company. *Poultry global market report 2021: Covid-19 impact and recovery to 2030*; 5240275; The Business Research Company: 2021.

23. Canadian Chicken Farmers. What is supply management? Available online: https://www.chickenfarmers.ca/what-is-supply-management/ (accessed on June 23 2021).

24. Alberta Chicken Producers. Chicken industry. Available online: https://chicken.ab.ca/chicken-industry/ (accessed on June 23 2021).

25. Lang, L. Supply management system. Available online: http://www.omafra.gov.on.ca/english/farmproducts/factsheets/4supply.htm (accessed on June 23 2021).

26. Ajaltouny, A. Customized report service – canadian exports of poultry by country Available online: https://www.agr.gc.ca/eng/international-trade/market-intelligence/reports/customized-report-service-canadian-exports-of-poultry-by-country/?id=1567695977652 (accessed on June 23 2021).

27. Alberta Chicken Producers. Market information. Available online: https://www.chicken.ab.ca/market-information/ (accessed on June 23 2021).

28. Chicken Farmers of Canada. *Chicken farmers of canada's sustainability report*; 2018.

29. Prescott, J.F. Veterinary antimicrobial stewardship in north america. *Aust. Vet. J.* **2019**, *97*, 243-248, doi:10.1111/avj.12811.

30. Chicken Farmers of Canada. Questions and answers. Available online: https://www.chickenfarmers.ca/faq/ (accessed on Sept 10 2021).

31. Cosby, D.E.; Cox, N.A.; Harrison, M.A.; Wilson, J.L.; Buhr, R.J.; Fedorka-Cray, P.J. *Salmonella* and antimicrobial resistance in broilers: A review. *Journal of Applied Poultry Research* **2015**, *24*, 408-426, doi:10.3382/japr/pfv038.

32. Dibner, J.J.; Richards, J.D. Antibiotic growth promoters in agriculture: History and mode of action. *Poult. Sci.* **2005**, *84*, 634-643, doi:10.1093/ps/84.4.634.

33. Hossan, S.; Huque, K.S.; Kamaruddin, K.M.; Haque Beg, A. Global restriction of using antibiotic growth promoters and alternative strategies in poultry production. *Sci. Prog.* **2018**, *101*, 52-75, doi:10.3184/003685018x15173975498947.

34. Economou, V.; Gousia, P. Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infection and Drug Resistance* **2015**, *8*, 49-61, doi:10.2147/IDR.S55778.

35. Moore, P.R.; Evenson, A.; Luckey, T.D.; Mccoy, E.; Elvehjem, C.A.; Hart, E.B. Use of sulfasuxidine, streptothricin, and streptomycin in nutritional studies with the chick. *J. Biol. Chem.* **1946**, *165*, 437-441, doi:10.1016/s0021-9258(17)41154-9.

36. Stokstad, E.L.R.; Jukes, T.H. Further observations on the "animal protein factor". *Proc. Soc. Exp. Biol. Med.* **1950**, *73*, 523-528, doi:10.3181/00379727-73-17731.

37. Jukes, H.G.; Hill, D.C.; Branion, H.D. Effect of feeding antibiotics on the intestinal tract of the chick. *Poult. Sci.* **1956**, *35*, 716-723, doi:10.3382/ps.0350716.

38. Brown, K.; Uwiera, R.R.E.; Kalmokoff, M.L.; Brooks, S.P.J.; Inglis, G.D. Antimicrobial growth promoter use in livestock: A requirement to understand their modes of action to develop effective alternatives. *Int. J. Antimicrob. Agents* **2017**, *49*, 12-24, doi:10.1016/j.ijantimicag.2016.08.006.

39. Ma, F.; Xu, S.; Tang, Z.; Li, Z.; Zhang, L. Use of antimicrobials in food animals and impact of transmission of antimicrobial resistance on humans. *Biosafety and Health* **2021**, *3*, 32-38, doi:10.1016/j.bsheal.2020.09.004.

40. Diarra, M.S.; Malouin, F. Antibiotics in canadian poultry productions and anticipated alternatives. *Front. Microbiol.* **2014**, *5*, 282-282, doi:10.3389/fmicb.2014.00282.

41. Food and drug regulations.

42. Regulations amending the food and drug regulations (veterinary drugs — antimicrobial resistance). **2017**, *151*.

43. Graham, J.P.; Boland, J.J.; Silbergeld, E. Growth promoting antibiotics in food animal production: An economic analysis. *Public Health Rep.* **2007**, *122*, 79-87, doi:10.1177/003335490712200111.

44. Shini, S.; Kaiser, P. Effects of stress, mimicked by administration of corticosterone in drinking water, on the expression of chicken cytokine and chemokine genes in lymphocytes. *Stress* **2009**, *12*, 388-399, doi:10.1080/10253890802526894.

45. Hangalapura, B.N.; Nieuwland, M.G.B.; Buyse, J.; Kemp, B.; Parmentier, H.K. Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. *Poult. Sci. Assoc.* **2004**, *83*, 1644-1649, doi:10.1093/ps/83.10.1644.

46. Zhao, F.Q.; Zhang, Z.W.; Qu, J.P.; Yao, H.D.; Li, M.; Li, S.; Xu, S.W. Cold stress induces antioxidants and hsps in chicken immune organs. *Cell Stress Chaperones* **2014**, *19*, 635-648, doi:10.1007/s12192-013-0489-9.

47. Zulkifli, I.; Najafi, P.; Nurfarahin, A.J.; Soleimani, A.F.; Kumari, S.; Aryani, A.A.; O'reilly, E.L.; Eckersall, P.D. Acute phase proteins, interleukin 6, and heat shock protein 70 in broiler chickens administered with corticosterone. *Poult. Sci.* **2014**, *93*, 3112-3118, doi:10.3382/ps.2014-04099.

48. Scanes, C.G. Biology of stress in poultry with emphasis on glucocorticoids and the heterophil to lymphocyte ratio. *Poult. Sci.* **2016**, *95*, 2208-2215, doi:10.3382/ps/pew137.

49. Bortolotti, G.R.; Marchant, T.A.; Blas, J.; German, T. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. *Funct. Ecol.* **2008**, *22*, 494-500, doi:10.1111/j.1365-2435.2008.01387.x.

50. Cockrem, J.F. Stress, corticosterone responses and avian personalities. *J. Ornithol.* **2007**, *148*, 169-178, doi:10.1007/s10336-007-0175-8.

51. Romero, L.M.; Reed, J.M. Collecting baseline corticosterone samples in the field: Is under 3 min good enough? *Comparative Biochemistry and Physiology Part A:*

Molecular & Integrative Physiology **2005**, *140*, 73-79, doi:10.1016/j.cbpb.2004.11.004.

52. Fulda, S.; Gorman, A.M.; Hori, O.; Samali, A. Cellular stress responses: Cell survival and cell death. *Int. J. Cell Biol.* **2010**, *2010*, 1-23, doi:10.1155/2010/214074.

53. Haussmann, M.F.; Longenecker, A.S.; Marchetto, N.M.; Juliano, S.A.; Bowden, R.M. Embryonic exposure to corticosterone modifies the juvenile stress response, oxidative stress and telomere length. *Proceedings of the Royal Society B: Biological Sciences* **2012**, *279*, 1447-1456, doi:10.1098/rspb.2011.1913.

54. Richter, K.; Haslbeck, M.; Buchner, J. The heat shock response: Life on the verge of death. *Mol. Cell* **2010**, *40*, 253-266, doi:10.1016/j.molcel.2010.10.006.

55. Lin, H.; Decuypere, E.; Buyse, J. Oxidative stress induced by corticosterone administration in broiler chickens (*gallus gallus domesticus*) 1. Chronic exposure. *Comp. Biochem. Physiol. B* **2004**, *139*, 737-744, doi:10.1016/j.cbpc.2004.09.013.

56. Lin, H.; Gao, J.; Song, Z.G.; Jiao, H.C. Corticosterone administration induces oxidative injury in skeletal muscle of broiler chickens. *Poult. Sci.* **2009**, *88*, 1044-1051, doi:10.3382/ps.2008-00312.

57. Dai, S.F.; Wang, L.K.; Wen, A.Y.; Wang, L.X.; Jin, G.M. Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. *Br. Poult. Sci.* **2009**, *50*, 333-340, doi:10.1080/00071660902806947.

58. Hayashi, K.; Nagai, Y.; Ohstuka, A.; Tomita, Y. Effects of dietary corticosterone and trilostane on growth and skeletal muscle protein turnover in broiler cockerels. *Br. Poult. Sci.* **1994**, *35*, 789-798, doi:10.1080/00071669408417743.

59. Zaytsoff, S.J.M.; Brown, C.L.J.; Montina, T.; Metz, G.a.S.; Abbott, D.W.; Uwiera, R.R.E.; Inglis, G.D. Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens. *Sci. Rep.* **2019**, *9*, 19225-19238, doi:10.1038/s41598-019-52267-6.

60. Jastrebski, S.F.; Lamont, S.J.; Schmidt, C.J. Chicken hepatic response to chronic heat stress using integrated transcriptome and metabolome analysis. *PLoS One* **2017**, *12*, e0181900, doi:10.1371/journal.pone.0181900.

61. Chowdhury, V.S. Heat stress biomarker amino acids and neuropeptide afford thermotolerance in chicks. *J. Poult. Sci.* **2019**, *56*, 1-11, doi:10.2141/jpsa.0180024.

62. Gross, W.B.; Siegel, P.B.; Dubose, R.T. Some effects of feeding corticosterone to chickens. *Poult. Sci.* **1980**, *59*, 516-522, doi:10.3382/ps.0590516 %J Poultry Science.

63. Roushdy, E.M.; Zaglool, A.W.; El-Tarabany, M.S. Effects of chronic thermal stress on growth performance, carcass traits, antioxidant indices and the expression of hsp70, growth hormone and superoxide dismutase genes in two broiler strains. *J. Therm. Biol.* **2018**, *74*, 337-343, doi:10.1016/j.jtherbio.2018.04.009.

64. Siegel, H.S. Physiological stress in birds. *Bioscience* **1980**, *30*, 529-534, doi:10.2307/1307973.

65. Azad, M.a.K.; Kikusato, M.; Maekawa, T.; Shirakawa, H.; Toyomizu, M. Metabolic characteristics and oxidative damage to skeletal muscle in broiler chickens exposed to chronic heat stress. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **2010**, *155*, 401-406, doi:10.1016/j.cbpa.2009.12.011.

66. Simoyi, M.F.; Van Dyke, K.; Klandorf, H. Manipulation of plasma uric acid in broiler chicks and its effect on leukocyte oxidative activity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2002**, *282*, R791-R796, doi:10.1152/ajpregu.00437.2001.

67. Eid, Y.Z.; Ohtsuka, A.; Hayashi, K. Tea polyphenols reduce glucocorticoid-induced growth inhibition and oxidative stress in broiler chickens. *Br. Poult. Sci.* **2003**, *44*, 127-132, doi:10.1080/0007166031000085427.

68. Zaytsoff, S.J.M.; Lyons, S.M.; Garner, A.M.; Uwiera, R.R.E.; Zandberg, W.F.; Abbott, D.W.; Inglis, G.D. Host responses to *clostridium perfringens* challenge in a chicken model of chronic stress. *Gut Pathog.* **2020**, *12*, 12-24, doi:10.1186/s13099-020-00362-9.

69. Hoerr, F.J. Clinical aspects of immunosuppression in poultry. *Avian Dis.* **2010**, *54*, 2-15, doi:10.1637/8909-043009-Review.1.

70. Moore, R.J. Necrotic enteritis predisposing factors in broiler chickens. *Avian Pathol.* **2016**, *45*, 275-281, doi:10.1080/03079457.2016.1150587.

71. Shini, S.; Huff, G.R.; Shini, A.; Kaiser, P. Understanding stress-induced immunosuppression: Exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes. *Poult. Sci.* **2010**, *89*, 841-851, doi:10.3382/ps.2009-00483.

72. Blas, J. Stress in birds. In *Sturkie's avian physiology*, 6th ed.; Scanes, Ed.; Elsevier: 2015; pp. 769-810.

73. Rettenbacher, S.; Palme, R.; Hackl, R.; Bamberg, E. Distribution and elimination of radiolabelled corticosterone in chicken broilers. *Wien. Tierarztl. Monatsschr.* **2006**, *93*, 283-287.

74. Rettenbacher, S.; Möstl, E.; Hackl, R.; Ghareeb, K.; Palme, R. Measurement of corticosterone metabolites in chicken droppings. *Br. Poult. Sci.* **2004**, *45*, 704-711, doi:10.1080/00071660400006156.

75. Alm, M.; Holm, L.; Tauson, R.; Wall, H. Corticosterone metabolites in laying hen droppings—effects of fiber enrichment, genotype, and daily variations. *Poult. Sci.* **2014**, *93*, 2615-2621, doi:10.3382/ps.2014-04193.

76. Zierer, J.; Jackson, M.A.; Kastenmueller, G.; Mangino, M.; Long, T.; Telenti, A.; Mohney, R.P.; Small, K.S.; Bell, J.T.; Steves, C.J.; et al. The fecal metabolome as a functional readout of the gut microbiome. *Nat. Genet.* **2018**, *50*, 790-795, doi:10.1038/s41588-018-0135-7.

77. Di-Poï, N.; Milinkovitch, M.C. The anatomical placode in reptile scale morphogenesis indicates shared ancestry among skin appendages in amniotes. *Sci. Adv.* **2016**, *2*, 1-8, doi:10.1126/sciadv.1600708.

78. Maderson, P.F.A.; Hillenius, W.J.; Hiller, U.; Dove, C.C. Towards a comprehensive model of feather regeneration. *J. Morphol.* **2009**, *270*, 1166-1208, doi:10.1002/jmor.10747.

79. Perry, R.J.; Wang, Y.; Cline, G.W.; Rabin-Court, A.; Song, J.D.; Dufour, S.; Zhang, X.M.; Petersen, K.F.; Shulman, G.I. Leptin mediates a glucose-fatty acid cycle to maintain glucose homeostasis in starvation. *Cell* **2018**, *172*, 234–248, doi:10.1016/j.cell.2017.12.001.

80. Stettenheim. The integumentary morphology of modern birds: An overview. *Am. Zool.* **2000**, *40*, 461-477, doi:10.1093/icb/40.4.461.

81. Harris, C.M.; Madliger, C.L.; Love, O.P. Temporal overlap and repeatability of feather corticosterone levels: Practical considerations for use as a biomarker. *Conservation Physiology* **2016**, *4*, 1-11, doi:10.1093/conphys/cow051.

82. Marsden, J.E.; May, B. Feather pulp: A non-destructive sampling technique for electrophoretic studies of birds. *The Auk* **1984**, *101*, 173-175, doi:10.1093/auk/101.1.173.

83. Zavala, G.; Jackwood, M.W.; Hilt, D.A. Polymerase chain reaction for detection of avian leukosis virus subgroup j in feather pulp. *Avian Dis.* **2002**, *46*, 971-978, doi:10.1637/0005-2086(2002)046[0971:PCRFDO]2.0.CO;2.

84. Docherty, D.E. *Corvidae* feather pulp and west nile virus detection. *Emerg. Infect. Dis.* **2004**, *10*, 907-909, doi:10.3201/eid1005.030825.

85. Cortes, A.L.; Montiel, E.R.; Lemiere, S.; Gimeno, I.M. Comparison of blood and feather pulp samples for the diagnosis of marek's disease and for monitoring marek's disease vaccination by real time-pcr. *Avian Dis.* **2011**, *55*, 302-310, doi:10.1637/9578-101510-ResNote.1.

86. Harris, C.M.; Madliger, C.L.; Love, O.P. An evaluation of feather corticosterone as a biomarker of fitness and an ecologically relevant stressor during breeding in the wild. *Oecologia* **2017**, *183*, 987-996, doi:10.1007/s00442-017-3836-1.

87. Fairhurst, G.D.; Marchant, T.A.; Soos, C.; Machin, K.L.; Clark, R.G. Experimental relationships between levels of corticosterone in plasma and feathers in a free-living bird. *J. Exp. Biol.* **2013**, *216*, 4071-4081, doi:10.1242/jeb.091280.

88. Greene, E.S.; Rajaei-Sharifabadi, H.; Dridi, S. Feather hsp70: A novel non-invasive molecular marker for monitoring stress induced by heat exposure in broilers. *Poult. Sci.* **2019**, *98*, 3400-3404, doi:10.3382/ps/pez120.

89. Chen, C.-F.; Foley, J.; Tang, P.-C.; Li, A.; Jiang, T.X.; Wu, P.; Widelitz, R.B.; Chuong, C.M. Development, regeneration, and evolution of feathers. *Annual Review of Animal Biosciences* **2015**, *3*, 169-195, doi:10.1146/annurev-animal-022513-114127.

90. Lin, C.Y.; Wu, H.; Tjeerdema, R.S.; Viant, M.R. Evaluation of metabolite extraction strategies from tissue samples using nmr metabolomics. *Metabolomics* **2007**, *3*, 55-67, doi:10.1007/s11306-006-0043-1.

91. Le Roy, C.I.; Mappley, L.J.; La Ragione, R.M.; Woodward, M.J.; Claus, S.P. Nmr-based metabolic characterization of chicken tissues and biofluids: A model for avian research. *Metabolomics* **2016**, *12*, 157-157, doi:10.1007/s11306-016-1105-7.

92. Abasht, B.; Mutryn, M.F.; Michalek, R.D.; Lee, W.R. Oxidative stress and metabolic perturbations in wooden breast disorder in chickens. *PLoS One* **2016**, *11*, e0153750, doi:10.1371/journal.pone.0153750.

93. Beauclercq, S.; Nadal-Desbarats, L.; Hennequet-Antier, C.; Gabriel, I.; Tesseraud, S.; Calenge, F.; Le Bihan-Duval, E.; Mignon-Grasteau, S. Relationships between digestive efficiency and metabolomic profiles of serum and intestinal contents in chickens. *Sci. Rep.* **2018**, *8*, 6678-6689, doi:10.1038/s41598-018-24978-9.

94. Inglis, G.D.; Wright, B.D.; Sheppard, S.A.; Abbott, D.W.; Oryschak, M.A.; Montina, T. Expeller-pressed canola (*brassica napus*) meal modulates the structure and function of the cecal microbiota, and alters the metabolome of the pancreas, liver, and breast muscle of broiler chickens. *Animals* **2021**, *11*, 1-22, doi:10.3390/ani11020577.

95. Vignoli, A.; Ghini, V.; Meoni, G.; Licari, C.; Takis, P.G.; Tenori, L.; Turano, P.; Luchinat, C. High-throughput metabolomics by 1d nmr. *Angew. Chem. Int. Ed. Engl.* **2019**, *58*, 968-994, doi:10.1002/anie.201804736.

96. Probert, C.S.J. A novel method for rapidly diagnosing the causes of diarrhoea. *Gut* **2004**, *53*, 58-61, doi:10.1136/gut.53.1.58.

97. Beckonert, O.; Keun, H.C.; Ebbels, T.M.; Bundy, J.; Holmes, E.; Lindon, J.C.; Nicholson, J.K. Metabolic profiling, metabolomic and metabonomic procedures for nmr spectroscopy of urine, plasma, serum and tissue extracts. *Nat. Protoc.* **2007**, *2*, 2692-2703, doi:10.1038/nprot.2007.376.

98. Emwas, A.H. The strengths and weaknesses of nmr spectroscopy and mass spectrometry with particular focus on metabolomics research. *Methods Mol. Biol.* **2015**, *1277*, 161-193, doi:10.1007/978-1-4939-2377-9_13.

99. Markley, J.L.; Bruschweiler, R.; Edison, A.S.; Eghbalnia, H.R.; Powers, R.; Raftery, D.; Wishart, D.S. The future of nmr-based metabolomics. *Curr. Opin. Biotechnol.* **2017**, *43*, 34-40, doi:10.1016/j.copbio.2016.08.001.

100. Nicholson, J.K.; Lindon, J.C.; Holmes, E. 'Metabonomics': Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological nmr spectroscopic data. *Xenobiotica* **1999**, *29*, 1181-1189, doi:10.1080/004982599238047.

101. Wishart, D.S. Quantitative metabolomics using nmr. *Trends Analyt. Chem.* **2008**, *27*, 228-237, doi:10.1016/j.trac.2007.12.001.

102. Moser, E.; Laistler, E.; Schmitt, F.; Kontaxis, G. Ultra-high field nmr and mri - the role of magnet technology to increase sensitivity and specificity. *Frontiers in physics* **2017**, *5*, doi:10.3389/fphy.2017.00033.

103. Nicholson, J.K.; Lindon, J.C. Metabonomics. *Nature* **2008**, *455*, 1054-1056, doi:10.1038/4551054a.

104. Le Belle, J.E.; Harris, N.G.; Williams, S.R.; Bhakoo, K.K. A comparison of cell and tissue extraction techniques using high-resolution ^{1}h-nmr spectroscopy. *NMR Biomed.* **2002**, *15*, 37-44, doi:10.1002/nbm.740.

105. Cui, M.; Trimigno, A.; Aru, V.; Khakimov, B.; Engelsen, S.B. Human faecal ^{1}h-nmr metabolomics: Evaluation of solvent and sample processing on coverage and reproducibility of signature metabolites. *Anal. Chem.* **2020**, doi:10.1021/acs.analchem.0c00606.

2 CORTICOSTERONE-MEDIATED PHYSIOLOGICAL STRESS ALTERS LIVER, KIDNEY, AND BREAST MUSCLE METABOLOMIC PROFILES IN CHICKENS[1]

2.1 Abstract

The impact of physiological stress on the metabolomes of liver, kidney, and breast muscle were investigated in chickens. To incite a stress response, birds were continuously administered corticosterone (CORT) in their drinking water at three doses (0, 10, and 30 mg L−1), and they were sampled 1, 5, and 12 days after the start of the CORT administration. To solubilize CORT, it was first dissolved in ethanol and then added to water. The administration of ethanol alone significantly altered branched chain amino acid metabolism in both the liver and the kidney, and amino acid and nitrogen metabolism in breast muscle. CORT significantly altered sugar and amino acid metabolism in all three tissues, but to a much greater degree than ethanol alone. In this regard, CORT administration significantly altered 11, 46, and 14 unique metabolites in liver, kidney, and breast muscle, respectively. Many of the metabolites that were affected by CORT administration, such as mannose and glucose, were previously linked to increases in glycosylation and gluconeogenesis in chickens under conditions of production stress. Moreover, several of these metabolites, such as dimethylglycine, galactose, and carnosine were also previously linked to reduced meat quality. In summary, the administration of CORT in chickens modulated host metabolism, and

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indicated that energy potentials may be diverted from muscle anabolism to muscle catabolism and gluconeogenesis during periods of stress.

2.2 Introduction

Chickens are exposed to many stressors during production. These include transportation from the hatchery to the barn, handling by people, and exposure to temperature fluctuations. When chickens experience a stressor, it stimulates the hypothalamic-pituitary-adrenal (HPA) axis and causes behavioral and physiological changes that help the chickens to cope with the stressor [1]. The glucocorticoid, corticosterone (CORT), is the major stress hormone in birds and is produced upon HPA activation [1,2]. Previous research has shown that an increase in the concentration of circulating CORT can lead to an altered cellular immune response [3] and that physiological stress negatively impacts the chicken immune system [4,5], which can have deleterious effects on production performance. For example, exposure to stressors can have a negative impact on poultry production metrics such as weight gain and feed conversion [6]. However, the systemic effects of stress on bird physiology, including impacts on metabolic processes, has not been extensively investigated to date.

One way to investigate the effects of stressors on metabolic processes is through metabolomics. Metabolomics is the study of the small molecular weight compounds, or metabolites, present in a tissue or biofluid, and allows for the analysis of the metabolic responses of living systems to external stimuli, such as stressors. An understanding of this response can provide valuable insight into the biochemical pathways that are potentially dysregulated due to physiological stress. Metabolomics can be used to study both pathological and physiological states, as well as to identify biomarkers in biological fluids

and tissues. The metabolomics research that has been conducted in chickens to date is often focused on the detection of drugs and drug metabolites such as anti-virals [7], antibiotics [8], pesticides [9], and banned feed additives such as animal growth promoters [10] and chicken bone meal [11] in chicken meat. In addition, there were chicken metabolomic studies that investigated biomarkers of spoiled meat [12], the effects of different cooking methods [13], the effects of diet [14], and the quality of the final meat [15-17] and cooked product [18].

Nuclear magnetic resonance spectroscopy (NMR) is an experimental technique used to characterize the metabolome of various biological tissues and fluids that is high throughput, non-destructive, and highly reproducible [19-21]. Recent research has explored the use of NMR-based metabolomics to examine chicken tissues for both model development [22] and to identify biomarkers of disease [23,24]. NMR-based metabolomics was also utilized to study the effects of stress in rat and human models, and focused largely on pre-natal maternal stress [25,26] or early life stress [27] and not lifetime stress or its effect on animal production. Previous work from our team has utilized a targeted approach to NMR based metabolomics analysis, in which only certain subsets of metabolites were analyzed, and combined this analysis with both genomics and histology to investigate the effect of physiological stress [28]. This study relied on the addition of ethanol to drinking water to administer CORT to the chickens as proposed in Post et al. [29]; however, it did not investigate the effects of this delivery method, nor did it comprehensively examine the metabolic effects of CORT administration in an untargeted manner whereby all metabolites present in the tissues were considered.

Understanding the effects of physiological stress on metabolism in a comprehensive and untargeted manner is an important step towards identifying biomarkers of stress. This

may facilitate the development of tools to help producers identify when stress may be adversely affecting their flocks so that mitigations can be employed early to prevent production losses. In the current study, we applied an untargeted NMR-based metabolomics approach to investigate the effects of both the ethanol-based delivery method of CORT administration and physiological stress, through dosage effects of CORT, on the metabolome of chicken kidney, liver, and breast muscle. We hypothesized that physiological stress modulates the metabolome of liver, kidney, and breast muscle due to increases in catabolism and gluconeogenesis.

2.3 Materials and methods

Ethics statement

The study was carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol Review #1526) before commencement of the research.

Experimental design

The study was designed as a factorial experiment with four levels of stress treatment and three levels of time arranged as a completely randomized design. The four stress treatments were: (1) control birds provided untreated drinking water (n = 9 chicks); (2) ethanol control birds provided with 0.2% ethanol in drinking water (n = 9); (3) 10 mg L^{-1} CORT in drinking water (n = 9); and (4) 30 mg L^{-1} CORT in drinking water (n = 9). The dose and method of CORT administration are detailed in a previous study [28]. Samples were obtained at 1, 5, and 12 days after initiation of the ethanol or CORT. Each replicate

included 12 birds, and replicates were conducted on three separate occasions to ensure independence.

Corticosterone administration

CORT (Sigma Aldrich Inc., Oakville, ON, Canada) was dissolved in 2 mL of anhydrous ethanol and added to 1 L of drinking water. Water containing CORT was prepared fresh each day and added to animal cages twice daily.

Bird husbandry

Thirty-six specific-pathogen-free white leghorn chickens were used in this study. Eggs were purchased from the Canadian Food Inspection Agency (Ottawa, ON, Canada), and upon arrival at Lethbridge (shipped by air) were incubated in a Brinsea Octagon 40 Advanced Digital Egg Incubator according to the manufacturer's recommendations (Brinsea Products Inc., Titusville, FL, USA). In this regard, eggs were maintained at 37.5 °C and 45% humidity with hourly turning for the first 18 days of incubation. Thereafter, eggs were set flat for hatching and humidity was increased to 60%. All hatched chicks were acclimatized in a group within one large animal pen (1.1 m^2) for 10 days and had access to a brooder (Brinsea Products Inc., Titusville, FL, USA). Birds had ad libitum access to a non-medicated starter diet (Hi-Pro Feeds, Lethbridge, AB, Canada) and water. Birds were maintained on a 12 h light: 12 h dark cycle. At 11 days-of-age, birds were randomly assigned to the four stress treatments and housed within individually ventilated cages (IVCs) (Techniplast, Montreal, QC, Canada). Each animal cage contained a companion bird to ensure no birds were socially isolated. Corticosterone treatment began when chicks reached 14 days-of-age and continued until the end of the experiment.

Sample collection

One bird per IVC was randomly sampled at each of the three sample times. Birds were anesthetized with isoflurane (5% isoflurane; 1 L O_2 min^{-1}) and euthanized by cervical dislocation under general anesthesia. The abdomen was opened with a ventral midline incision and the liver, a kidney, and a sample of breast muscle were aseptically removed and stored at –80 °C until processing. Liver, kidney, and breast muscle were examined because the metabolome of these tissues was previously shown to be significantly altered in response to stress in chickens [14,28].

Sample preparation

Tissues from liver, kidney, and breast muscle were homogenized in 4 mL g^{-1} methanol and 1.6 mL g^{-1} deionized water. Tissues were homogenized with 6-mm-diameter steel beads for 5 min intervals using a Qiagen Tissue Lyser at 50 Hz followed by 1 min of vortexing. This step was repeated two additional times to ensure complete tissue homogenization. To each sample, 2 mL g^{-1} chloroform was added and vortexed thoroughly. Next, 2 mL g^{-1} chloroform and 4 mL g^{-1} deionized water were added to each sample and vortexed until thoroughly mixed. Samples were then incubated at 4 °C for 15 min to allow for protein precipitation and then centrifuged at 1,000× *g* for 15 min at 4 °C. Next, 700 µL of the supernatant was removed and left until evaporated. Samples were rehydrated in 480 µL of metabolomics buffer (0.125 M KH_2PO_4, 0.5 M K_2HPO_4, 0.00375 M NaN_3, and 0.375 M KF; pH 7.4). A 120 µL aliquot of deuterium oxide containing 0.05% *v*/*v* trimethylsilylpropanoic acid (TSP) was added to each sample (final total volume of 600 µL); TSP was used as a chemical shift reference for ^{1}H-NMR spectroscopy. The solution was vortexed and then centrifuged at 12,000× *g* for 5 min at 4 °C to pellet any particulate matter. Following centrifugation, a 550 µL aliquot was loaded

into a 5 mm NMR tube and run on a 700 MHz Bruker Avance III HD spectrometer (Bruker, Milton, ON, Canada) for spectral collection.

Nuclear magnetic resonance data acquisition and processing

Spectra were collected on a 700 MHz Bruker Avance III HD spectrometer (Bruker, Milton, ON, Canada). The Bruker 1-D NOESY gradient water suppression pulse sequence 'noesygppr1d' was used with 10 ms mixing time. Each sample was run for 512 scans to a total acquisition size of 128 k, a spectral window of 20.5 ppm, a transmitter offset of 4.7 ppm, and a recycle delay of 4 s. All measurements were recorded using a Bruker triple resonance TBO-Z probe. The Bruker automation program "pulsal" was used on each sample before data acquisition to guarantee that the 90-degree pulse was calibrated correctly, ensuring quantitative and comparable data across samples [26]. The spectra were zero filled to 256 k, automatically phased, baseline corrected, and line-broadened by 0.3 Hz [25]. Spectra were then exported to MATLAB (MathWorks, Natick, MA, USA) as ASCII files, and underwent dynamic adaptive binning [30], followed by manual inspection and correction. Spectral binning resulted in 439, 460, and 379 spectral bins for kidney, liver, and breast muscle, respectively. The dataset was then normalized to the total metabolome, excluding the region containing the water peak, and pareto scaled.

Statistical analysis

Spectral bins were subjected to both a univariate paired *t*-test and a multivariate variable importance analysis based on random variable combination (VIAVC) [31] analysis in MATLAB (Math Works, Natick, MA, USA) to determine which metabolites were significantly altered between treatments. For the paired *t*-test each comparison was paired within each replicate, and samples from all three time points, (1, 5, and 12 days after CORT administration) were pooled to provide an adequate sample size. The

univariate measures were calculated using a decision tree algorithm as described by Goodpaster et al. [32]. All *p*-values obtained from this analysis were Bonferroni-Holm corrected for multiple comparisons. The VIAVC algorithm uses both Partial Least Squares Discriminant Analysis (PLS-DA) and the area under the Receiver Operating Characteristics (ROC) curve to determine the best subset of bins for group classification while also accounting for the significance of the bins; thus the algorithm considers the synergistic effects of bins [31]. MATLAB was also used to calculate the percent difference of the bins between treatments. The R package, MetaboanalystR [33] was used to carry out the Principle Component Analysis (PCA) and Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA). Metabolites were identified using the Chenomx 8.2 NMR Suite (Chenomx Inc, Edmonton, AB, Canada) and the complete list of significant metabolites was used to carry out pathway topology analysis using the Metaboanalyst's Metabolomics Pathway Analysis (MetPA) web-based tool. The Hypergeometric Test was used to calculate relative betweenness centrality and out degree centrality measures to determine the importance of the compounds identified in the samples [34]; the KEGG database [35] for chicken pathways was selected for this analysis.

2.4 Results

Ethanol alters the metabolome of kidneys, liver, and breast muscle

Paired *t*-test and VIAVC tests revealed the bins that were significantly altered ($p \leq 0.05$) between the control and ethanol control treatments in kidney (18 bins by *t*-test, 9 bins by VIAVC, no bins in common between the two tests), liver (53 bins by *t*-test, 9 bins by VIAVC, 5 common bins), and breast (no bins by *t*-test, 9 bins by VIAVC, no common

bins). Examination of bins that were observed to be significantly altered via the paired *t*-test and/or the VIAVC analysis was done using a PCA score plot which showed little to no unsupervised group separation in any of the tissues (**Figure 2-1**). Subsequent OPLS-DA analyses for the ethanol control treatment showed supervised group separation and a good model fit (as indicated by the Q^2) in liver ($Q^2 = 0.85$, $p < 0.001$, $R^2 = 0.97$, $p < 0.050$) and breast ($Q^2 = 0.66$, $p < 0.001$, $R^2 = 0.76$, $p < 0.050$), but not kidney ($Q^2 = 0.16$, $p = 0.076$, $R^2 = 0.65$, $p = 0.062$) tissues (**Figure 2-2**).

Double cross-validation and permutation tests validated the observed supervised separation results as a function of ethanol in the drinking water in liver and breast ($p < 0.001$), but not kidney ($p = 0.076$) tissues. Quantities of specific metabolites differed ($p < 0.050$) between the control and ethanol control treatments as determined by paired *t*-test and/or VIAVC analysis. In the kidney, 5/10 (50%), in liver, 11/27 (40%), and in breast muscle, 1/5 (20%) unique metabolites were significantly up-regulated (**Figure 2-3**). Pathway topology analysis showed that the inclusion of ethanol in the drinking water affected aminoacyl-tRNA biosynthesis ($p < 0.001$), and alanine, aspartate, and glutamate metabolism ($p < 0.050$) in kidney and liver, as well as D-glutamine and D-glutamate metabolism and nitrogen metabolism ($p < 0.050$) in kidney and breast muscle (**Figure 2-4**). Only in the kidney was the branched chain amino acid (BCAA) (e.g., valine, leucine and isoleucine) degradation pathway altered ($p < 0.050$). In the liver, other amino acid metabolism pathways that were altered included the phenylalanine metabolism pathway ($p < 0.050$) and the arginine and proline metabolism pathway ($p < 0.050$).

Corticosterone substantively alters the metabolome of kidneys, liver, and breast muscle

To ascertain the impact of CORT, the metabolome of birds administered CORT at a dose of 10 mg L^{-1} or 30 mg L^{-1} was compared to birds administered ethanol without CORT in drinking water to eliminate metabolite bias as a result of the ethanol carrier. Paired *t*-test and VIAVC tests were applied to each CORT treatment. The liver tissues had the largest number of significantly altered bins across all tissues and dosages (**Table 2-1**). The relative concentration changed for 46 metabolites in kidney, 71 in liver, and 75 in breast with CORT administration (**Tables A1–A3**). A PCA score plot shows that at the lower dose of CORT there was no unsupervised separation in kidney tissue (**Figure 2-5A**), but there was unsupervised separation in liver and breast muscle tissues (**Figure 2-5C,E**) with some confidence interval overlap. At the higher dose of CORT there was unsupervised separation observed in all three tissues (**Figure 2-5B,D,F**) with complete separation of the confidence intervals for breast muscle and less overlaps for both the kidney and liver tissues. Subsequent OPLS-DA analyses showed supervised group separation and a good model fit for 10 mg L^{-1} CORT in liver ($Q^2 = 0.80$, $p < 0.001$, $R^2 = 0.99$, $p < 0.050$) and breast muscle ($Q^2 = 0.44$, $p = 0.050$, $R^2 = 0.63$, $p < 0.050$), but not in kidney ($Q^2 = 0.02$, $p = 0.151$, $R^2 = 0.66$, $p = 0.111$) (**Figure 2-6A,C,E**). Likewise, supervised separation was observed for the 30 mg L^{-1} CORT dosage in liver ($Q^2 = 0.81$, $p < 0.001$, $R^2 = 0.93$, $p < 0.001$), breast muscle ($Q^2 = 0.87$, $p < 0.001$, $R^2 = 0.92$, $p < 0.001$), and kidney ($Q^2 = 0.71$, $p < 0.001$, $R^2 = 0.92$, $p < 0.050$) (**Figure 2-6B,D,F**). At 10 mg L^{-1}, CORT significantly up-regulated 39/43 (90%) of the unique metabolites in the kidney, 20/115 (17%) in the liver, and 20/25 (80%) in breast muscle. At 30 mg L^{-1}, CORT significantly up-regulated 13/36 (36%) of the unique metabolites in the kidney, 26/93

(28%) in the liver, and 17/96 (18%) in breast muscle (data not shown). There were 11 metabolites that were significantly altered at both the 10 and 30 mg L^{-1} CORT doses in kidney, 46 in liver, and 14 in breast muscle (**Figure 2-7**). Glucose and N-methylhydatoin were significantly altered in all three tissues. In kidney and breast muscle, all metabolites that were altered at both doses showed a greater percentage difference at 30 mg L^{-1} CORT than at 10 mg L^{-1} CORT. In liver there were 16 metabolites that showed a greater change in regulation at 10 mg L^{-1} CORT than at 30 mg L^{-1} CORT, and 27 metabolites that followed the same trend observed in kidney and breast muscle, with three metabolites that had equal changes in regulation at both concentrations of CORT.

Pathway topology analysis indicates that the inclusion of 10 mg L^{-1} CORT in the drinking water affected galactose ($p < 0.001$); starch and sucrose ($p < 0.050$); and inositol phosphate ($p < 0.050$) metabolism. In liver and kidney alanine, aspartate, and glutamate ($p < 0.050$); and histidine ($p < 0.050$) metabolism were altered (data not shown). For the 30 mg L^{-1} CORT, treatment pathway topology analysis indicated that two pathways were altered in all three tissues: these were the aminoacyl-tRNA biosynthesis ($p < 0.001$) and galactose metabolism ($p < 0.050$) pathways (**Figure 2-8**). In liver and kidney, glutathione ($p < 0.050$), and starch and sucrose ($p < 0.050$) metabolism were also altered. In liver and breast muscle, pantothenate and CoA biosynthesis ($p < 0.050$), histidine ($p < 0.050$), and purine ($p < 0.050$) metabolism were also all significantly altered. In kidney and breast muscle, alanine, aspartate, and glutamate metabolism ($p < 0.050$), pyruvate metabolism ($p < 0.050$), and glycine, serine, and threonine metabolism ($p < 0.050$) were also altered.

2.5 Discussion

Metabolomics provides a comprehensive understanding of all the responses of whole living systems to pathophysiological stimuli, genetic modification, diet, and the environment. Furthermore, metabolomics is particularly adapted to identify biomarkers as it provides a snapshot of all the metabolic activity occurring in a system [21,36]. For these reasons, identifying biomarkers of physiological stress and disease using metabolomics is gaining more momentum in human biomedical research, as well as in poultry science [23,37,38].

When chickens are exposed to a stressor, the HPA axis is activated to help the bird cope with the stressor [3,39]. This activation leads to an increased concentration of circulating CORT, the main glucocorticoid in birds. Factors that stimulate CORT release are relevant to poultry producers as the hormone can be linked to detrimental effects on bird health [23,40], growth rate [41], and the overall quality of the meat [17,42]. Previous studies showed that CORT decreases mass gain [41,43,44], increases the amount of lipids in the liver [43], decreases skeletal muscle growth [41], and decreases nitrogen retention [45] by increasing the degradation of muscle proteins [41,44]. In addition, CORT has previously been shown to cause an increase in plasma glucose [41] and fatty acid concentrations [45]. In the current study, we investigated the effect of CORT administered at two doses in drinking water (10 and 30 mg L^{-1}) on white leghorn chickens using ^{1}H-NMR metabolomics, and we observed distinct changes in the metabolomes of liver, kidney, and breast muscle regardless of the dose administered. Moreover, CORT administration was observed to alter several metabolite concentrations that can be linked to alterations in metabolic pathways related to both bird health and meat quality.

Ethanol impacts on kidneys

Previous studies reported that rats administered ethanol in drinking water exhibit reduced renal function due to kidney injury [46,47]. Both the liver and the kidney have the ability to catabolize ethanol, but the formation of free radicals occurs during catabolism, which can damage these organs and cause energy-intensive inflammatory responses [48]. As a result of increased energy demand, the process of gluconeogenesis can be utilized by the kidney to convert glycerol into glucose and to provide energy for cells in need [49,50]. The decrease in glycerol concentrations observed in the kidney of birds administered ethanol supports this mechanism, and suggests that gluconeogenesis was increased in the kidney to provide more energy due to the higher energy demands due to the inflammatory response caused by the ethanol. In chickens, gluconeogenesis occurs in both the liver and the kidney; however, the kidney is the primary site of gluconeogenesis in chickens [51]. A further indication that the kidney indeed had higher energy demands is supported by the observed decrease in alanine. The Cahill cycle, or the glucose-alanine cycle, occurs in most extrahepatic tissues, and the resultant molecules from the breakdown of BCAA are converted to alanine, which can more easily be transported to the liver where it can be converted into glucose [50-53]. Although we did not observe an increase in alanine within the livers of birds administered ethanol, the accumulation of alanine could have been obscured by the rapid conversion of alanine to urea via the urea cycle within the liver.

Ethanol impacts on liver

The liver is the primary site of ethanol catabolism, and chronic ethanol ingestion and metabolism causes mild damage to the liver through oxidative stress [48] and can induce fatty liver disease [48,54]. Furthermore, the administration of ethanol to chickens has

been shown in previous studies to decrease liver antioxidant enzyme activities [55]. The combination of increased oxidative stress and reduced antioxidant enzymes would be expected to result in an increase in energy demands in the liver. The liver is the major site of the Cori cycle in chickens, in which lactate is converted to glucose to supply these energy demands [45,51]. The observed down-regulation of lactate in the livers of the ethanol control birds in the current study is consistent with an increase in the use of energy in chickens [14]. We also observed that phenylalanine was down-regulated in the livers of birds administered ethanol. Phenylalanine is a precursor for tyrosine, which is then used for the synthesis of neurotransmitters [56], and a decrease in phenylalanine concentration may adversely affect a chicken's ability to respond to stimuli [14].

Ethanol impacts on breast muscle

In breast muscle of birds administered ethanol, the metabolites of the glutamine and glutamate metabolism pathways, along with the metabolites of the nitrogen metabolism pathway, were significantly altered. Glutamine is normally the most abundant amino acid in healthy skeletal muscle; however, when muscle is stressed, the concentration of glutamine drops significantly. Therefore, glutamine is designated as a conditionally essential amino acid during periods of stress [57,58]. Studies have shown the glutamine supplementation in broilers was associated with improved growth, carcass characteristics, and overall meat quality while under stress [57,59]. Under stress conditions, there is a depletion of glutamine [59], as was observed in breast muscle of birds administered ethanol in the current study, suggesting that ethanol can lead to a decrease in breast meat quality.

Corticosterone impacts on the kidney metabolome

The concentration of glutamate in the kidney was decreased in CORT stressed birds regardless of the concentration administered. Glutamate is a neurotransmitter, but is also present in peripheral tissues, like kidney, where it plays an important role in energy production, nitrogen metabolism, and the body's response to oxidative stress [50,60]. Glutamate receptors in the kidney are coupled to a number of G-protein cascades [60]. There are many N-methyl-D-aspartate (NMDA) receptors in the kidney to which glutamate can bind. Although glutamate spectra are included in the Chenomx database for identification, glutamate bound to its receptor is not, meaning that bound glutamate cannot be identified in ^{1}H-NMR spectra. The lower concentration of glutamate that we observed in the kidney of birds administered CORT could be caused by greater amounts of the metabolite being bound to its receptor. The activation of these receptors affects renal function, and in some cases may induce renal dysfunction; for example, a previous study showed that prolonged exposure to NMDA caused excessive NMDA receptor activation that led to an accumulation of reactive oxygen species [60] which are toxic for renal cells; and this agrees with the metabolite changes observed in the current study. In addition, the concentration of glucose in the kidneys of the CORT birds was increased, and this agrees with the previously suggested increased role of the Cahill cycle. In the Cahill cycle, alanine is converted to glucose, and one key source of alanine is the conversion of glutamate and pyruvate into alanine and alpha-ketoglutarate. Previous studies also showed an increase in glucose in kidneys under stressed conditions [50-53]. This suggests that the combination of relative changes in both glucose and glutamate might serve as a strong biomarker of stress in chickens.

All metabolites altered in the kidney following CORT administration were affected to a greater extent in birds administered CORT at the 30 mg L^{-1} dose relative to the 10 mg L^{-1} dose. For example, the concentration of 4-hydroxyproline was substantially increased for birds administered CORT at the higher dose. In most animals, including chickens, hydroxyproline is required for the synthesis of glycine. Glycine is known to scavenge oxidants and help regulate the redox state of cells [61]. Moreover, up-regulation of hydroxyproline has been observed in response to many different kinds of stress, such as inflammatory and genotoxic stresses [62,63]. Elevated concentrations of malate were linked to kidney dysfunction in rats [64], and the increased concentration of malate observed in the kidneys of the birds administered CORT could indicate that the CORT treatment is a biomarker of renal dysfunction and this warrants further investigation.

Corticosterone impacts on the liver metabolome

A key function of the liver is to provide glucose to tissues. Under conditions of stress the liver increases rates of both glycogenolysis and gluconeogenesis to meet the body's increased energy demands [65]. Amino acids represent the main metabolic source of energy for the liver [66,67]. The essential amino acids that were identified in the liver of CORT-treated chickens in the current study (i.e., threonine, valine, isoleucine, methionine, phenylalanine, and tryptophan) were all down-regulated, indicating that energy demands in the liver were higher in birds administered CORT (i.e., relative to birds administered ethanol alone). This agrees with previous research that showed that concentrations of amino acids drop in liver under stress due to increases in metabolic demands [65,67,68]

In contrast to essential amino acids, mannose concentrations were significantly increased in the livers of birds administered CORT at 10 and 30 mg L^{-1} as compared to

the ethanol control treatments. Previous studies observed up-regulation of mannose-6-phosphase, which is indistinguishable from mannose in 1D ^{1}H-NMR spectra; mannose-6-phosphate is an integral metabolite in the formation of GDP-mannose which is important for N-linked glycosylation [67]. Glycosylation has been shown to be increased under acute stress conditions [67], and mannose is often used as the substrate for glycosylation if the cell experiences stress [69]. The function of glycosylation under acute stress is poorly understood at present, but is likely involved in the regulation of heat shock proteins, which are often up-regulated under various conditions of stress [69].

Previous studies observed an increase in glucose in chicken livers when the birds were stressed by high temperatures [67]. The up-regulation of glucose observed in the livers of chickens administered CORT in the current study is consistent with these findings, thereby suggesting that increases in the concentration of glucose in the liver may be indicative of physiological stress regardless of the incitant. Furthermore, excessive CORT exposure was shown to cause fatty liver in chickens [70,71]. Steatosis, which is a buildup of fat in the liver, was noted in the livers of the birds receiving CORT after 5 days, which was previously published on the same cohort of birds from which tissues were obtained and analyzed in the current study [28].

Corticosterone impacts on the breast muscle metabolome

We observed that BCAAs in breast muscle were significantly down-regulated in CORT-treated birds as compared to control treatment birds. BCAAs are mostly metabolized in skeletal muscle and other peripheral tissues [72], and the decrease in BCAAs observed in the current study is consistent with an increase in metabolic demands imparted due to stress. Protein synthesis is an energy intensive process, and much more so than proteolysis [73,74]. Thus, insufficient energy to build protein, as would be the case if

the birds' energy was used for other metabolic processes, may have contributed to the lower body weights that were previously observed on this cohort of stressed chickens [28]. It is noteworthy that stress is known to reduce feed intake [67,75]. Although we did not measure feed intake in the current study, it is possible that the decrease in bird weights could be caused entirely or partly by a reduced appetite in the birds, as seen in previous studies [67]. However, reduced food intake would still be expected to result in the observed changes in BCAAs related to muscle metabolism and increased energy demand. It is widely accepted that high concentrations of circulating CORT suppress muscle growth and protein synthesis [65], and can even lead to muscle atrophy [76], which would also contribute to the lack of mass gain in the CORT chickens.

We observed an increased concentration of glucose in breast muscle of birds administered CORT as compared to control treatment birds. As indicated previously, stress increases gluconeogenesis in the liver [17,67]. This results in increased levels of glucose in liver tissue, as well as transport of glucose to other tissues [77]. Stress was shown to increase the level of glucose in breast muscle [28,78], and previous studies have correlated meat quality to glucose levels [16], with higher quality breast meat possessing lower levels of glucose than poorer quality meat [16,42]. Thus, the findings of higher concentrations of glucose in breast muscle of birds administered CORT suggest that stress contributes to poor meat quality. Consistent with this conclusion, anserine, carnosine, proline, dimethylglycine, and phenylalanine concentrations were significantly reduced, while mannose-6-phosphate was significantly increased in CORT-treated birds. This agrees with previous research that showed similar concentration changes in these metabolites associated with overall meat quality measures [16,38,79].

Galactose metabolism is a pathway that has previously been determined to be significantly up-regulated in lower quality breast meat samples [16]. Metabolites associated with the galactose metabolism pathway, including glucose and glucose-1-phosphate, were observed to be significantly up-regulated in birds administered CORT as compared to the control birds in the current study, further implicating stress as an important contributor to meat quality. Overall, we identified and confirmed a variety of breast muscle metabolites that were significantly altered due to physiological stress incited by CORT, which may be of value as biomarkers of stress and impacts of stress on breast meat quality.

2.6 Conclusions

In the current study we showed that ethanol altered the metabolomes of the liver, kidney, and breast muscle of chickens; however, the effects of ethanol alone were substantially less than the metabolomic effects of CORT orally administered in ethanol. It is possible that CORT and ethanol together exerted a larger effect than either alone would have. Study results indicate that CORT should be administered in an alternate fashion (e.g., incorporated into the feed) in future studies to remove the confounding effects of ethanol on the metabolome. Despite the observed effects of ethanol, the data from this study showed that the stress hormone, CORT, was linked to changes in metabolite concentrations across several important tissues in chickens that could be linked to overall health and production. This includes kidney glutamate concentrations, liver amino acid concentrations, and increases in glucose concentrations in the kidney, liver, and breast muscle of white leghorn chickens. Significantly, several stress induced changes in metabolite concentrations observed in breast muscle may be associated with meat quality

which warrants further investigation. Moreover, alterations to the metabolome of chickens incited by CORT were consistent with increases in metabolism due to stress induction, which were consistent with a previously published reduction in the mass of CORT administered birds relative to control treatments in the same chicken cohort. Future research should focus on relating metabolite changes in tissues to non-destructive markers like blood, feces, or feathers relative to metrics of birds' health and meat quality with the goal of developing new diagnostic tools to better monitor on-farm stress. Ultimately, the identification of quantitative biomarkers of stress will allow producers to utilize the markers to develop and objectively evaluate mitigation strategies to proactively enhance bird health and flock performance.

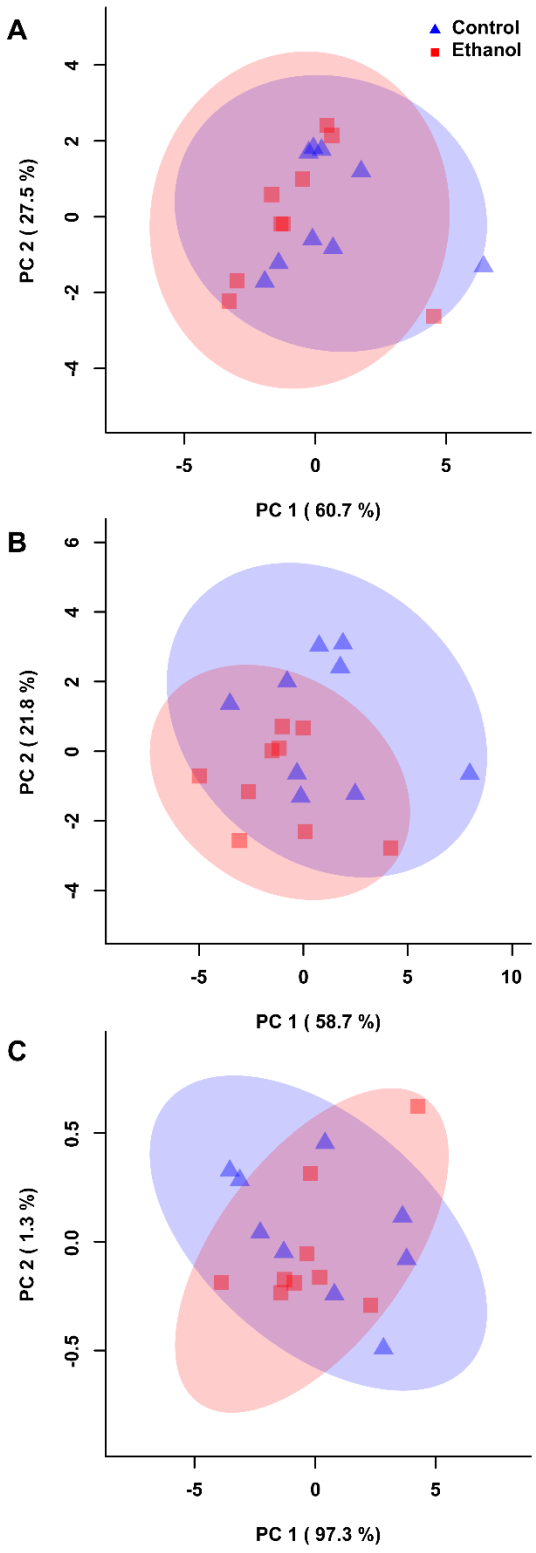

Figure 2-1: Principal component analysis score plots for the control treatment vs. the ethanol treatment. (A) Kidney. (B) Liver. (C) Breast muscle. The X and Y axes show principal components with brackets indicating percent variance and the shaded ellipse represents the 95% confidence interval

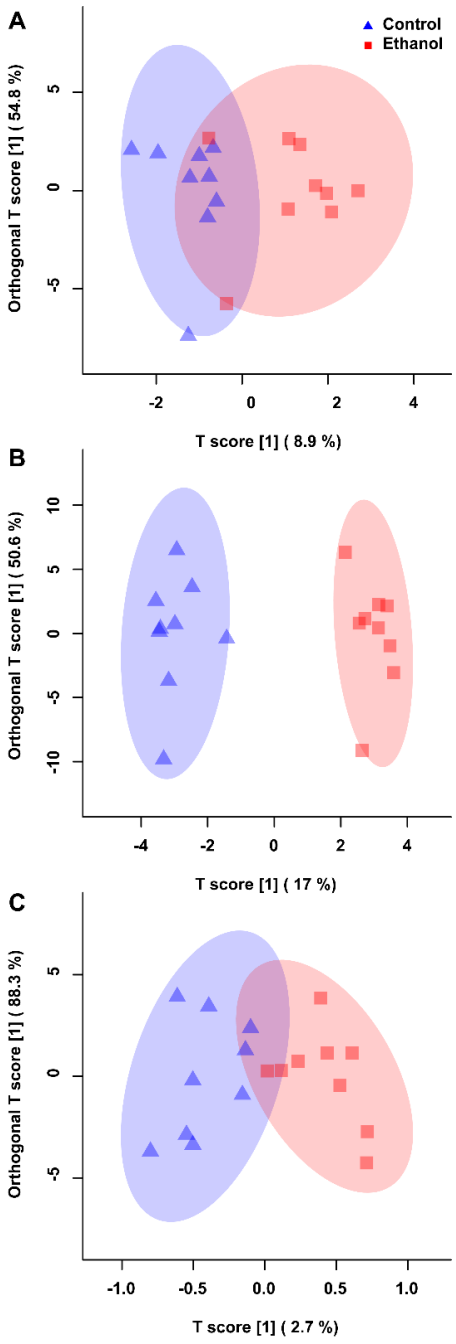

Figure 2-2: Orthogonal partial least squares determinant analysis score plots for the control treatment vs. the ethanol control treatment. **(A)** Kidney ($Q^2 = 0.16$, $p = 0.076$, $R^2 = 0.65$, $p = 0.062$). **(B)** Liver ($Q^2 = 0.85$, $p < 0.001$, $R^2 = 0.97$, $p < 0.050$). **(C)** Breast muscle ($Q^2 = 0.66$, $p < 0.001$, $R^2 = 0.76$, $p < 0.050$). Each triangle or square represents one chicken under study ($n = 9$), plotted using a list of bins found to be statistically significant via paired *t*-test and/or multivariate variable importance analysis based on random variable combination analysis. The x- and y-axis represent the predictive (between group separation) and orthogonal (within group variation) components of the data, respectively. The shaded ellipse represents the 95% confidence interval

Table 2-1: Number of significantly altered bins across all tissues and corticosterone (CORT) doses

Kidney	Paired T-test	VIAVC	Common to both tests
10 mg CORT/L water	63	12	4
30 mg CORT/L water	45	7	1
Liver			
10 mg CORT/L water	236	5	3
30 mg CORT/L water	217	3	3
Breast			
10 mg CORT/L water	31	10	1
30 mg CORT/L water	251	75	72

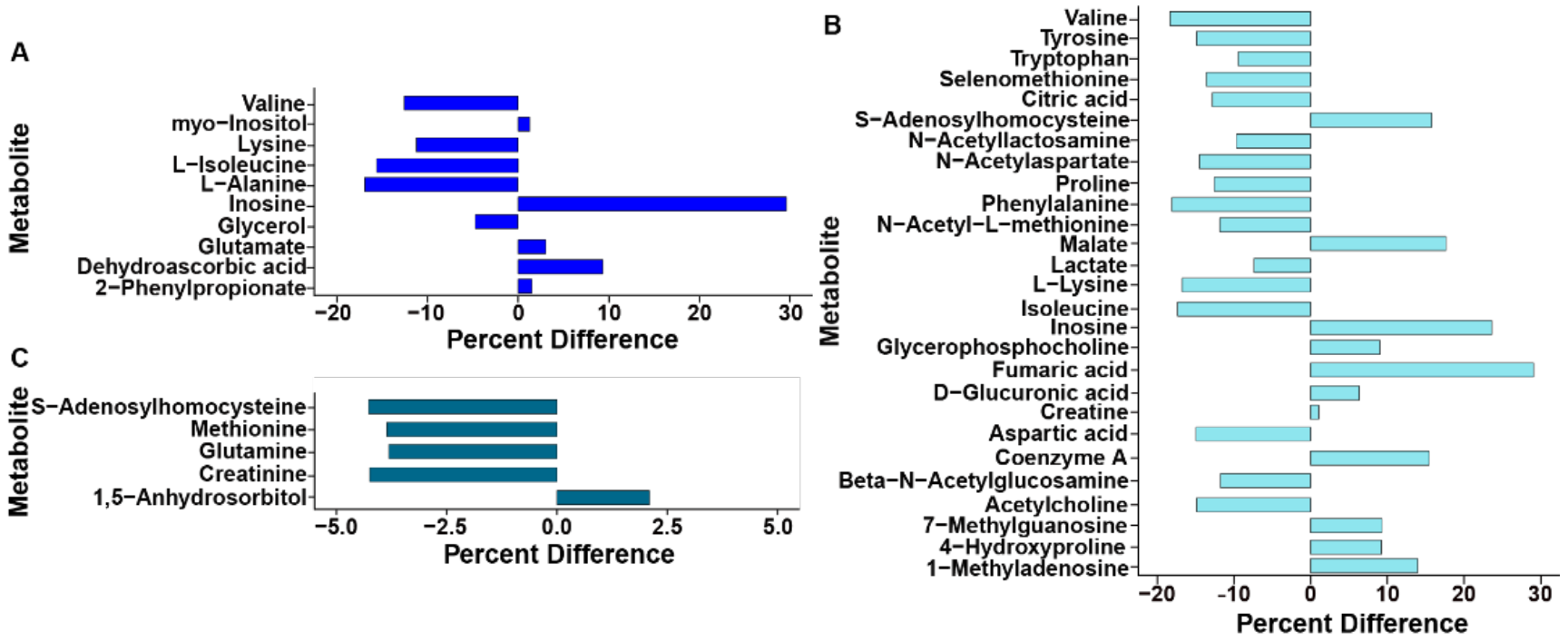

Figure 2-3: Percent difference for metabolites significantly altered in the control treatment when compared to the ethanol treatment. **(A)** Kidney. **(B)** Liver. **(C)** Breast muscle. All metabolites were significantly altered based on paired *t*-test and/or multivariate variable importance analysis based on random variable combination analysis

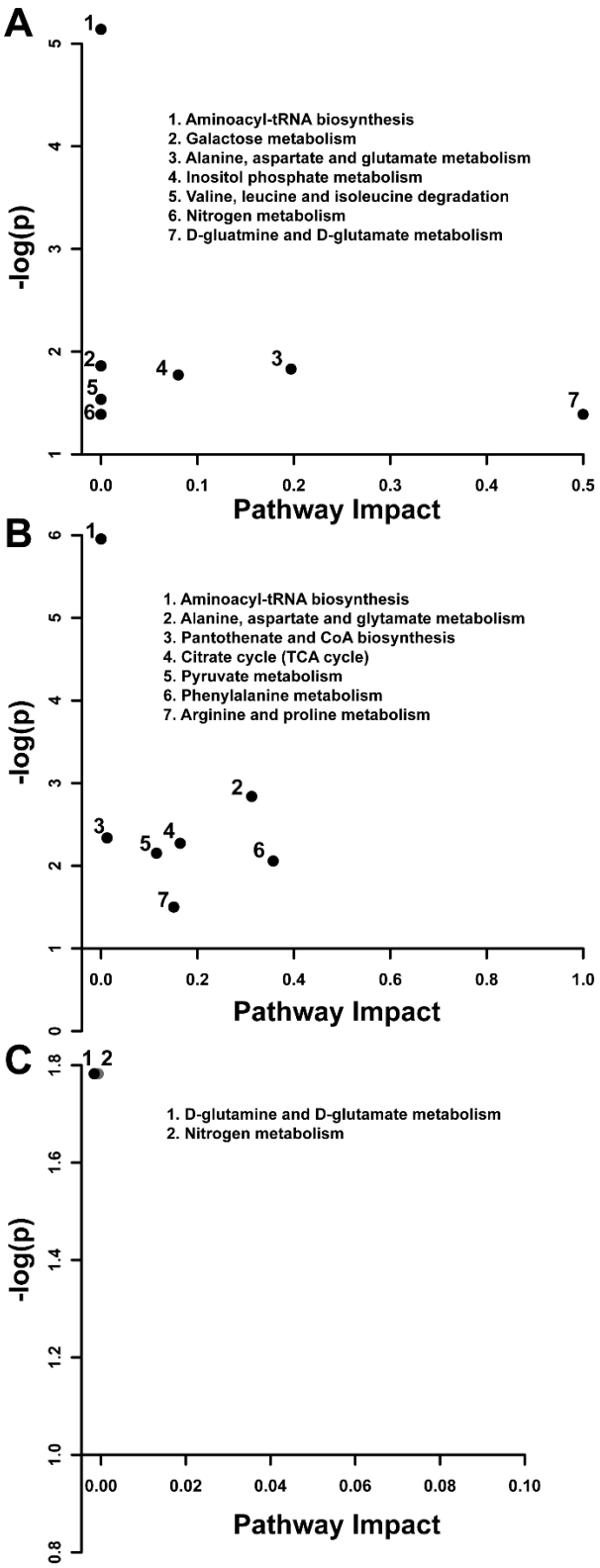

Figure 2-4: Metabolomic pathway analysis showing all matched pathways according to p-values from pathway enrichment analysis and pathway impact values for the control treatment vs. the ethanol treatment. **(A)** Kidney. **(B)** Liver. **(C)** Breast muscle

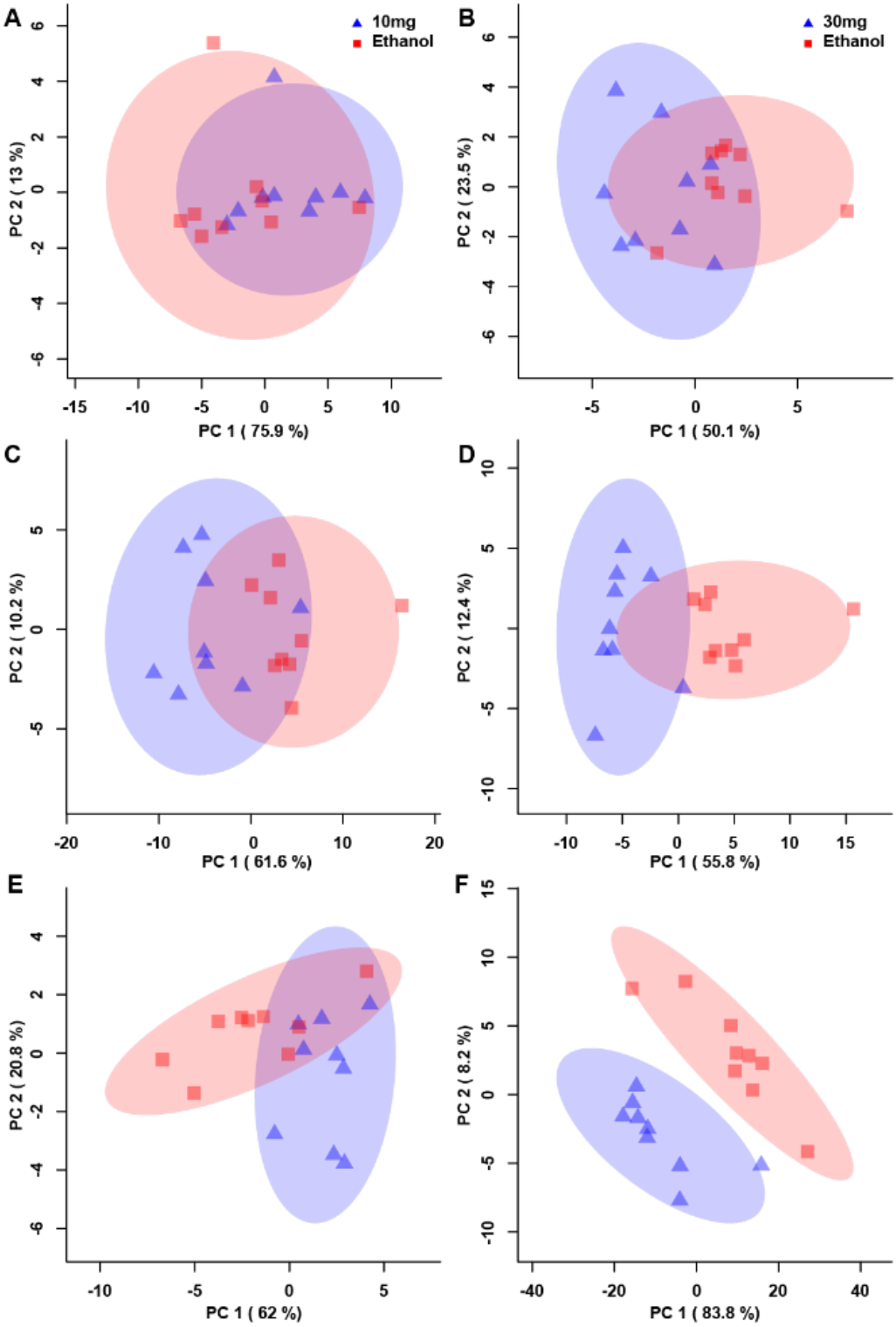

Figure 2-5: Principal component analysis score plots for the corticosterone treatments vs. the ethanol control treatment. **(A)** Kidney—10 mg L^{-1} corticosterone. **(B)** Kidney—30 mg L^{-1} corticosterone. **(C)** Liver—10 mg L^{-1} corticosterone. **(D)** Liver—30 mg L^{-1} corticosterone. **(E)** Breast muscle—10 mg L^{-1} corticosterone. **(F)** Breast muscle—30 mg L^{-1} corticosterone. The shaded ellipse represents the 95% confidence interval

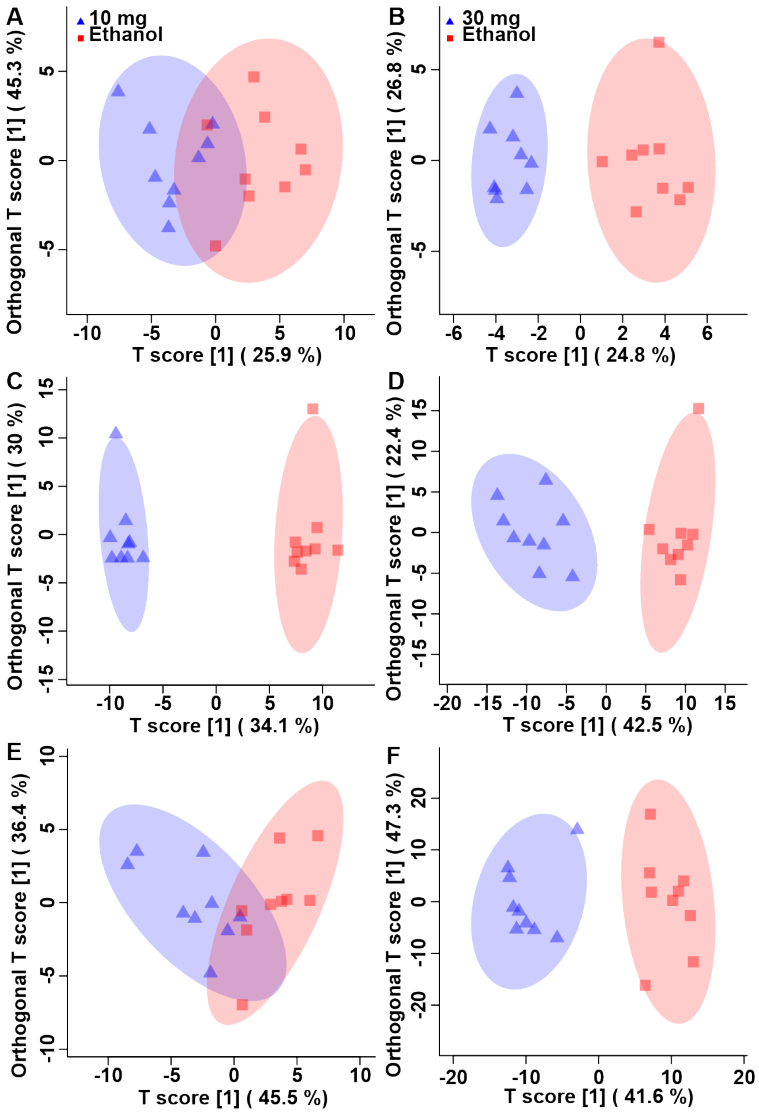

Figure 2-6: Orthogonal partial least squares determinant analysis score plots for the corticosterone treatments vs. the ethanol control treatment. **(A)** Kidney—10 mg L^{-1} corticosterone ($Q^2 = 0.02$, $p = 0.151$, $R^2 = 0.66$, $p = 0.111$). **(B)** Kidney—30 mg L^{-1} corticosterone ($Q^2 = 0.71$, $p < 0.001$, $R^2 = 0.92$, $p < 0.050$). **(C)** Liver—10 mg L^{-1} CORT ($Q^2 = 0.80$, $p < 0.001$, $R^2 = 0.99$, $p < 0.050$). **(D)** Liver—30 mg L^{-1} corticosterone ($Q^2 = 0.81$, $p < 0.001$, $R^2 = 0.93$, $p < 0.001$). **(E)** Breast muscle—10 mg L^{-1} corticosterone ($Q^2 = 0.44$, $p = 0.050$, $R^2 = 0.63$, $p < 0.050$). **(F)** Breast muscle—30 mg L^{-1} corticosterone ($Q^2 = 0.87$, $p < 0.001$, $R^2 = 0.92$, $p < 0.001$). Each triangle or square represents one chicken under study ($n = 9$), plotted using a list of bins found to be statistically significant via paired *t*-test and/or multivariate variable importance analysis based on random variable combination analysis. All three tissues show supervised separation at the highest corticosterone dose. All but kidney also show supervised separation at the lower dose. The x- and y-axis represent the predictive (between group separation) and orthogonal (within group variation) component of the data, respectively. The shaded ellipse represents the 95% confidence interval

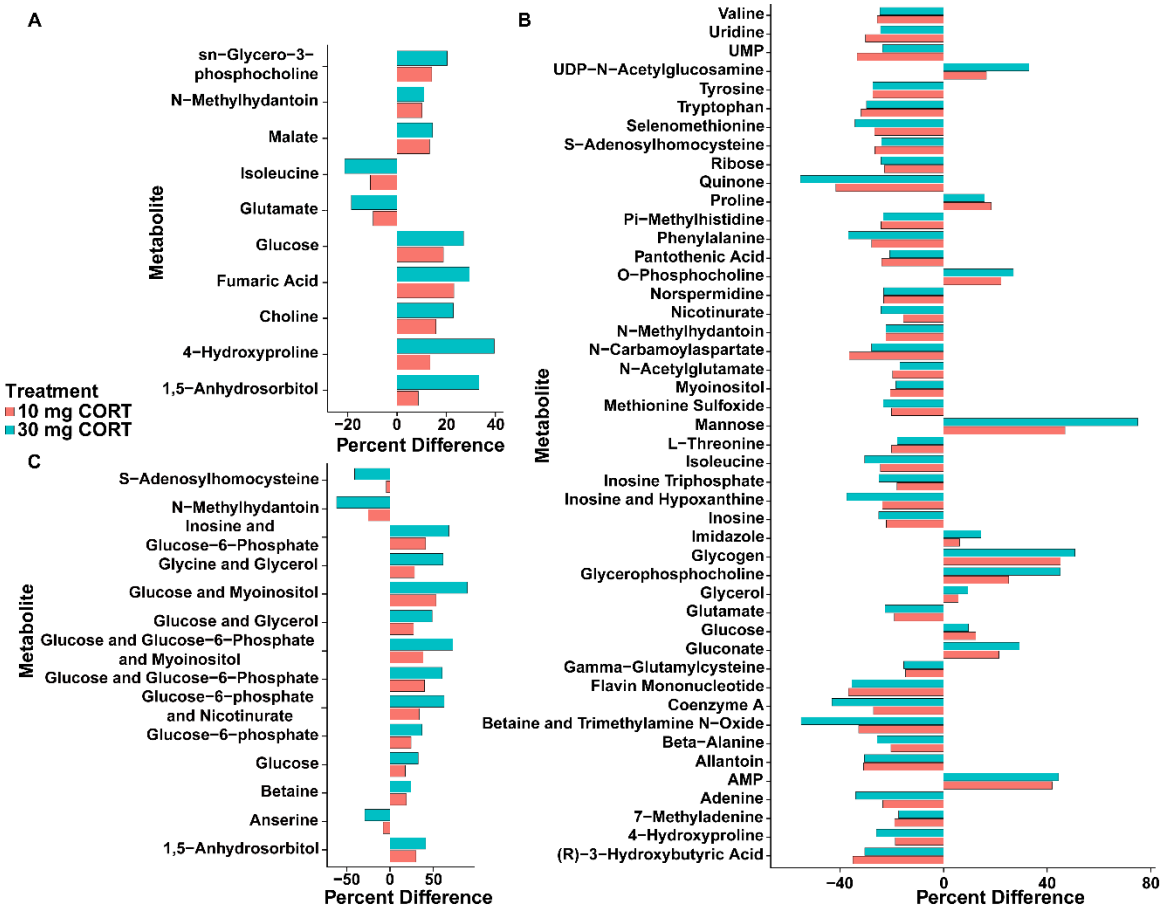

Figure 2-7: Metabolites significantly altered in both the 10 and 30 mg L^{-1} corticosterone treatments. **(A)** Kidney. **(B)** Liver. **(C)** Breast muscle. All metabolites were significantly altered ($p < 0.050$) as compared to the ethanol control treatment based on paired *t*-test and/or multivariate variable importance analysis based on random variable combination analysis

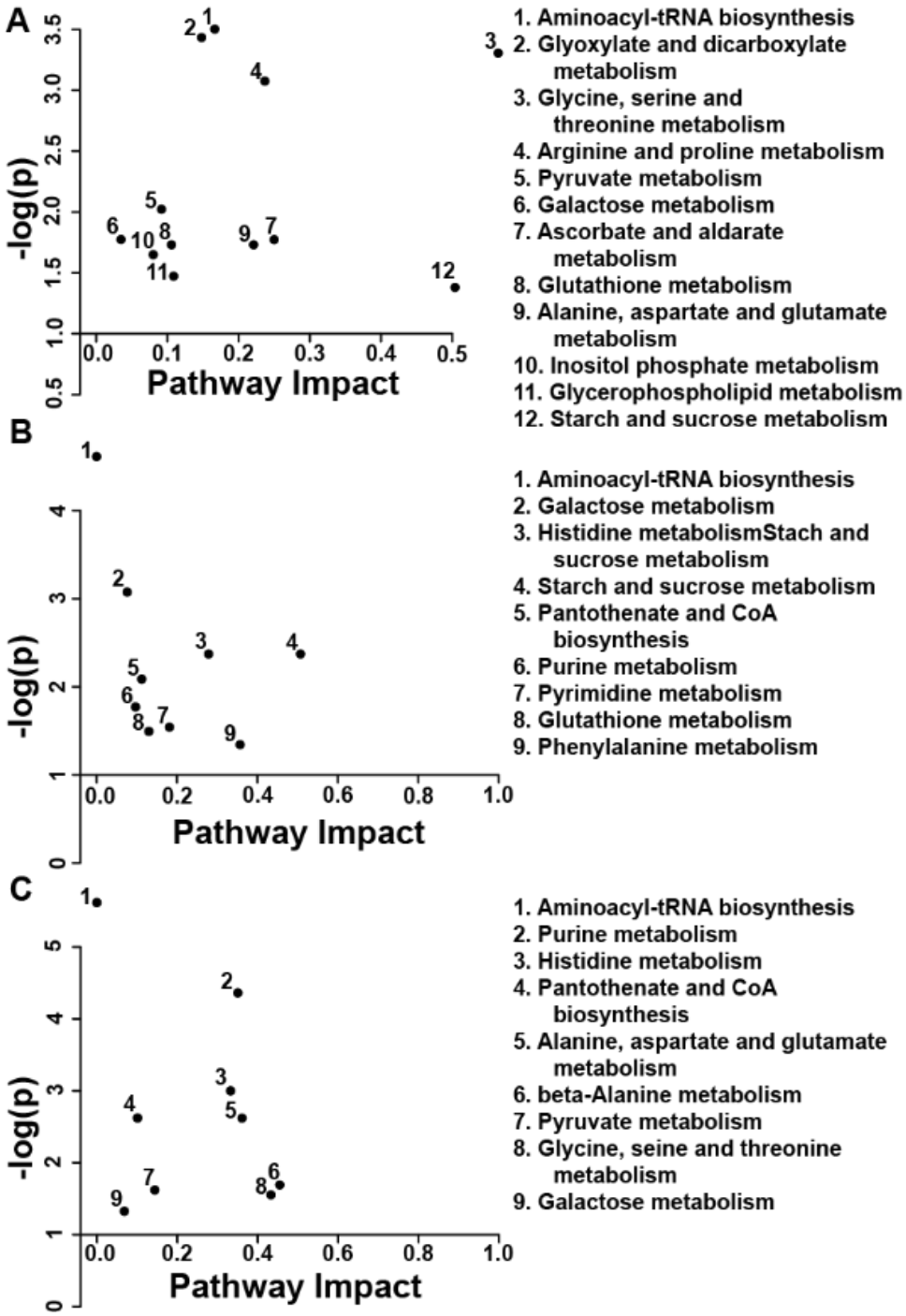

Figure 2-8: Metabolomic pathway topology analysis showing all matched pathways according to *p*-values from pathway enrichment analysis and pathway impact values for corticosterone administered at a dose of 30 mg L^{-1} vs. the ethanol control treatment. **(A)** Kidney. **(B)** Liver. **(C)** Breast. A larger value on the *y*-axis indicates a lower *p*-value. The *x*-axis gives the pathway impact as calculated using the hypergeometric test. All metabolic pathways with $p < 0.050$ are shown. This figure was created using the lists of metabolites identified as significant by paired *t*-test or multivariate variable importance analysis based on random variable combination testing

2.7 References

1. Cockrem, J.F. Stress, corticosterone responses and avian personalities. *J. Ornithol.* **2007**, *148*, 169-178, doi:10.1007/s10336-007-0175-8.

2. Lattin, C.R.; Reed, J.M.; Desrochers, D.W.; Romero, L.M. Elevated corticosterone in feathers correlates with corticosterone-induced decreased feather quality: A validation study. *J. Avian Biol.* **2011**, *42*, 247-252, doi:10.1111/j.1600-048x.2010.05310.x.

3. Shini, S.; Kaiser, P. Effects of stress, mimicked by administration of corticosterone in drinking water, on the expression of chicken cytokine and chemokine genes in lymphocytes. *Stress* **2009**, *12*, 388-399, doi:10.1080/10253890802526894.

4. Hangalapura, B.N.; Nieuwland, M.G.B.; Buyse, J.; Kemp, B.; Parmentier, H.K. Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. *Poult. Sci. Assoc.* **2004**, *83*, 1644-1649, doi:10.1093/ps/83.10.1644.

5. Zhao, F.Q.; Zhang, Z.W.; Qu, J.P.; Yao, H.D.; Li, M.; Li, S.; Xu, S.W. Cold stress induces antioxidants and Hsps in chicken immune organs. *Cell Stress Chaperon.* **2014**, *19*, 635-648, doi:10.1007/s12192-013-0489-9.

6. Zulkifli, I.; Najafi, P.; Nurfarahin, A.J.; Soleimani, A.F.; Kumari, S.; Aryani, A.A.; O'Reilly, E.L.; Eckersall, P.D. Acute phase proteins, interleukin 6, and heat shock protein 70 in broiler chickens administered with corticosterone. *Poult. Sci.* **2014**, *93*, 3112-3118, doi:10.3382/ps.2014-04099.

7. Mu, P.; Xu, N.; Chai, T.; Jia, Q.; Yin, Z.; Yang, S.; Qian, Y.; Qiu, J. Simultaneous determination of 14 antiviral drugs and relevant metabolites in chicken muscle by UPLC–MS/MS after QuEChERS preparation. *J. Chromatogr. B* **2016**, *1023-1024*, 17-23, doi:10.1016/j.jchromb.2016.04.036.

8. Li, Y.; Liu, K.; Beier, R.C.; Cao, X.; Shen, J.; Zhang, S. Simultaneous determination of mequindox, quinocetone, and their major metabolites in chicken and pork by UPLC-MS/MS. *Food Chem.* **2014**, *160*, 171-179, doi:10.1016/j.foodchem.2014.03.094.

9. Monajjemzadeh, F.; Farajzadeh, M.A.; Mogaddam, M.R.A. Combination of pressurised liquid extraction with dispersive liquid–liquid microextraction method for the extraction of some pesticides and their related metabolites from chicken liver. *Int. J. Environ. Anal. Chem.* **2021**, 1-15, doi:10.1080/03067319.2020.1865329.

10. Yong, Y.; Liu, Y.; He, L.; Xu, L.; Zhang, Y.; Fang, B. Simultaneous determination of quinocetone and its major metabolites in chicken tissues by high-

performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. B* **2013**, *919-920*, 30-37, doi:10.1016/j.jchromb.2012.12.038.

11. Cajka, T.; Danhelova, H.; Zachariasova, M.; Riddellova, K.; Hajslova, J. Application of direct analysis in real time ionization–mass spectrometry (DART–MS) in chicken meat metabolomics aiming at the retrospective control of feed fraud. *Metabolomics* **2013**, *9*, 545-557, doi:10.1007/s11306-013-0495-z.

12. Alexandrakis, D.; Brunton, N.P.; Downey, G.; Scannell, A.G.M. Identification of spoilage marker metabolites in Irish chicken breast muscle using HPLC, GC–MS coupled with SPME and traditional chemical techniques. *Food Bioprocess Tech.* **2012**, *5*, 1917-1923, doi:10.1007/s11947-010-0500-8.

13. Lytou, A.E.; Nychas, G.-J.E.; Panagou, E.Z. Effect of pomegranate based marinades on the microbiological, chemical and sensory quality of chicken meat: A metabolomics approach. *Int. J. Food Microbiol.* **2018**, *267*, 42-53, doi:10.1016/j.ijfoodmicro.2017.12.023.

14. Inglis, G.D.; Wright, B.D.; Sheppard, S.A.; Abbott, D.W.; Oryschak, M.A.; Montina, T. Expeller-pressed canola (*Brassica napus*) meal modulates the structure and function of the cecal microbiota, and alters the metabolome of the pancreas, liver, and breast muscle of broiler chickens. *Animals* **2021**, *11*, 1-22, doi:10.3390/ani11020577.

15. Aggrey, S.E.; Milfort, M.C.; Fuller, A.L.; Yuan, J.; Rekaya, R. Effect of host genotype and *Eimeria acervulina* infection on the metabolome of meat-type chickens. *PLoS One* **2019**, *14*, e0223417, doi:10.1371/journal.pone.0223417.

16. Beauclercq, S.; Nadal-Desbarats, L.; Hennequet-Antier, C.; Collin, A.; Tesseraud, S.; Bourin, M.; Le Bihan-Duval, E.; Berri, C. Serum and muscle metabolomics for the prediction of ultimate pH, a key factor for chicken-meat quality. *J. Proteome Res.* **2016**, *15*, 1168-1178, doi:10.1021/acs.jproteome.5b01050.

17. Zhang, L.; Yue, H.Y.; Zhang, H.J.; Xu, L.; Wu, S.G.; Yan, H.J.; Gong, Y.S.; Qi, G.H. Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality. *Poult. Sci.* **2009**, *88*, 2033-2041, doi:10.3382/ps.2009-00128.

18. Xiao, Z.; Luo, Y.; Wang, G.; Ge, C.; Zhou, G.; Zhang, W.; Liao, G. ^{1}H-NMR-based water-soluble low molecular weight compound characterization and fatty acid composition of boiled Wuding chicken during processing. *J. Sci. Food Agric.* **2019**, *99*, 429-435, doi:10.1002/jsfa.9204.

19. Nicholson, J.K.; Lindon, J.C.; Holmes, E. 'Metabonomics': Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999**, *29*, 1181-1189, doi:10.1080/004982599238047.

20. Wishart, D.S. Quantitative metabolomics using NMR. *Trends Analyt. Chem.* **2008**, *27*, 228-237, doi:10.1016/j.trac.2007.12.001.

21. Markley, J.L.; Bruschweiler, R.; Edison, A.S.; Eghbalnia, H.R.; Powers, R.; Raftery, D.; Wishart, D.S. The future of NMR-based metabolomics. *Curr. Opin. Biotechnol.* **2017**, *43*, 34-40, doi:10.1016/j.copbio.2016.08.001.

22. Le Roy, C.I.; Mappley, L.J.; La Ragione, R.M.; Woodward, M.J.; Claus, S.P. NMR-based metabolic characterization of chicken tissues and biofluids: A model for avian research. *Metabolomics* **2016**, *12*, 157-157, doi:10.1007/s11306-016-1105-7.

23. Abasht, B.; Mutryn, M.F.; Michalek, R.D.; Lee, W.R. Oxidative stress and metabolic perturbations in wooden breast disorder in chickens. *PLoS One* **2016**, *11*, e0153750, doi:10.1371/journal.pone.0153750.

24. Beauclercq, S.; Nadal-Desbarats, L.; Hennequet-Antier, C.; Gabriel, I.; Tesseraud, S.; Calenge, F.; Le Bihan-Duval, E.; Mignon-Grasteau, S. Relationships between digestive efficiency and metabolomic profiles of serum and intestinal contents in chickens. *Sci. Rep.* **2018**, *8*, 6678, doi:10.1038/s41598-018-24978-9.

25. Kiss, D.; Ambeskovic, M.; Montina, T.; Metz, G.A.S. Stress transgenerationally programs metabolic pathways linked to altered mental health. *Cell. Mol. Life Sci.* **2016**, *73*, 4547-4557, doi:10.1007/s00018-016-2272-4.

26. Paxman, E.J.; Boora, N.S.; Kiss, D.; Laplante, D.P.; King, S.; Montina, T.; Metz, G.A.S. Prenatal maternal stress from a natural disaster alters urinary metabolomic profiles in project ice storm participants. *Sci. Rep.* **2018**, *8*, 12932, doi:10.1038/s41598-018-31230-x.

27. Poplawski, J.; Radmilovic, A.; Montina, T.D.; Metz, G.A.S. Cardiorenal metabolic biomarkers link early life stress to risk of non-communicable diseases and adverse mental health outcomes. *Sci. Rep.* **2020**, *10*, doi:10.1038/s41598-020-69866-3.

28. Zaytsoff, S.J.M.; Brown, C.L.J.; Montina, T.; Metz, G.A.S.; Abbott, D.W.; Uwiera, R.R.E.; Inglis, G.D. Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens. *Sci. Rep.* **2019**, *9*, 19225, doi:10.1038/s41598-019-52267-6.

29. Post, J.; Rebel, J.M.; ter Huurne, A.A. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress. *Poult. Sci.* **2003**, *82*, 1313-1318, doi:10.1093/ps/82.8.1313.

30. Anderson, P.E.; Mahle, D.A.; Doom, T.E.; Reo, N.V.; Delraso, N.J.; Raymer, M.L. Dynamic adaptive binning: An improved quantification technique for NMR

spectroscopic data. *Metabolomics* **2011**, *7*, 179-190, doi:10.1007/s11306-010-0242-7.

31. Yun, Y.-H.; Liang, F.; Deng, B.-C.; Lai, G.-B.; Vicente Gonçalves, C.M.; Lu, H.-M.; Yan, J.; Huang, X.; Yi, L.-Z.; Liang, Y.-Z. Informative metabolites identification by variable importance analysis based on random variable combination. *Metabolomics* **2015**, *11*, 1539-1551, doi:10.1007/s11306-015-0803-x.

32. Goodpaster, A.M.; Romick-Rosendale, L.E.; Kennedy, M.A. Statistical significance analysis of nuclear magnetic resonance-based metabonomics data. *Anal. Biochem.* **2010**, *401*, 134-143, doi:10.1016/j.ab.2010.02.005.

33. Chong, J.; Xia, J. MetaboAnalystR: An R package for flexible and reproducible analysis of metabolomics data. *Bioinformatics* **2018**, *34*, 4313-4314, doi:10.1093/bioinformatics/bty528.

34. Xia, J.; Wishart, D.S. Metpa: A web-based metabolomics tool for pathway analysis and visualization. *Bioinformatics* **2010**, *26*, 2342-2344, doi:10.1093/bioinformatics/btq418.

35. Kanehisa, M. Toward understanding the origin and evolution of cellular organisms. *Protein Sci.* **2019**, *28*, 1947-1951, doi:10.1002/pro.3715.

36. Emwas, A.-H.M.; Salek, R.M.; Griffin, J.L.; Merzaban, J. NMR-based metabolomics in human disease diagnosis: Applications, limitations, and recommendations. *Metabolomics* **2013**, *9*, 1048-1072, doi:10.1007/s11306-013-0524-y.

37. Hazard, D.; Fernandez, X.; Pinguet, J.; Chambon, C.; Letisse, F.; Portais, J.-C.; Wadih-Moussa, Z.; Rémignon, H.; Molette, C. Functional genomics of the muscle response to restraint and transport in chickens. *J. Anim. Sci.* **2011**, *89*, 2717-2730, doi:10.2527/jas.2010-3288.

38. Tomonaga, S.; Okuyama, H.; Tachibana, T.; Makino, R. Effects of high ambient temperature on plasma metabolomic profiles in chicks. *Anim. Sci. J.* **2018**, *89*, 448-455, doi:10.1111/asj.12951.

39. Shini, S.; Huff, G.R.; Shini, A.; Kaiser, P. Understanding stress-induced immunosuppression: Exploration of cytokine and chemokine gene profiles in chicken peripheral leukocytes. *Poult. Sci.* **2010**, *89*, 841-851, doi:10.3382/ps.2009-00483.

40. Zeng, Z.K.; Li, Q.Y.; Piao, X.S.; Liu, J.D.; Zhao, P.F.; Xu, X.; Zhang, S.; Niu, S. *Forsythia suspensa* extract attenuates corticosterone-induced growth inhibition, oxidative injury, and immune depression in broilers. *Poult. Sci.* **2014**, *93*, 1774-1781, doi:10.3382/ps.2013-03772.

41. Dong, H.; Lin, H.; Jiao, H.C.; Song, Z.G.; Zhao, J.P.; Jiang, K.J. Altered development and protein metabolism in skeletal muscles of broiler chickens (*Gallus gallus domesticus*) by corticosterone. *Comp. Biochem. Physiol. A* **2007**, *147*, 189-195, doi:10.1016/j.cbpa.2006.12.034.

42. Lu, Z.; He, X.; Ma, B.; Zhang, L.; Li, J.; Jiang, Y.; Zhou, G.; Gao, F. Dietary taurine supplementation improves breast meat quality in chronic heat-stressed broilers via activating the Nrf2 pathway and protecting mitochondria from oxidative attack. *J. Sci. Food Agric.* **2019**, *99*, 1066-1072, doi:10.1002/jsfa.9273.

43. Bartov, I. Effects of dietary protein concentration and corticosterone injections on energy and nitrogen balances and fat deposition in broiler chicks. *Br. Poult. Sci.* **1985**, *26*, 311-324, doi:10.1080/00071668508416819.

44. Hayashi, K.; Nagai, Y.; Ohstuka, A.; Tomita, Y. Effects of dietary corticosterone and trilostane on growth and skeletal muscle protein turnover in broiler cockerels. *Br. Poult. Sci.* **1994**, *35*, 789-798, doi:10.1080/00071669408417743.

45. Scanes, C.G.; Braun, E. Avian metabolism: Its control and evolution. *Front. Biol.* **2012**, *8*, 134-159, doi:10.1007/s11515-012-1206-2.

46. Elkomy, N.M.I.M.; Ibrahim, I.A.A.E.-H.; Elshazly, S.M.; El-Fayoumi, H.M. Ameliorative effects of clonidine on ethanol induced kidney injury in rats: Potential role for imidazoline-1 receptor. *Eur. J. Pharmacol.* **2018**, *824*, 148-156, doi:10.1016/j.ejphar.2018.02.001.

47. Van Thiel, D.H.; Gavaler, J.S.; Little, J.M.; Lester, R. Alcohol: Its effect on the kidney. *Metabolism* **1977**, *26*, 857-866, doi:10.1016/0026-0495(77)90004-x.

48. Latchoumycandane, C.; Nagy, L.E.; Mcintyre, T.M. Chronic ethanol ingestion induces oxidative kidney injury through taurine-inhibitable inflammation. *Free Radic. Biol. Med.* **2014**, *69*, 403-416, doi:10.1016/j.freeradbiomed.2014.01.001.

49. Neeland, I.J.; Hughes, C.; Ayers, C.R.; Malloy, C.R.; Jin, E.S. Effects of visceral adiposity on glycerol pathways in gluconeogenesis. *Metabolism* **2017**, *67*, 80-89, doi:10.1016/j.metabol.2016.11.008.

50. Shen, J.-c.; Lian, L.-y.; Zhang, Y.; He, Q.-d.; Chen, J.-l.; Zhang, L.-b.; Huang, M.-s.; Liu, M.; Qian, L.-c.; Liu, C.-c.; et al. Dynamic analysis of metabolic response in gastric ulcer (GU) rats with electroacupuncture treatment using ^{1}H NMR-based metabolomics. *Evid. Based Complement. Alternat. Med.* **2019**, *2019*, doi:10.1155/2019/1291427.

51. Watford, M. Gluconeogenesis in the chicken: Regulation of phosphoenolpyruvate carboxykinase gene expression. *Fed. Proc.* **1985**, *44*, 2469-2474.

52. Karmen, A.; Wróblewski, F.; Ladue, J.S. Transaminase activity in human blood. *J. Clin. Invest.* **1955**, *34*, 126-133, doi:10.1172/jci103055.

53. Perry, R.J.; Wang, Y.; Cline, G.W.; Rabin-Court, A.; Song, J.D.; Dufour, S.; Zhang, X.M.; Petersen, K.F.; Shulman, G.I. Leptin mediates a glucose-fatty acid cycle to maintain glucose homeostasis in starvation. *Cell* **2018**, *172*, 234-248.e217, doi:10.1016/j.cell.2017.12.001.

54. Latchoumycandane, C.; Nagy, L.E.; Mcintyre, T.M. Myeloperoxidase formation of PAF receptor ligands induces PAF receptor-dependent kidney injury during ethanol consumption. *Free Radic. Biol. Med.* **2015**, *86*, 179-190, doi:10.1016/j.freeradbiomed.2015.05.020.

55. Afkhami, M.; Kermanshahi, H.; Majidzadeh Heravi, R. Evaluation of whey protein sources on performance, liver antioxidants and immune responses of broiler chickens challenged with ethanol. *J. Anim. Physiol. Anim. Nutr. (Berl.)* **2020**, *104*, 898-908, doi:10.1111/jpn.13327.

56. Fernstrom, J.D.; Fernstrom, M.H. Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. *J. Nutr.* **2007**, *137*, 539S–1547S, doi:10.1093/jn/137.6.1539S.

57. Dai, S.F.; Wang, L.K.; Wen, A.Y.; Wang, L.X.; Jin, G.M. Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. *Br. Poult. Sci.* **2009**, *50*, 333-340, doi:10.1080/00071660902806947.

58. Askanazi, J.; Carpentier, Y.A.; Michelsen, C.B.; Elwyn, D.H.; Furst, P.; Kantrowitz, L.R.; Gump, F.E.; Kinney, J.M. Muscle and plasma amino acids following injury: Influence of intercurrent infection. *Ann. Surg.* **1980**, *192*, 78-85.

59. Hu, H.; Bai, X.; Shah, A.A.; Dai, S.; Wang, L.; Hua, J.; Che, C.; He, S.; Wen, A.; Jiang, J. Interactive effects of glutamine and gamma-aminobutyric acid on growth performance and skeletal muscle amino acid metabolism of 22–42-day-old broilers exposed to hot environment. *Int. J. Biometeorol.* **2016**, *60*, 907-915, doi:10.1007/s00484-015-1084-9.

60. Dryer, S.E. Glutamate receptors in the kidney. *Nephrol. Dial. Transplant.* **2015**, *30*, 1630-1638, doi:10.1093/ndt/gfv028.

61. Wu, G.; Bazer, F.W.; Burghardt, R.C.; Johnson, G.A.; Kim, S.W.; Knabe, D.A.; Li, P.; Li, X.; Mcknight, J.R.; Satterfield, M.C.; et al. Proline and hydroxyproline metabolism: Implications for animal and human nutrition. *Amino Acids* **2011**, *40*, 1053-1063, doi:10.1007/s00726-010-0715-z.

62. Phang, J.M.; Donald, S.P.; Pandhare, J.; Liu, Y. The metabolism of proline, a stress substrate, modulates carcinogenic pathways. *Amino Acids* **2008**, *35*, 681-690, doi:10.1007/s00726-008-0063-4.

63. Phang, J.M.; Liu, W.E.I.; Zabirnyk, O. Proline metabolism and microenvironmental stress. *Annu. Rev. Nutr.* **2010**, *30*, 441-463, doi:10.1146/annurev.nutr.012809.104638.

64. Roginski, A.C.; Cecatto, C.; Wajner, S.M.; Camera, F.D.M.; Castilho, R.F.; Wajner, M.; Amaral, A.U. Experimental evidence that maleic acid markedly compromises glutamate oxidation through inhibition of glutamate dehydrogenase and α-ketoglutarate dehydrogenase activities in kidney of developing rats. *Mol. Cell. Biochem.* **2019**, *458*, 99-112, doi:10.1007/s11010-019-03534-7.

65. Ma, B.; Zhang, L.; Li, J.; Xing, T.; Jiang, Y.; Gao, F. Heat stress alters muscle protein and amino acid metabolism and accelerates liver gluconeogenesis for energy supply in broilers. *Poult. Sci.* **2021**, *100*, 215-223, doi:10.1016/j.psj.2020.09.090.

66. Rui, L. Energy metabolism in the liver. *Compr. Physiol,* **2014**, *4*, 177-197, doi:10.1002/cphy.c130024.

67. Jastrebski, S.F.; Lamont, S.J.; Schmidt, C.J. Chicken hepatic response to chronic heat stress using integrated transcriptome and metabolome analysis. *PLoS One* **2017**, *12*, e0181900, doi:10.1371/journal.pone.0181900.

68. Hubbard, A.H.; Zhang, X.; Jastrebski, S.; Singh, A.; Schmidt, C. Understanding the liver under heat stress with statistical learning: An integrated metabolomics and transcriptomics computational approach. *BMC Genomics* **2019**, *20*, doi:10.1186/s12864-019-5823-x.

69. Henle, K.J.; Kaushal, G.P.; Nagle, W.A.; Nolen, G.T. Prompt protein glycosylation during acute heat stress. *Exp. Cell Res.* **1993**, *207*, 245-251, doi:10.1006/excr.1993.1190.

70. Hu, Y.; Sun, Q.; Liu, J.; Jia, Y.; Cai, D.; Idriss, A.A.; Omer, N.A.; Zhao, R. In ovo injection of betaine alleviates corticosterone-induced fatty liver in chickens through epigenetic modifications. *Sci. Rep.* **2017**, *7*, 40251, doi:10.1038/srep40251.

71. Hu, Y.; Sun, Q.; Hu, Y.; Hou, Z.; Zong, Y.; Omer, N.A.; Abobaker, H.; Zhao, R. Corticosterone-induced lipogenesis activation and lipophagy inhibition in chicken liver are alleviated by maternal betaine supplementation. *J. Nutr.* **2018**, *148*, 316-325, doi:10.1093/jn/nxx073.

72. Monirujjaman, M.; Ferdouse, A. Metabolic and physiological roles of branched-chain amino acids. *Adv. Mol. Biol.* **2014**, *2014*, 1-6, doi:10.1155/2014/364976.

73. Bequette, B.J. Amino acid metabolism in animals: An overview. *Amino Acids in Animal Nutrition* **2003**, 87-102.

74. Urdaneta-Rincon, M.; Leeson, S. Muscle (pectoralis major) protein turnover in young broiler chickens fed graded levels of lysine and crude protein. *Poult. Sci.* **2004**, *83*, 1897-1903, doi:doi.org/10.1093/ps/83.11.1897.

75. Chowdhury, V.S. Heat stress biomarker amino acids and neuropeptide afford thermotolerance in chicks. *J. Poult. Sci.* **2019**, *56*, 1-11, doi:10.2141/jpsa.0180024.

76. Girón, M.D.; Vílchez, J.D.; Shreeram, S.; Salto, R.; Manzano, M.; Cabrera, E.; Campos, N.; Edens, N.K.; Rueda, R.; López-Pedrosa, J.M. B-hydroxy-β-methylbutyrate (HMB) normalizes dexamethasone-induced autophagy-lysosomal pathway in skeletal muscle. *PLoS One* **2015**, *10*, e0117520, doi:10.1371/journal.pone.0117520.

77. Faseleh Jahromi, M.; Wesam Altaher, Y.; Shokryazdan, P.; Ebrahimi, R.; Ebrahimi, M.; Idrus, Z.; Tufarelli, V.; Liang, J.B. Dietary supplementation of a mixture of *Lactobacillus* strains enhances performance of broiler chickens raised under heat stress conditions. *Int. J. Biometeorol.* **2016**, *60*, 1099-1110, doi:10.1007/s00484-015-1103-x.

78. Savenije, B.; Lambooij, E.; Gerritzen, M.A.; Venema, K.; Korf, J. Effects of feed deprivation and transport on preslaughter blood metabolites, early postmortem muscle metabolites, and meat quality. *Poult. Sci.* **2002**, *81*, 699-708, doi:10.1093/ps/81.5.699.

79. Yang, P.; Hao, Y.; Feng, J.; Lin, H.; Feng, Y.; Wu, X.; Yang, X.; Gu, X. The expression of carnosine and its effect on the antioxidant capacity of *longissimus dorsi* muscle in finishing pigs exposed to constant heat stress. *Asian. Austral. J. Anim.* **2014**, *27*, 1763-1772, doi:10.5713/ajas.2014.14063.

3 FECAL ^{1}H-NMR METABOLOMICS: A COMPARISON OF SAMPLE PREPARATION METHODS FOR NMR AND NOVEL *IN SILICO* BASELINE CORRECTION

3.1 Abstract

Analysis of the functioning of the enteric microbiota indirectly through analysis of the fecal metabolome has the potential to be a cost-effective and informative diagnostic tool. However, metabolomic analysis of feces is hampered by the difficulty of effectively removing high concentrations of fats and large macromolecules like proteins and fiber from samples. Using rat fecal samples, the efficacy of four different sample preparation methods for ^{1}H-NMR analysis were investigated. The three most promising methods, ultrafiltration (UF), Bligh-Dyer methanol-chloroform extraction (BD), and no extraction (samples added directly to buffer, vortexed, and centrifuged) were then tested on fecal samples obtained from multiple rats (n=10) and chickens (n=8) to ascertain whether the methods chosen worked equally well across species and individuals within species. An *in silico* baseline correction method was also evaluated to determine if a cost-effective data processing algorithm could be utilized to obtain reproducible results similar to those obtained via UF. For both rat and chicken feces, UF removed all macromolecules and produced no baseline distortion among samples. In contrast, the BD and no extraction methods did not remove all the macromolecules and produced significant baseline distortions. The application of *in silico* baseline correction removed the effects of the distortion and produced spectra comparable to the UF spectra. In the case of no extraction, spectra with more intense peaks were produced. This suggests that baseline correction may be a cost-effective and reproducible method for metabolomic analysis of

fecal samples as an alternative to the more costly UF method. UF was the most versatile, reproducible, and efficient method to extract metabolites from fecal samples; however, BD and no extraction followed by baseline correction can produce comparable results.

3.2 Introduction

The enteric microbiota plays a critical role in the health of mammals and avians, and the structure and function of the microbiota can be an indicator of the health status of the host. The enteric microbiota impacts host health in a number of ways, including digestion of foods (e.g. fermentation of plant fiber), production of short-chain fatty acids and vitamins, stimulation and modulation of immune responses, and protecting the host from pathogens to name a few [1,2]. Alterations to the normal structure of the microbiota (i.e. dysbiosis) is associated with a number of adverse acute and chronic health conditions such as pseudomembraneous colitis [3,4], Inflammatory Bowel Disease (IBD) [5], and Irritable Bowel Syndrome (IBS) [6]. Autoimmune disorders such as Multiple Sclerosis (MS) [7], mood disorders such as Major Depressive Disorder (MDD) [8], Autism Spectrum Disorder (ASD) [9], and neurodegenerative diseases such as human Motor Neuron Disease (MND) [10] have also been linked to dysbioses in the enteric microbiota. However, whether this is cause or effect, and the mechanisms by which the microbiome affects host health are currently enigmatic.

In recent years an emphasis has been placed on characterizing the composition and structure of the enteric microbiota using 16S rRNA gene sequencing [11]. However, this technique does not directly measure bacterial function such as metabolic processes [12]. Another disadvantage of 16S rRNA gene sequencing is that it currently delivers poor taxonomic resolution (i.e. family or genus), it is relatively time and bioinformatically

intensive, and it can be cost prohibitive [12,13]. Using metabolomics to ascertain the function of the microbiota, including the impact of enteric dysbioses on host health has considerable benefits, and it is receiving increasing attention for applications in human and non-human medicine. Metabolomics, the study of the small molecular weight molecules forming and resulting from the biochemical pathways of an organism, can be used to characterize the metabolome in a variety of tissues and biofluids [14-16]. The fecal metabolome reflects host-microbiome interactions. However, up to 68% of its variance arises from the enteric microbiota, and not the host [17]. As the relationship between the enteric microbiome and health of the host becomes more evident, there is increasing interest in using fecal metabolomics as an indirect and non-invasive diagnostic indicator of host health.

Despite the presence of established methods and databases for proton nuclear magnetic resonance (^{1}H-NMR)-based metabolomic analysis of serum [18], urine [19], and saliva [20], characterization of the fecal metabolome is an emerging field with a large amount of variation in sample preparation methods reported among studies [21-25]. The analysis of fecal samples using metabolomic methods presents a series of unique challenges, namely the solid nature of the sample, and the presence of varying and large amounts of molecules such as water, proteins, fats, and fiber. Notably, inconsistent and incomplete removal of fats and macromolecules from fecal samples can result in an increase in the number of broad signals in the baseline of ^{1}H-NMR spectra, and these signals can often span several parts per million on the NMR chemical shift scale. As a direct result of this, there is a great deal of inaccuracy in the quantification of any metabolite that overlaps with these broad signals [26].

Previous studies have examined the effects of various sample processing and metabolite extraction methods of feces to determine which are the most unbiased, efficient, and reproducible. These include but are not limited to fresh *versus* frozen fecal samples [27,28], pooled *versus* un-pooled samples [29], freeze-dried *versus* frozen samples [21,24,25,27,29-32], refrigerated *versus* frozen samples [33], different homogenization methods, and the ratio of feces to buffer (W_f:V_b) [34]. Although currently published studies present important information, there is currently no universal consensus on the best methods by which to prepare and analyze fecal samples for metabolomic analysis using ^{1}H-NMR [35]. In this regard, Cui *et al*. [35] evaluated several different methods for preparation of fecal samples for ^{1}H-NMR, including a comparison of extraction solvents and processing on the reproducibility of signature metabolites. However, they did not test ultrafiltration (UF), which has been recommended for many types of samples [18,36]; UF provides good signal-to-noise, provides good reproducibility without unwanted evaporation of volatile metabolites, and avoids loss of metabolites by dissolution in solvents or the hydrolysis of metabolites due to the need to neutralize the solution [36]. An alternative approach for the consistent removal of broad signals from NMR spectra is *in silico* baseline correction. With respect to metabolomics, these methods have only been applied to smoothing or flattening distortions in the baseline of a spectrum, such as rolling baselines, and they have not been applied to the removal of the large broad signals that may be present due to residual macromolecules [37,38].

The goal of the current study was to compare a number of sample preparation methods for fecal metabolomics analysis by ^{1}H-NMR. The methods tested were: (1) no extraction in which the fecal sample is added to buffer, vortexed, and centrifuged

(hereafter referred to as “no extraction”); (2) UF [25]; (3) the traditional Bligh-Dyer (BD) fat and protein extraction method [34,39]; and (4) a modified version of the BD (M-BD) method that was intended for samples which have high fatty acid content [40]. Lastly, the most effective of these methods, UF, was compared to results achieved using both the no-extraction and BD methods in which the spectra were processed using a *in silico* baseline correction. These methods were evaluated both qualitatively and quantitatively based on the least amount of baseline variation and the most consistent results across samples.

3.3 Materials and methods

Ethics approval

Procedures involving the handling of rats were approved by the University of Lethbridge Animal Care Committee under guidelines put forth by the Canadian Council on Animal Care. The chicken component of this project was reviewed and approved by the Agriculture and Agri-Food Canada (AAFC) Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol Review 1903). The study was carried out in strict accordance with the recommendations established in the Canadian Council on Animal Care Guidelines.

Animal protocol

Long-Evans rats were born and housed in the University of Lethbridge vivarium on 12:12 hr day/night cycle in standard polycarbonate cages on corn chip bedding. The ambient temperature in the animal room was 22°C, and animals had access to food and water *ad libitum*. White Leghorn chickens were born and housed at the LeRDC vivarium on 16:8 hr day/night cycle. Chickens were maintained in individually ventilated cages (Techniplast, Montreal, QC) on autoclaved wood shavings (United Farmers of Alberta

Co-operative Ltd., Lethbridge, AB). The ambient temperature in the animal room was 30°C for the first 2 days, 28°C for the next 2 days, and 26°C thereafter and birds had access to food (Hi-Pro Feeds, Lethbridge, AB) and water *ad libitum*.

Sample preparation – Comparing methods without variation across individuals

Recently excreted fecal pellets were collected from a single adult Long-Evans rat at a single time point, and placed at -80ºC within 20 min of collection. Frozen samples were later divided into four equal aliquots with the goal of conducting analyses on samples with identical metabolomes. The four fecal subsamples were thawed on ice, mixed with metabolomics buffer at a 2:1 volume to mass ratio, vortexed until the sample became homogenous, centrifuged at 14,000 x g for 20 min, and the supernatant was removed and is hereafter is referred to as fecal water. The metabolomics buffer consists of a 4:1 ratio of mono and dibasic potassium phosphate salts (K_2HPO_4/KH_2PO_4) in H_2O with 3 mM sodium azide (NaN_3). The resulting pH of this buffer was 7.4; sodium azide was added as an anti-microbial agent. Fecal water samples were then stored at -80ºC until processed. As previously indicated, water soluble metabolites were isolated from the fecal water using the following four techniques: (1) no extraction (2) UF, (3) BD extraction, and (4) fatty acid favoring extraction. In the case of the no extraction method, 275 µL of each fecal water sample was used to prepare the NMR sample without further processing.

Ultrafiltration

Amicon Ultra 0.5 mL Centrifugal Filters (Merck Millipore, Cork, IRL) with a molecular weight cutoff of 3 kDa were utilized. Each filter was washed by adding 500 µL of Millipore water (Merck Millipore) to the filter, and centrifuging at 14,000 x g for 5 min. This washing step was repeated ten times in order to ensure that all the glycerol in the filter had been removed [36,41]. Following the wash step, 350 µL of the fecal water

was added to the filter and centrifuged at 14,000 x g for 30 min at 4ºC. A 275 µL aliquot of the fecal water filtrate was set aside for NMR sample preparation.

Bligh-Dyer methanol-chloroform extraction

In a 2 mL tube, 275 µL of each fecal water sample was combined with 387.5 µL of methanol and 343.8 µL of chloroform. The tube was then vortexed and stored at -20ºC for 15 min to precipitate proteins, and then centrifuged at 15,300 x g for 15 min at 4ºC. The supernatant from each sample was decanted into a new tube containing equal volumes of 343.8 µL of chloroform and deionized water. This mixture was then vortexed briefly, and centrifuged at 6,700 x g for 5 min at 4°C. Following centrifugation, 1 mL of the top aqueous layer containing the water-soluble metabolites of each sample was pipetted into new tube, and samples were placed in a nitrogen gas flow box for 5-6 days to dry. Once dry, samples were rehydrated in 275 µL of deionized water, and the rehydrated samples were set aside for NMR sample preparation.

Fatty acid removal favoring liquid-liquid extraction

In a 2 mL tube, 275 µL of each fecal water sample was combined with 1,500 µL of methanol and 1,500 µL of chloroform. The sample tube was vortexed and stored at -20°C for 15 min to allow precipitation of proteins, and then centrifuged at 15,000 x g for 15 min at 4°C. The supernatant from each sample was then transferred to a new microcentrifuge tube containing 375 µL of methanol, 375 µL of chloroform, and 475 µL of deionized water. Each sample was vortexed and centrifuged at 6,800 x g for 5 min at 4°C. After centrifugation, 1 mL of the top layer containing the water-soluble metabolites of each sample was pipetted into a new tube, left to dry in a nitrogen gas flow box, and rehydrated as described above.

Sample preparation – Replication of preparation methods across multiple samples

Individual fecal samples were obtained from rats (n = 10) and chickens (n = 8). Each fecal sample was split into three aliquots (i.e. subsamples) with each having a mass of at least 150 mg, and each aliquot was used to prepare fecal water as described previously. The following three methods were evaluated for variation within and across fecal samples by species: (1) no extraction; (2) UF; and (3) BD extraction as described previously. *In silico* baseline correction was applied to the spectra obtained from fecal water samples processed by the BD and no-extraction methods. This allowed for a comparison of the application of *in silico* baseline correction filter to the spectra obtained using the UF method.

Sample preparation for ^{1}H-NMR spectroscopy

From each sample, 275 μL was mixed with a 120 μL of deuterium oxide (D_2O) containing 0.027% w/v sodium 3-trimethylsilylpropanoate-2,2,3,3-d_4 (TSP), and 205 μL of metabolomics buffer (total volume of 600 μL); TSP was used as a chemical shift reference for ^{1}H-NMR spectroscopy. The solution was vortexed and then centrifuged at 12,000 x g for 5 min at 4 °C to pellet particulate matter. Following centrifugation, a 550 μL aliquot of the supernatant was loaded into a 5 mm NMR tube.

NMR data acquisition and processing

Spectra were obtained on a 700 MHz Bruker Avance III HD spectrometer using the 1-D NOESY gradient pulse pre-saturation water suppression pulse sequence 'noesygppr1d' with 10 ms mixing time. Each sample was run for 512 scans to a total acquisition size of 128 k, a spectral window of 20.5 ppm, a transmitter offset of 4.7 ppm, and a recycle delay of 4 sec. All measurements were recorded using a Bruker triple resonance TBO-Z probe. The Bruker automation program "pulsecal" was used on each

sample before data acquisition to guarantee that the 90-degree pulse was calibrated correctly, ensuring quantitative and comparable data across samples [42]. The spectra were zero filled to 256 k, automatically phased, baseline corrected and line-broadened by 0.3 Hz [43].

***In silico* baseline correction**

The baseline correction algorithm provided with the Bruker AssureNMR software (Bruker, Billerica MA), referenced as underground baseline correction by the manufacturer, was used to create a python macro that was applied in Bruker's TopSpin software. The first step in this macro was to enter a filter width in hertz (Hz) applicable to the baseline correction. This width is then converted from Hz to the number of spectral data points by dividing the width by the digital resolution (number of spectral points per Hz). For each datum point in the spectrum, the algorithm determines a minimum intensity value (baseline) by searching to the left and right of the datum point by the number or points set as the filter width. This minimum value was then subtracted from the current datum point, and the process was repeated until all data points had been searched. Following this process, an average value was calculated from the first corrected point to that point plus $1/16^{th}$ of the total spectral points. This average value was then subtracted from every datum point in the spectrum.

Metabolite quantification

Metabolites were identified and quantified using Chenomx NMR Suite 8.5 standard (Edmonton, AB) with custom libraries of metabolites that were previously identified in mammalian [21] and chicken [44] feces. The mammalian library contained 415 metabolites previously detected in feces and present as NMR spectra in either the Human Fecal Metabolome Database (HFMDB) [45] or Chenomx database. The chicken library

contained 37 metabolites previously detected in chicken feces [44] and present as NMR spectra in the Chenomx database. The concentration of each metabolite was calculated using the known internal concentration of the TSP peak for each sample (0.37 mM).

3.4 Results and discussion

Quantifying changes in the fecal metabolome can provide valuable insights into the functioning of the enteric microbiota, and interactions with the host. However, fecal samples present a unique challenge due to the varying amounts of water and macromolecules, such as proteins, fats, and fiber that are present and interfere with quantification in NMR-based metabolomics. Currently, there are many different methods proposed for the preparation of fecal samples for ^{1}H-NMR; however, there is no universal consensus on the most cost-effective and reproducible method for water-soluble metabolite extraction. Thus, an overarching goal of the current study was to investigate various sample preparation methods in order to determine the best way to effectively and reproducibly remove unwanted macromolecules, while preserving the small molecule water-soluble metabolites [35]. We evaluated four different extraction methods, as well as the application of *in silico* baseline correction.

Comparison of different metabolite extraction methods

The fatty acid favoring extraction method (M-BD) provided a spectrum with a greatly reduced number of peaks, and the signals were much less intense when compared to the other methods (**Figure 3-1A**). This was particularly evident for the peaks corresponding to glucose and succinic acid at ~5.3 and 2.4 ppm, respectively. The only exception was the formic acid peak at 8.4 ppm, which was most intense when using the M-BD extraction. Overall, the M-BD extraction removed or reduced almost all of the

metabolite peaks, and thus it was not pursued further. The peaks in the no extraction spectrum (**Figure 3-1B**) were more intense than the majority of the peaks in the BD extraction (**Figure 3-1C**) and UF (**Figure 3-1D**) spectra. For example, the argininosuccinic acid, glucose, and butyric acid peaks at 6.5 ppm, 5.3/4.7 ppm, and 1.6 ppm, respectively, were noticeably more intense. In addition, a conspicuous reduction in the spectral intensity of all metabolites was observed for the BD extraction method *versus* the no extraction and UF methods. The UF, no extraction, and BD extraction methods were all chosen for analysis of inter-sample variability, as in contrast to the M-BD method, all three contained sufficient metabolite information to conduct non-targeted metabolomic analyses.

Inter-sample variability and baseline distortion

All of the fecal samples that were extracted using the UF method showed a consistent baseline (**Figure 3-2A**). However, upon close examination, the samples that were processed using both the BD (**Figure 3-2B**) and no extraction (**Figure 3-2C**) methods displayed a baseline distortion that was most prominent in the 1-5 ppm region of the spectra, with this effect being most pronounced for the BD method (**Figure 3-2B**). The BD and no extraction spectra were produced from the same samples that were used for testing the UF method, where a consistent baseline was observed, suggesting that the observed variability is due to the extraction method and is most likely caused by residual macromolecules in the samples. For example, lipids in the sample give broad signals mainly at 0.8-0.9 ppm, 1.1-1.4 ppm, and 5.2-5.4 ppm from CH_3 groups, $(CH_2)_n$ groups, and = CH groups, respectively [26]. Thus, even though the no extraction method provided more intense metabolite peaks as compared to the UF method (**Figure 3-1**), the quantification of these results will not be reliable due to baseline distortions, as broad

signals cause the integral of the peaks to be increased, making quantification inaccurate [26].

To investigate the effects of inter-species variability on metabolite extraction from fecal samples, the same test was conducted with fecal samples obtained from chickens (**Figure 3-3**). A similar trend to rats was observed. That is, the baseline of the UF spectra showed little to no distortions, while the BD and no extraction methods exhibited significant baseline distortions in the 1-5 ppm range of the spectra. This suggests that this problem is not species specific, and it needs to be considered when processing fecal samples regardless of the species under examination.

***In silico* baseline correction removed the baseline distortions from macromolecules**

A relatively unexplored option for removing the baseline distortions observed in the spectra obtained for the BD or no extraction methods is the application of *in silico* baseline correction. Baseline correction is an attractive alternative to the molecular cut-off filters used in the UF method, as these filters can be quite expensive on a per sample basis as compared to baseline correction which has no per-sample cost. However, the correct filter width must be selected to achieve optimal baseline correction (i.e. that most closely resembles the baseline obtained with the UF method). Several different filter widths for *in silico* baseline correction were tested and a filter width of 175 Hz was observed to produce a spectral baseline that was most similar to the UF spectrum (**Figure 3-4**). Subsequently, baseline correction was applied using the 175 Hz filter width to each of the spectra obtained for rat fecal samples processed with the BD and no extraction methods (**Figure 3-5**). Both the BD and no extraction methods followed by *in silico* baseline correction (**Figure 3-5B** and **3-5C**, respectively) produced baselines with little to no distortions across samples, and both closely resembled the spectrum acquired for the UF

method (**Figure 3-5A**). It is noteworthy that the application of baseline correction appeared to cause two additional changes. Firstly, the application of baseline correction resulted in a larger section of the spectrum around the water peak being unusable, which is a result of the way in which the filter is applied (see methods section). Secondly, the overall height of the baseline between 1 and 5 ppm was slightly reduced compared to the UF method. The latter of these two could be seen as a benefit with respect to consistent metabolite quantification.

In silico baseline correction with a filter width of 175 Hz was also applied to the chicken fecal samples prepared using the BD and no extraction methods (**Figure 3-6B** and **3-6C**, respectively). In the case of chicken feces, when combined with *in silico* baseline correction, both the BD and no extraction methods provided more consistent spectral baselines than BD and no extraction alone. However, the spectral baselines were not as consistent as the spectral baseline produced by UF. This indicates that *in silico* baseline correction can be effectively applied to correct baseline distortions regardless of the species under investigation, but the choice of filter width is most likely species specific.

Another method that is frequently used to accommodate samples with large quantities of macromolecules, such as whole blood samples, is the Carr Purcell Meiboom Gill (CPMG) pulse sequence for data acquisition. This pulse sequence functions through the application of multiple repeated Hahn echoes, whereby the NMR signals from macromolecules, which have much more rapid transverse relaxation times (T_2), are fully relaxed during the echo evolution period whereas small molecule metabolites are not (i.e. they have much longer T_2 times) [46]. This is known as relaxation editing or filtering and the resulting spectra do not contain the signals from the macromolecules. However, there

are some issues with the application of the CPMG pulse sequence. Firstly, if the sample contains a lot of proteins, the proteins can bind to small molecules, and this subsequently reduces the T_2 times and also broadens the small molecule metabolite NMR peaks leading to an overestimation of metabolite quantities [47,48]. Secondly, the application of the pulse sequence-based relaxation method inherently removes the ability to accurately quantify absolute concentrations of metabolites, as their signals are differentially reduced by the application of the Hahn echo train (i.e. each metabolite is differentially affected by this relaxation editing) [49-51]. As a result, the application of the CPMG pulse sequence leads to data that can only be quantified on a relative scale. For example, only the metabolite concentration normalized to the total metabolome can be reported. For these reasons, our findings show that both the no extraction and BD method followed by *in silico* baseline correction offer a cost-effective choice as an alternative to the UF method.

Different extraction methods produce different fecal metabolomes

In total, 372 unique metabolites were identified from the rat fecal spectra (**Table A2-1**). A total of 133 of these metabolites were common to the five extraction methods and only 53 had an occurrence of 50 percent or higher (**Table 3-1**). The average rat fecal metabolome is shown in **Figure 3-7**, with the numbers corresponding to the metabolite numbering shown in **Table 3-1**. Thirty-four metabolites were identified in the chicken fecal spectra from the custom library of 37 metabolites (**Figure 3-8**, **Table 3-2**).

The concentrations observed for the no extraction and UF methods were similar for most metabolites, and higher relative to the other three methods. However, in some cases, metabolite concentrations were higher for the no extraction method as compared to UF. In the case of chickens, the concentrations of metabolites obtained using the no extraction method were consistently higher than those obtained using both the UF and BD methods

(**Table 3-2**). The higher concentrations observed for the no extraction method could be caused by the incomplete removal of fats, which makes the peak areas appear larger than they are. Alternatively, the decreased concentrations observed for the UF extraction method could be caused by incomplete filtration, whereby the proteins that concentrate on the membrane bind to metabolites and prevent them from passing through the filter. This is exacerbated by the hydrophobic nature of the membranes, as the membranes adsorb proteins both on the surface and inside the pores due to membrane-protein interactions resulting from higher retention [52]. In addition, as filters are used they accumulate the impermeable macromolecules that have been filtered out on top of the filter, and this can cause both concentration polarization and membrane fouling which prevents the metabolites from passing through the filter [52]. This may be a bigger issue in samples that contain higher concentrations of macromolecules to be removed. For example, this might be the case for fecal samples obtained from animals that are being fed a protein enriched diet. Lastly, the concentrations of metabolites from the BD methanol chloroform extraction were consistently lower than concentrations of metabolites for the UF and no extraction methods, likely due to the metabolites of interest being removed during the extraction. For example, the solvents used can result in the loss of some metabolites. In this regard, liquid-liquid extractions depend on the variable solubility of the metabolites in two immiscible solvents; however, there is no guarantee that the metabolites may not be more soluble in the chloroform than the water, meaning that they may be lost when the chloroform evaporates. Volatile metabolites can also be lost during the drying step. Conversely, the no extraction method does not remove any of the interfering macromolecules, and it is possible that proteins in the samples will bind to the chemical shift reference TSP. If the TSP is fully bound to proteins it can cause broadening of the

chemical shift reference peak. Moreover, partial binding of proteins can lead to a splitting of the chemical shift indicator peak [53]. In the case of the baseline correction, the concentrations obtained via the no extraction and BD methods generally decreased, which supports the possibility that incomplete removal of macromolecules increased the calculated concentrations. However, there are a few key exceptions to this that were observed. In this regard, the concentration of galactose was increased for both methods followed by baseline correction, while glucose only exhibited an increase for the BD method. Similarly, the concentration of betaine increased only for the no extraction method with baseline correction. Lastly, following the application of baseline correction, a limited number of metabolites either became quantifiable or not. For example, methylamine, citrate, and valerate were quantified following the application of baseline correction; however, only valerate was detected at a level greater than 10 mM, with both methylamine and citrate quantified at levels that were near the detection threshold of NMR. Lastly, 2-hydroxybutyrate, which was quantifiable for the no extraction method, was undetectable following the application of baseline correction. These changes should be considered when choosing the extraction method of choice for a targeted characterization of the fecal metabolome using ^{1}H-NMR.

When determining the best extraction method to use, it is also important to consider if the samples being extracted potentially contain pathogens (e.g. if the samples were obtained from animals inoculated with a pathogen). In the case of samples containing pathogens, UF will physically remove pathogen cells rendering samples sterile. It is also possible that the solvents used in the BD extraction will effectively kill pathogens, but this should be ascertained for individual pathogens of interest (e.g. endospores produced by bacterial pathogens may be resistant to solvents).

A diversity of metabolites were quantified in rat fecal samples by each method (**Figure 3-9**). The following number of metabolites were identified by each method in descending order: (i) BD method followed by baseline correction (313); (ii) the BD method with no baseline correction (267); (iii) the UF method (260); (iv) no extraction followed by baseline correction (229); and (v) no extraction (213). There were 46 metabolites that were unique to a single extraction method, with the largest number, 28, unique to the BD method followed by baseline correction. Furthermore, more metabolites were identified for both the BD and no extraction methods when they were followed by *in silico* baseline correction in comparison to the same method without baseline correction (313 and 229 *versus* 267 and 213, respectively). These findings further support the use of either the UF method or the BD method followed by *in silico* baseline correction for NMR-based metabolomic analysis of feces, as they provide more metabolite information than the no extraction methods, and both have the potential to be applied to the study samples obtained from animals infected with pathogens.

Principal Component Analysis (PCA) of the different extraction methods that we evaluated showed unsupervised separation between metabolite bins among the UF, BD, and no extraction methods for both rats (**Figure 3-10A**) and chickens (**Figure 3-10B**). This indicates that the extraction method chosen alters the metabolome obtained, and is concordant with the variation observed in the quantification of metabolites reported in **Table 3-1**, **Table A2- 1**, and **Table 3-2**. For feces obtained from both species, the variation observed in the metabolite spectra obtained for the no extraction method more closely resemble the spectra obtained for the UF method. The spectra generated from the BD method showed a higher degree of separation relative to the no extraction and UF methods, as evidenced by minimal to no overlap in the 95% confidence intervals. These

findings further support the need to carefully choose an extraction method, and reinforces that any fecal metabolomic findings reported in the literature must take the extraction method used into consideration. The similarity between the spectra obtained using the no extraction and UF methods further supports the use of either UF or the no extraction with *in silico* baseline correction to characterize the water-soluble metabolome in feces using ^{1}H-NMR metabolomics.

3.5 Conclusions

The aims of the current study were to: (i) examine the variance observed in the fecal metabolomic data obtained from NMR following the application of the most common small molecule water-soluble metabolite extraction methods; (ii) assess the utility of applying *in silico* baseline correction as a means to deal with incomplete removal of macromolecules from fecal samples; and (iii) to provide a recommendation for the best extraction method, in terms of efficiency, reproducibility, and cost, to be utilized for NMR-based metabolomic studies of feces. To this end, results obtained showed that the no extraction method provided the best signal to noise ratio as compared to either the UF or BD extraction methods. However, an examination of inter-sample variability showed that both the no extraction and BD methods resulted in baseline distortions that were caused by the incomplete removal of macromolecules during extraction. Notably, baseline distortions were not evident for samples extracted using the UF method. In addition, the baseline distortions observed for both the no extraction and BD methods were shown to be species independent, as they were observed for fecal samples obtained from both rats and chickens. The application of *in silico* baseline correction with a filter width of 175 Hz to the spectra obtained following the no extraction and BD method

effectively removed the distortions and produced spectra with a consistent baseline similar to that obtained with the UF method, regardless of the species investigated. However, the UF method can be utilized in the case of both normal and pathogen-containing samples, whereas the no extraction method cannot be used on samples potentially containing pathogens, and the BD method must be tested on each pathogen to ensure that the solvents used effectively kill the pathogen. To this end, this study found that the most versatile, reproducible, and efficient method to extract water soluble small molecule metabolites from fecal samples was the UF method. However, salient disadvantages of the UF method were its higher cost and the potential for membrane fouling. Although the no extraction and BD methods were subject to significant baseline distortion, the application of *in silico* baseline correction largely negated the distortion. Thus, these two methods with baseline correction should be explored further, including their applicability to characterization the fecal metabolome of other species as well as non-fecal samples using ^{1}H-NMR-based metabolomics.

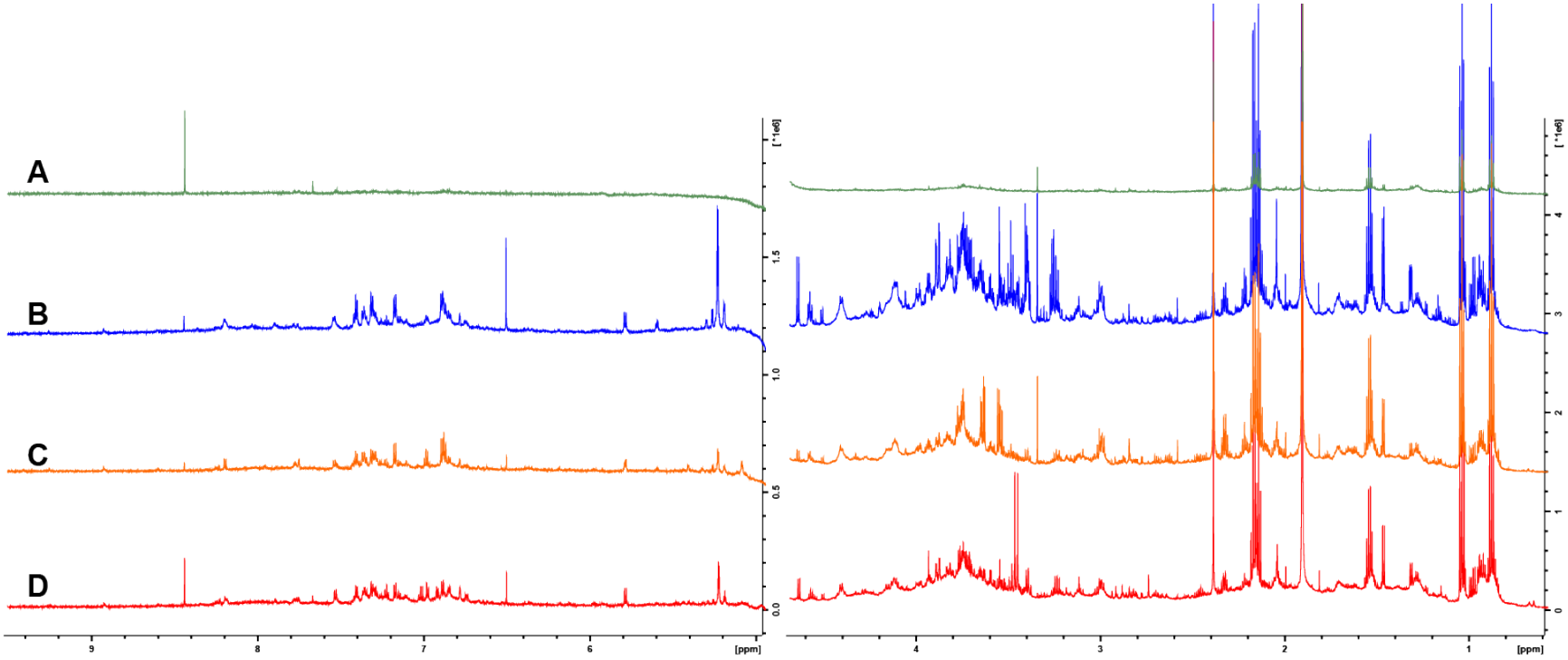

Figure 3-1: NMR spectra of rat fecal samples that were processed using four different techniques: **(A)** fatty acid favoring extraction; **(B)** no extraction; **(C)** ultrafiltration; and **(D)** Bligh-Dyer extraction. The spectra have been split at the water peak and the vertical scale has been increased to better illustrate the metabolites that are present

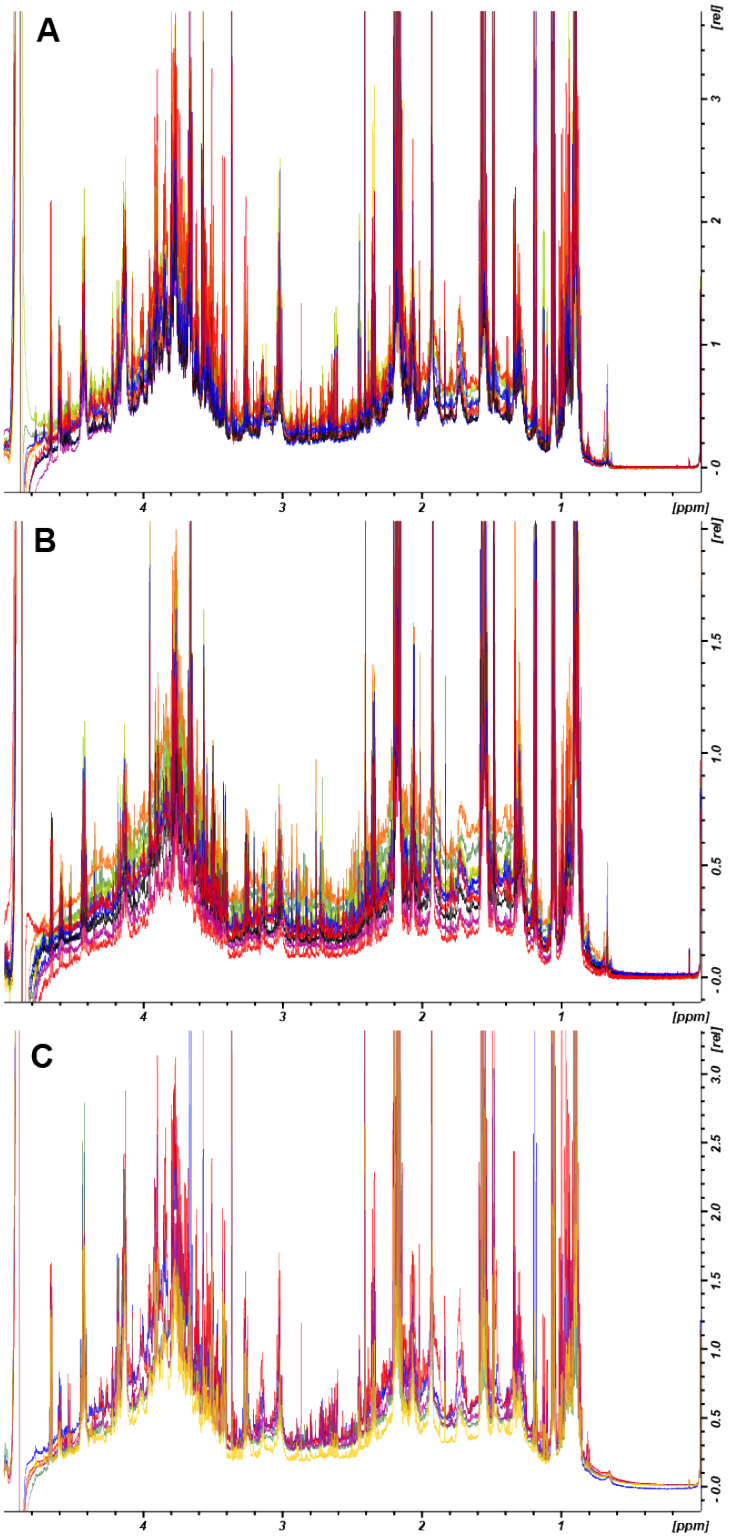

Figure 3-2: Overlaid NMR spectra in the 1-5 ppm range of rat feces that were processed by: **(A)** ultrafiltration; **(B)** Bligh-Dyer extraction; and **(C)** no extraction. The fanning of the spectra baseline indicates variability across samples. The vertical scale has been increased to better illustrate spectral fanning

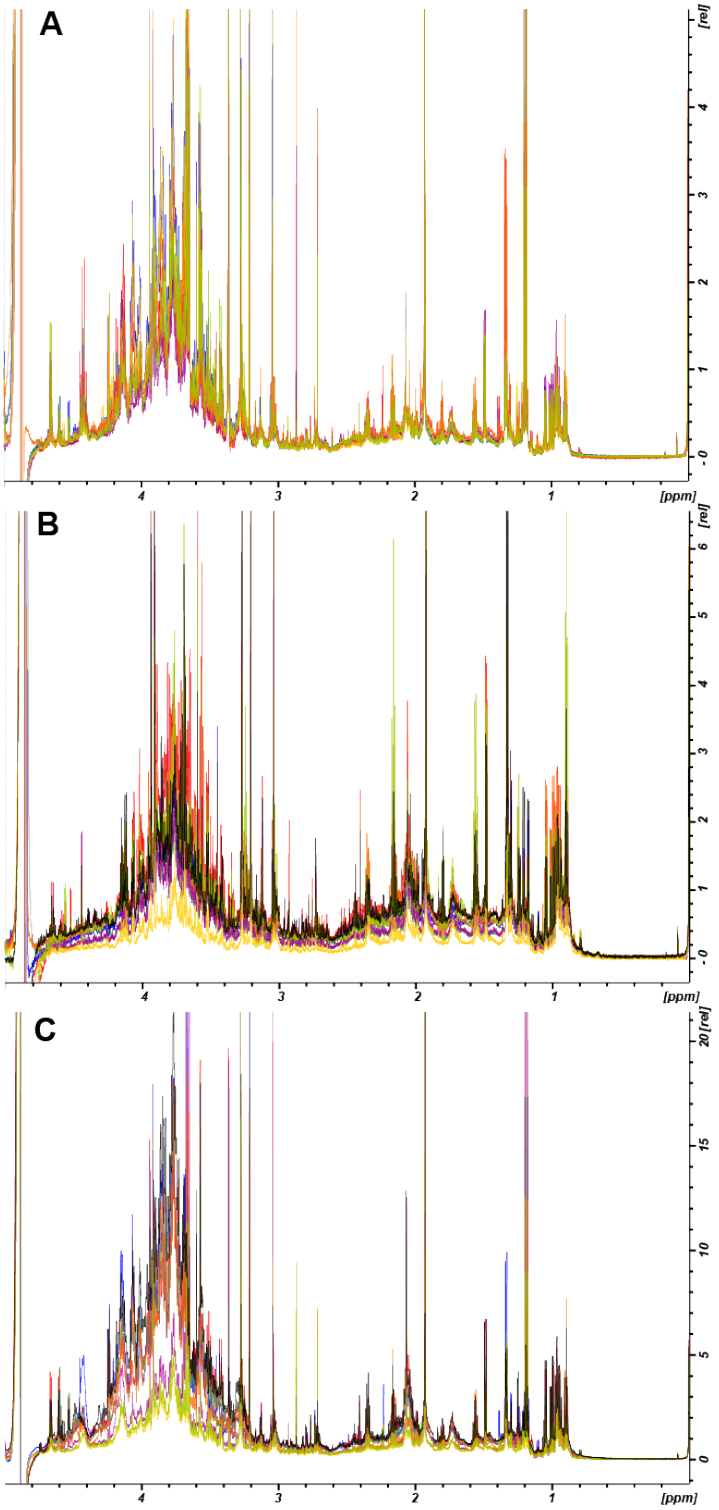

Figure 3-3: Overlaid NMR spectra of water-soluble metabolites in the 1-5 ppm region of chicken feces that was processed by: **(A)** ultrafiltration; **(B)** Bligh-Dyer extraction; and **(C)** no extraction. The fanning of the spectra baseline indicates variability in the same method across samples. The spectra have been split at the water peak, and the vertical scale has been increased to better illustrate spectral fanning

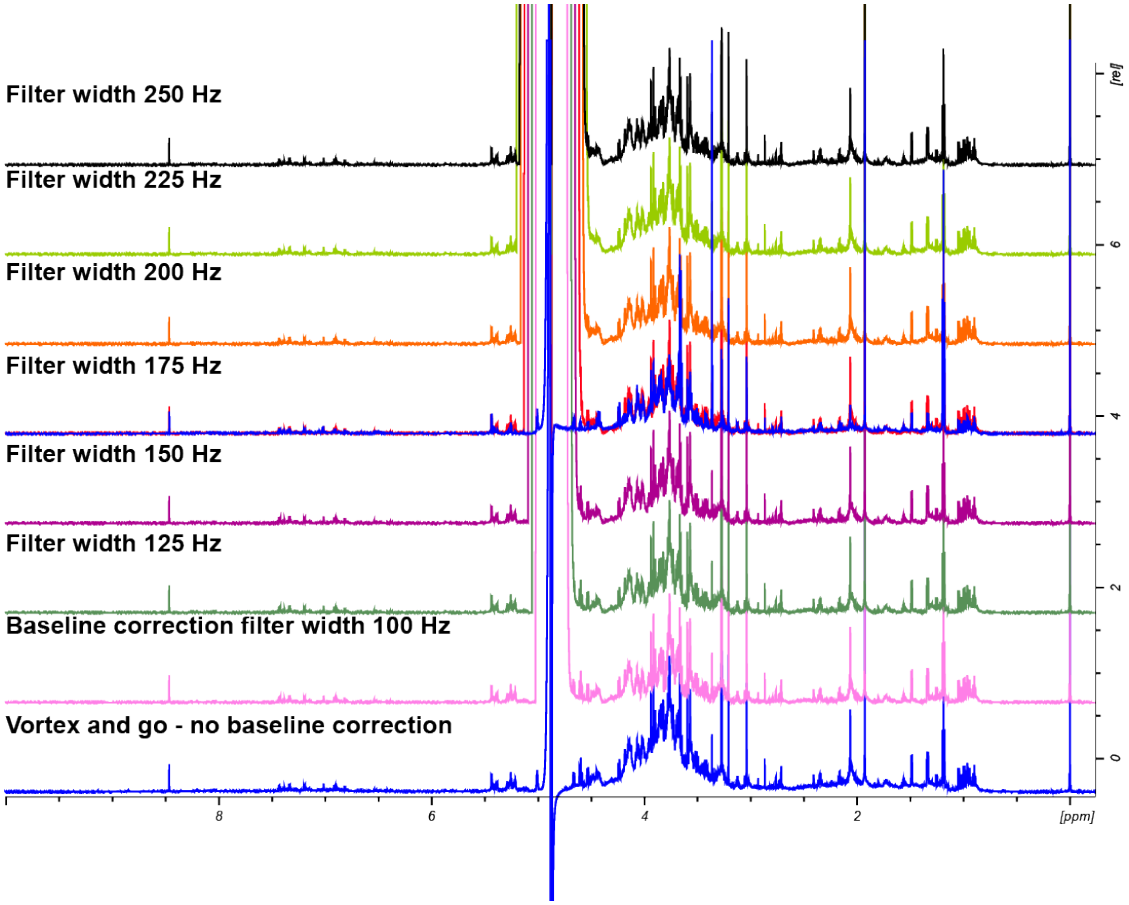

Figure 3-4: The effect of different in silico baseline correction filter widths on a representative rat fecal spectrum. A filter width of 175 Hz was chosen as it most closely resembled the spectrum produced by ultrafiltration and is shown in blue superimposed on top of the spectrum obtained using a filter width of 175 Hz

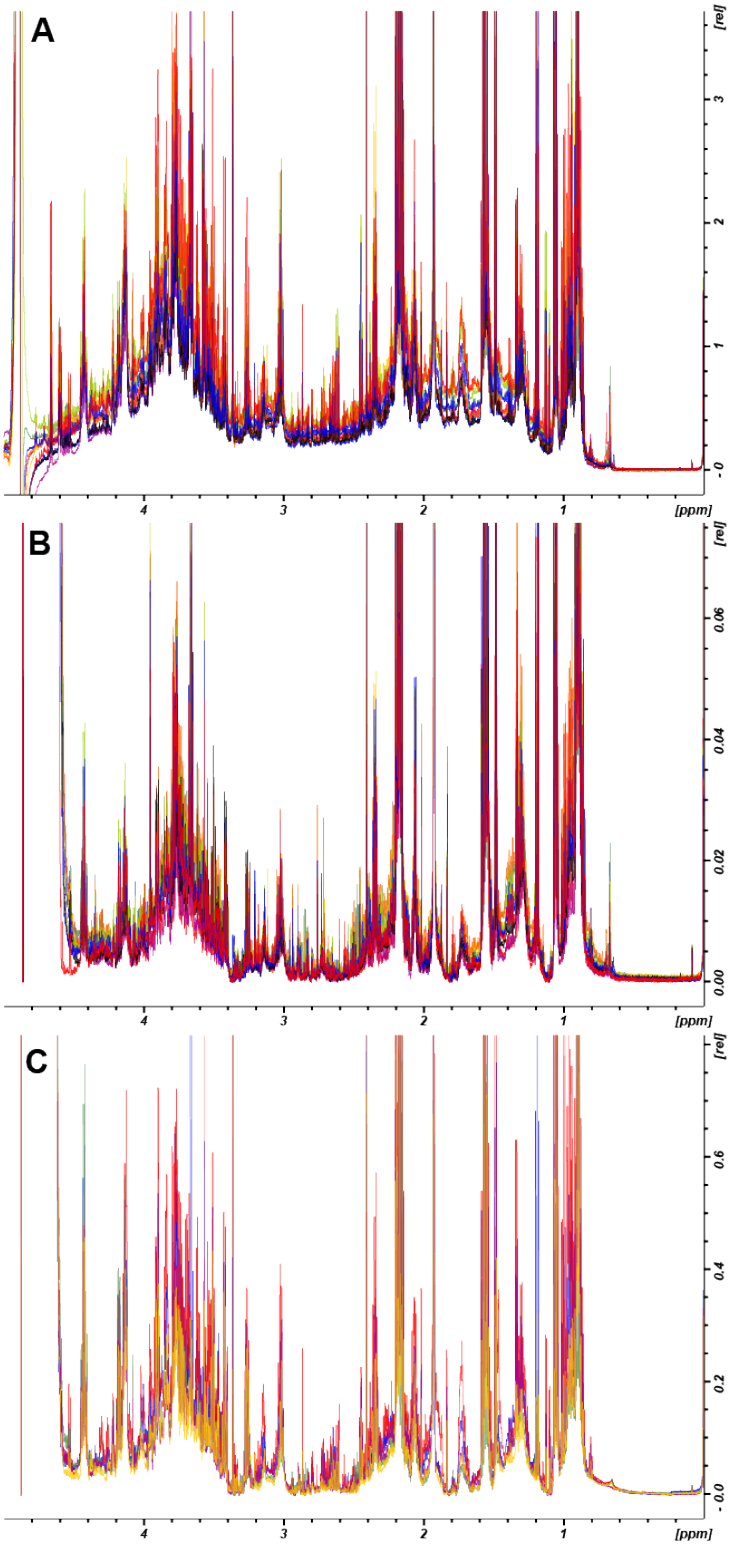

Figure 3-5: Comparison of NMR water-soluble metabolite spectra in the 1-5 ppm of range of rat feces that were processed by: **(A)** ultrafiltration; **(B)** Bligh-Dyer extraction; and **(C)** no extraction. In the case of B and C, spectra were also processed after data collection using in silico baseline correction with a filter width of 175 Hz

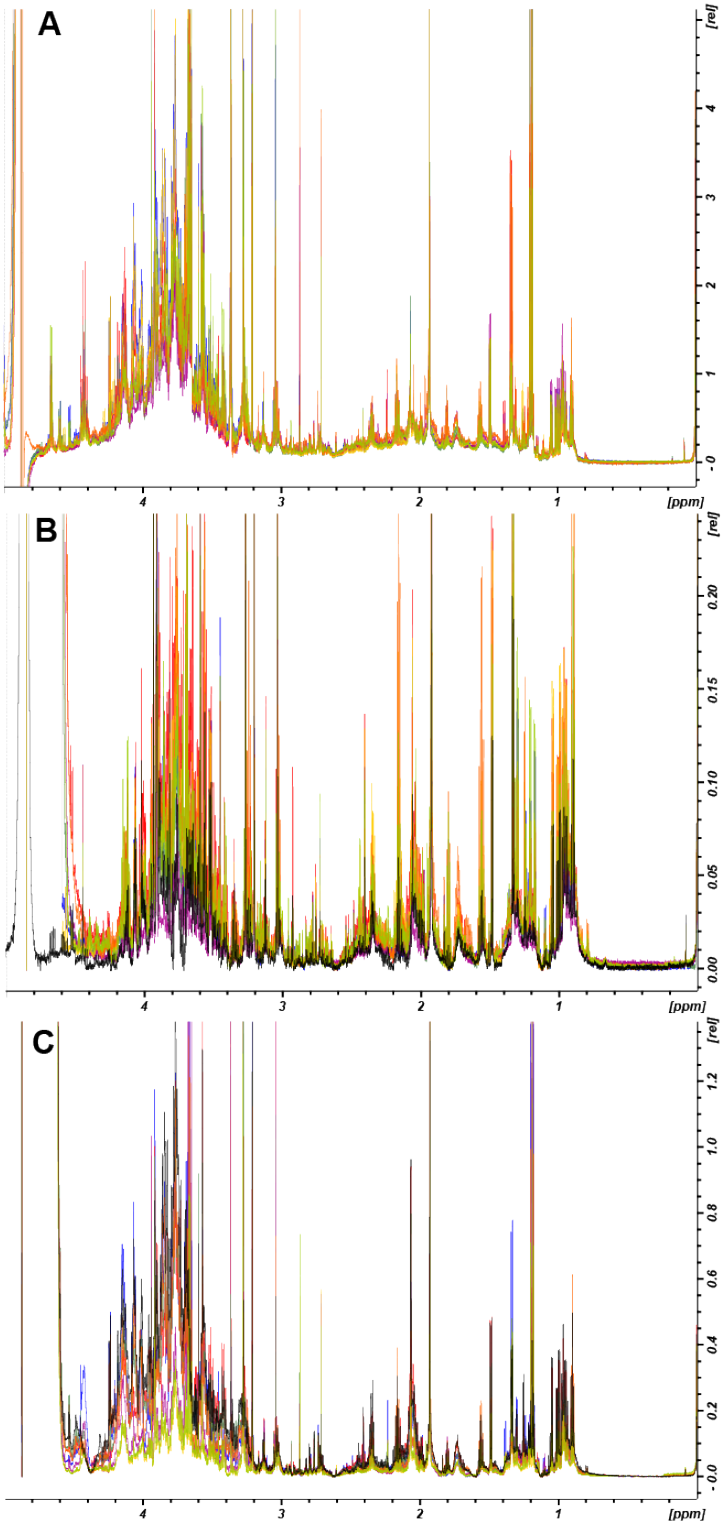

Figure 3-6: Comparison of NMR water-soluble metabolite spectra in the 1-5 ppm range of chicken feces that was processed by: **(A)** ultrafiltration; **(B)** Bligh-Dyer extraction; and **(C)** no extraction. In the case of B and C, the spectra were processed after data collection using in silico baseline correction with a filter width of 175 Hz

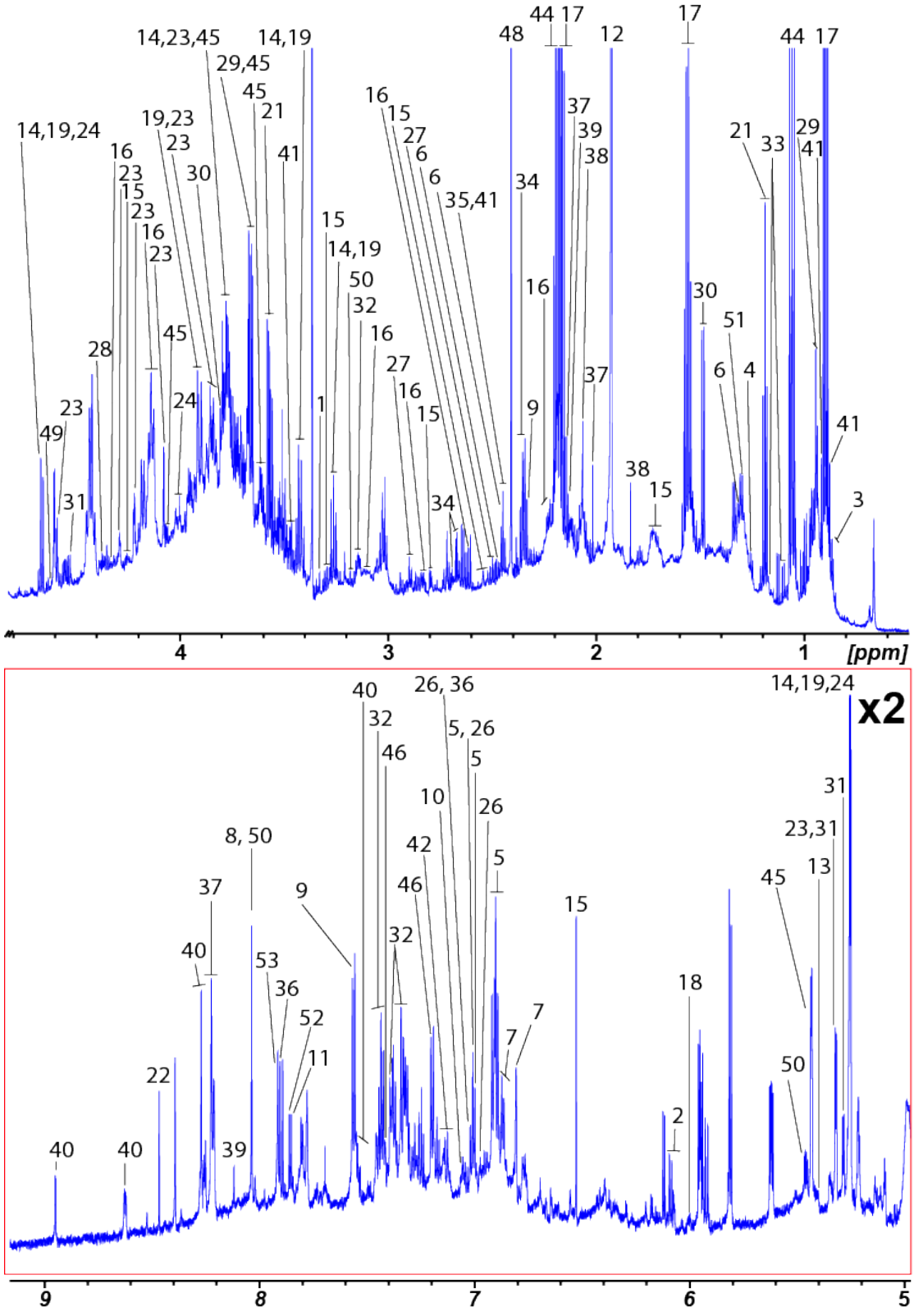

Figure 3-7: Average rat fecal metabolome. Numbers correspond to the metabolites listed in **Table 3-1**

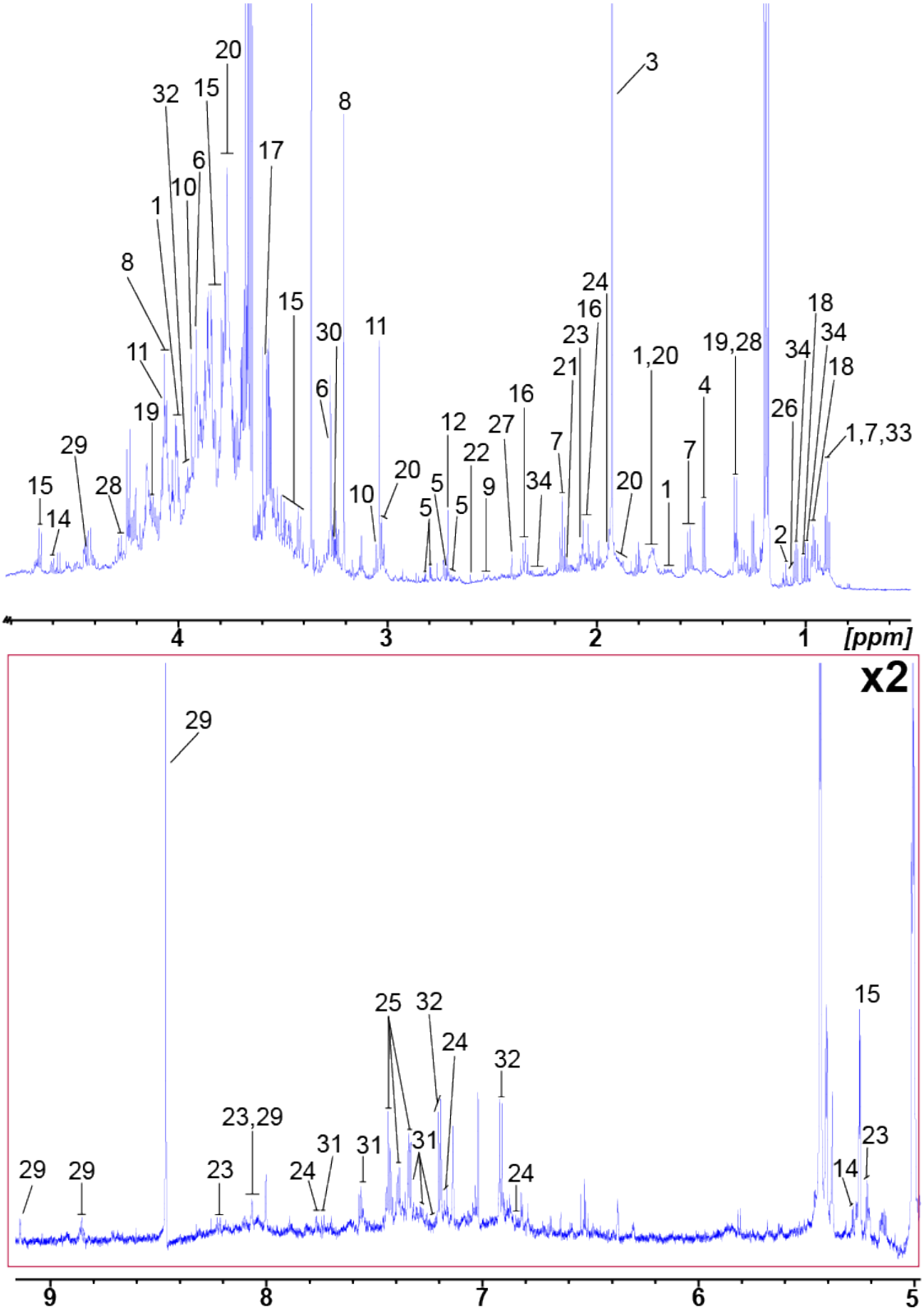

Figure 3-8: Average chicken fecal metabolome. Numbers correspond to the metabolites listed in **Table 3-2**

Table 3-1: Average concentrations (mM) of select metabolites in rat feces that were processed by ultrafiltration, no extraction without baseline correction, Bligh-Dyer (BD) extraction without baseline correction, no extraction with baseline correction, and BD with baseline correction. Metabolites were identified and quantified using Chenomx, and only metabolites with a percent occurrence of 50 or higher are shown. Metabolite numbers correspond to **Figure 3-7**, and the numbers presented in brackets correspond to the standard deviation and percent occurrence of each metabolite, respectively

#	Compound Name	No Extraction	Bligh-Dyer	Ultra-filtration	No Extraction BC	Bligh-Dyer BC
1	1,3-Dimethyluric acid	0.078 (0.041/100)	0.039 (0.011/80)	0.080 (0.033/100)	0.058 (0.029/100)	0.036 (0.014/80)
2	1-Methyladenosine	1.837 (0.645/75)	0.445 (0.117/70)	1.171 (0.639/80)	1.013 (0.462/75)	0.350 (0.139/80)
3	2-Isopropylmalic acid	0.058 (0.025/50)	0.019 (0.005/60)	0.049 (0.020/90)	0.026 (0.013/100)	0.012 (0.003/100)
4	3-Hydroxyisovaleric acid	0.101 (0.034/50)	0.033 (0.012/90)	0.138 (0.061/80)	0.070 (0.034/75)	0.031 (0.012/90)
5	3-Hydroxymandelic acid	0.037 (0.010/50)	0.013 (0.006/80)	0.035 (0.006/90)	0.031 (0.003/100)	0.029 (0.058/70)
6	3-Hydroxymethylglutaric acid	0.112 (0.010/75)	0.036 (0.012/90)	0.101 (0.019/90)	0.063 (0.014/100)	0.032 (0.008/90)
7	3-Hydroxyphenylacetic acid	2.543 (0.247/100)	1.005 (0.177/70)	1.793 (0.436/90)	2.544 (0.246/50)	1.039 (0.159/80)
8	3-Methylxanthine	0.042 (0.010/75)	0.009 (0.004/80)	0.022 (0.009/70)	0.008 (0.005/100)	0.002 (0.001/80)
9	4-Pyridoxic acid	0.054 (0.030/75)	0.028 (0.009/100)	0.055 (0.023/100)	0.042 (0.031/100)	0.020 (0.014/90)
10	5-Hydroxyindoleacetic acid	0.115 (0.044/50)	0.030 (0.009/80)	0.113 (0.046/90)	0.110 (0.056/75)	0.033 (0.008/90)
11	7-Methylxanthine	11.815 (1.319/75)	4.602 (0.774/90)	8.367 (1.864/90)	11.802 (1.312/100)	4.652 (0.798/90)
12	Acetic acid	0.521 (0.445/100)	0.194 (0.100/100)	0.565 (0.306/100)	0.531 (0.433/100)	0.189 (0.094/90)
13	Allantoin	0.010 (0.002/100)	0.004 (0.001/60)	0.007 (0.002/100)	0.006 (0.002/100)	0.008 (0.011/60)
14	Alpha-D-Glucose	0.752 (0.425/75)	0.200 (0.109/50)	0.350 (0.180/90)	0.079 (0.038/75)	0.126 (0.070/70)
15	Argininosuccinic acid	1.147 (0.310/50)	0.167 (0.057/70)	1.000 (0.251/60)	1.381 (0.319/100)	0.300 (0.043/90)
16	Biotin	0.186 (0.147/100)	0.050 (0.035/100)	0.085 (0.061/100)	0.188 (0.171/50)	0.079 (0.028/90)
17	Butyric acid	0.037 (0.015/100)	0.011 (0.004/100)	0.034 (0.011/100)	0.049 (0.013/100)	0.038 (0.013/90)
18	Cytidine monophosphate	1.129 (0.188/75)	0.333 (0.106/50)	0.887 (0.210/80)	0.117 (0.053/50)	0.112 (0.059/60)
19	D-Glucose	0.080 (0.043/50)	0.015 (0.010/90)	0.046 (0.017/100)	0.014 (0.002/100)	0.010 (0.008/60)

20	D-Maltose	0.028 (0.017/75)	0.004 (0.003/50)	0.011 (0.005/80)	0.004 (0.002/50)	0.003 (0.001/70)
21	Ethanol	0.634 (0.122/75)	0.121 (0.055/90)	0.239 (0.262/100)	0.378 (0.231/75)	0.285 (0.211/90)
22	Formic acid	0.751 (0.254/50)	0.132 (0.078/80)	0.413 (0.166/100)	0.142 (0.074/75)	0.442 (1.226/70)
23	Galacturonic acid	0.147 (0.051/100)	0.026 (0.014/100)	0.086 (0.013/100)	0.022 (0.001/100)	0.131 (0.313/90)
24	Glucose 6-phosphate	0.747 (0.137/75)	0.236 (0.078/80)	1.155 (0.971/100)	0.922 (0.775/75)	0.227 (0.115/50)
25	Glycine	3.848 (1.948/50)	1.324 (0.467/90)	2.551 (1.217/90)	3.734 (2.067/75)	1.305 (0.457/90)
26	Homoveratric acid	0.033 (0.005/100)	0.007 (0.001/50)	0.022 (0.012/80)	0.016 (0.007/50)	0.007 (0.001/100)
27	Hydrocinnamic acid	0.166 (0.027/100)	0.068 (0.024/90)	0.094 (0.058/90)	0.132 (0.022/100)	0.035 (0.023/90)
28	Hydroxyacetone	5.395 (1.241/100)	1.411 (0.457/90)	5.416 (0.907/80)	2.046 (1.035/75)	0.691 (0.397/90)
29	Isovalerylglycine	0.036 (0.018/50)	0.004 (0.002/60)	0.012 (0.013/80)	0.004 (0.001/75)	0.004 (0.002/60)
30	L-Alanine	0.320 (0.116/75)	0.045 (0.014/100)	0.213 (0.069/100)	0.108 (0.067/100)	0.048 (0.012/90)
31	L-Arabinose	0.448 (0.015/100)	0.086 (0.033/50)	0.372 (0.077/90)	0.334 (0.058/50)	0.097 (0.047/50)
32	L-Phenylalanine	6.985 (2.362/100)	0.460 (0.288/70)	0.576 (0.638/100)	2.502 (0.997/100)	0.719 (0.405/70)
33	L-Valine	0.325 (0.184/100)	0.124 (0.040/100)	0.176 (0.126/90)	0.279 (0.140/100)	0.153 (0.065/100)
34	Malic acid	0.179 (0.043/100)	0.019 (0.008/100)	0.124 (0.056/100)	0.159 (0.047/100)	0.023 (0.013/100)
35	Methyl isobutyl ketone	0.072 (0.059/100)	0.074 (0.019/70)	0.156 (0.041/100)	0.157 (0.050/100)	0.038 (0.026/50)
36	Methylimidazole acetic acid	0.214 (0.115/100)	0.154 (0.076/100)	0.199 (0.083/100)	0.211 (0.117/100)	0.165 (0.072/90)
37	N-Acetyl-L-methionine	0.441 (0.090/100)	0.113 (0.058/70)	0.329 (0.132/60)	0.227 (0.062/100)	0.074 (0.030/100)
38	N-Acetylneuraminic acid	0.106 (0.069/100)	0.073 (0.039/100)	0.091 (0.053/100)	0.076 (0.038/100)	0.062 (0.019/100)
39	N-Formyl-L-methionine	1.099 (0.181/75)	0.343 (0.141/50)	0.979 (0.191/100)	0.351 (0.189/100)	0.236 (0.063/70)
40	Nicotinic acid	0.181 (0.053/100)	0.038 (0.010/80)	0.118 (0.041/100)	0.116 (0.011/100)	0.035 (0.009/70)
41	Pantothenic acid	0.091 (0.016/100)	0.035 (0.011/100)	0.074 (0.013/100)	0.076 (0.017/100)	0.037 (0.010/100)
42	p-Cresol sulfate	2.040 (0.679/100)	0.594 (0.304/90)	0.927 (0.465/100)	0.184 (0.062/100)	0.129 (0.069/90)
43	Phenylacetic acid	0.099 (0.030/50)	0.024 (0.010/80)	0.045 (0.021/80)	0.053 (0.001/75)	0.024 (0.007/90)
44	Propionic acid	0.158 (0.077/100)	0.015 (0.005/100)	0.058 (0.037/100)	0.020 (0.003/100)	0.037 (0.022/90)
45	Raffinose	0.338 (0.152/75)	0.159 (0.054/80)	0.477 (0.166/70)	0.340 (0.134/100)	0.168 (0.052/90)
46	Riboflavin	0.220 (0.064/100)	0.050 (0.011/100)	0.164 (0.028/100)	0.663 (0.132/100)	0.147 (0.053/90)

47	Sebacic acid	0.028 (0.001/100)	0.088 (0.027/90)	0.034 (0.017/100)	0.026 (0.004/75)	0.091 (0.026/80)
48	Succinic acid	0.264 (0.166/100)	0.094 (0.030/100)	0.163 (0.128/100)	0.081 (0.016/100)	0.054 (0.008/90)
49	Sumiki's acid	0.399 (0.157/75)	0.160 (0.027/80)	0.331 (0.090/80)	0.402 (0.153/50)	0.164 (0.026/50)
50	Thiamine	0.151 (0.083/75)	0.034 (0.018/70)	0.094 (0.030/70)	0.173 (0.058/75)	0.060 (0.021/60)
51	Undecanedioic acid	4.792 (2.549/100)	1.785 (1.351/100)	4.081 (1.655/100)	0.396 (0.097/100)	0.357 (0.243/80)
52	Urocanic acid	0.016 (0.003/100)	0.012 (0.002/70)	0.021 (0.004/90)	0.022 (0.008/75)	0.014 (0.004/60)
53	Xanthine	0.121 (0.107/75)	0.057 (0.030/100)	0.197 (0.155/100)	0.330 (0.073/100)	0.065 (0.014/90)

Table 3-2: Average concentrations (mM) of all metabolites identified in chicken feces that were processed by ultrafiltration, no extraction without baseline correction, Bligh-Dyer (BD) extraction without baseline correction, no extraction with baseline correction, and BD with baseline correction. Metabolites were identified and quantified using Chenomx. Metabolite numbers correspond to **Figure 3-8**, and the numbers presented in brackets correspond to the standard deviation and percent occurrence of each metabolite, respectively

#	Compound Name	No Extraction Average	Bligh-Dyer Average	Ultra-filtration Average	No Extraction with Baseline Correction Average	Bligh-Dyer with Baseline Correction Average
1	2-Hydroxy-butyrate	0.121 (0.051/75)	0.051 (0.022/75)	0.059 (0.028/75)	- (-/0)	0.037 (0.007/50)
2	3-Hydroxyiso-butyrate	0.010 (0.005/37.5)	- (-/0)	0.007 (0.000/12.5)	0.009 (0.003/37.5)	- (-/0)
3	Acetate	2.046 (0.647/100)	1.124 (0.492/100)	0.864 (0.322/87.5)	2.045 (0.648/100)	1.124 (0.491/100)
4	Alanine	0.134 (0.055/100)	0.070 (0.026/100)	0.059 (0.019/87.5)	0.134 (0.055/100)	0.070 (0.026/100)
5	Aspartate	0.105 (0.050/50)	0.034 (0.012/50)	0.044 (0.011/87.5)	0.072 (0.023/62.5)	0.034 (0.012/50)
6	Betaine	0.117 (0.047/87.5)	0.092 (0.058/100)	0.076 (0.056/87.5)	0.132 (0.120/100)	0.092 (0.058/100)
7	Butyrate	0.117 (0.123/50)	0.083 (0.095/87.5)	0.033 (0.015/37.5)	0.154 (0.090/100)	0.092 (0.087/100)
8	Choline	0.140 (0.096/100)	0.127 (0.040/100)	0.076 (0.040/87.5)	0.139 (0.096/100)	0.127 (0.040/100)
9	Citrate	0.017 (0.000/12.5)	- (-/0)	- (-/0)	0.021 (0.007/37.5)	0.008 (0.000/12.5)
10	Creatine	0.097 (0.125/75)	0.129 (0.110/100)	0.067 (0.059/75)	0.109 (0.120/100)	0.130 (0.107/100)
11	Creatinine	0.166 (0.070/100)	0.035 (0.016/100)	0.074 (0.037/87.5)	0.117 (0.073/100)	0.034 (0.023/100)
12	Dimethylamine	0.066 (0.032/100)	0.009 (0.004/25)	0.043 (0.017/87.5)	0.054 (0.039/100)	0.007 (0.004/75)
13	Formate	0.560 (0.187/100)	0.309 (0.115/100)	0.295 (0.081/87.5)	0.560 (0.187/100)	0.309 (0.115/100)
14	Galactose	0.288 (0.221/37.5)	0.050 (0.028/50)	0.088 (0.034/87.5)	0.331 (0.000/12.5)	0.230 (0.043/50)
15	Glucose	0.371 (0.176/87.5)	0.059 (0.040/87.5)	0.333 (0.136/87.5)	0.370 (0.117/62.5)	0.125 (0.067/62.5)
16	Glutamate	- (-/0)	0.160 (0.095/25)	- (-/0)	- (-/0)	0.146 (0.057/50)
17	Glycine	0.080 (0.000/12.5)	0.050 (0.023/100)	0.112 (0.045/75)	0.080 (0.000/12.5)	0.050 (0.023/100)
18	Isoleucine	0.080 (0.033/100)	0.033 (0.013/100)	0.031 (0.011/87.5)	0.080 (0.033/100)	0.032 (0.011/75)
19	Lactate	0.185 (0.000/12.5)	0.169 (0.050/62.5)	0.117 (0.000/12.5)	0.175 (0.000/12.5)	0.135 (0.061/100)

20	Lysine	0.191 (0.000/12.5)	0.032 (0.000/12.5)	0.074 (0.000/12.5)	0.114 (0.033/37.5)	0.049 (0.015/25)
21	Methionine	0.026 (0.011/50)	0.009 (0.006/37.5)	0.014 (0.006/75)	0.015 (0.006/75)	0.008 (0.004/100)
22	Methylamine	0.012 (0.000/12.5)	- (-/0)	0.007 (0.002/62.5)	0.004 (0.004/100)	0.001 (0.000/12.5)
23	N-Acetylglucos-amine	0.145 (0.066/100)	0.056 (0.012/62.5)	0.054 (0.027/87.5)	0.106 (0.047/100)	0.046 (0.014/75)
24	N-Acetyltyrosine	0.019 (0.006/37.5)	0.015 (0.005/87.5)	0.015 (0.002/25)	0.015 (0.007/100)	0.013 (0.005/87.5)
25	Phenylalanine	0.084 (0.026/100)	0.040 (0.012/62.5)	0.039 (0.009/87.5)	0.079 (0.026/100)	0.040 (0.012/62.5)
26	Propionate	0.071 (0.000/12.5)	0.030 (0.017/25)	0.024 (0.000/12.5)	0.037 (0.025/50)	0.017 (0.007/50)
27	Succinate	0.020 (0.009/87.5)	0.013 (0.005/100)	0.008 (0.002/87.5)	0.018 (0.009/100)	0.013 (0.005/100)
28	Threonine	0.226 (0.120/75)	0.067 (0.043/100)	0.106 (0.063/87.5)	0.218 (0.119/75)	0.044 (0.035/100)
29	Trigonelline	0.032 (0.015/100)	0.021 (0.007/87.5)	0.016 (0.007/87.5)	0.027 (0.014/100)	0.020 (0.007/87.5)
30	Trimethyl-amine N-oxide	0.167 (0.154/37.5)	0.021 (0.018/50)	0.011 (0.006/75)	0.125 (0.139/62.5)	0.013 (0.016/87.5)
31	Tryptophan	0.019 (0.006/87.5)	0.012 (0.003/87.5)	0.013 (0.004/75)	0.018 (0.005/87.5)	0.013 (0.004/100)
32	Tyrosine	0.085 (0.024/50)	0.039 (0.014/75)	0.040 (0.010/62.5)	0.082 (0.024/62.5)	0.041 (0.012/62.5)
33	Valerate	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.042 (0.000/12.5)
34	Valine	0.107 (0.048/100)	0.046 (0.018/100)	0.039 (0.014/87.5)	0.107 (0.048/100)	0.047 (0.019/100)

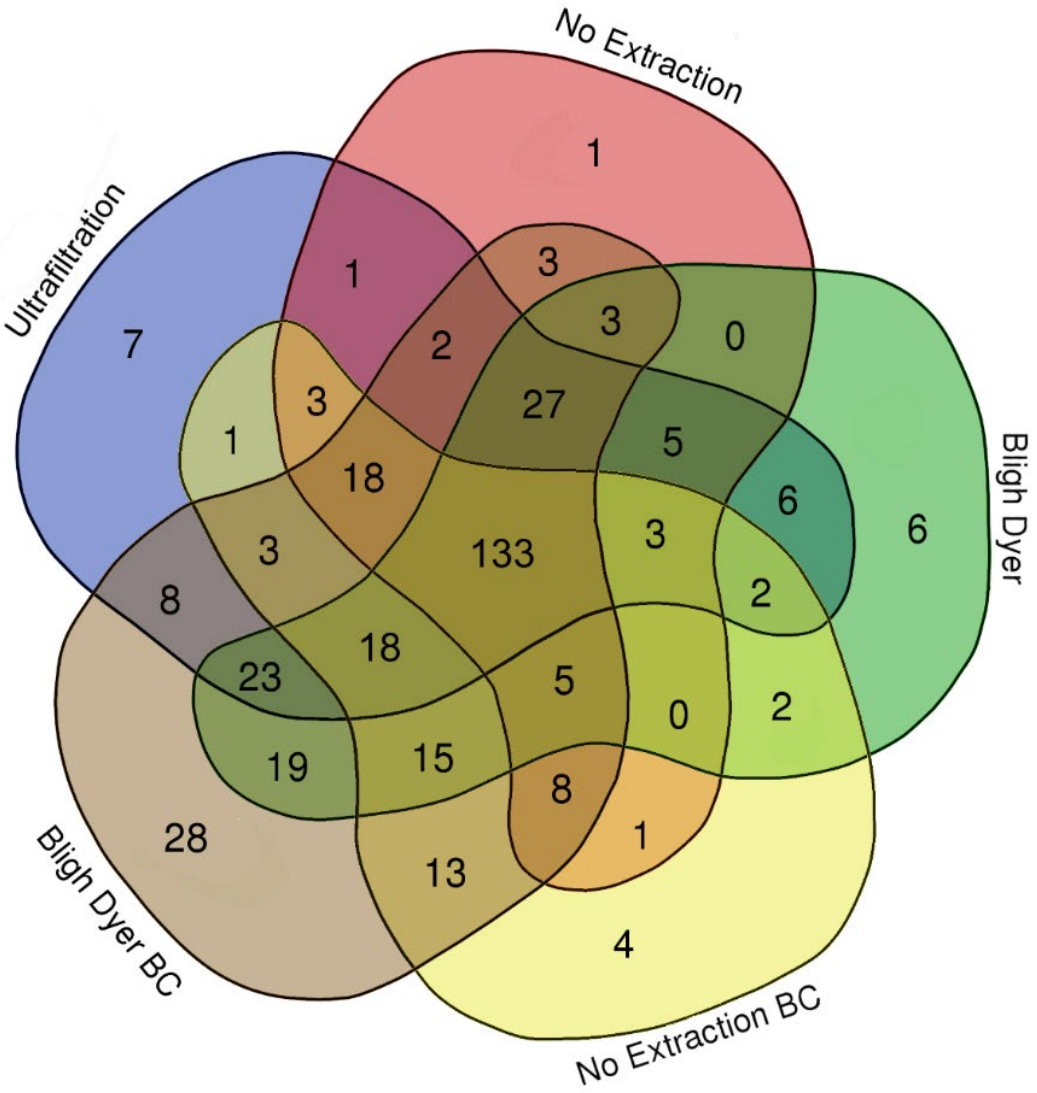

Figure 3-9: Venn diagram showing the number of water-soluble metabolites identified in rat feces that was processed by ultrafiltration, no extraction, Bligh-Dyer (BD) extraction, no extraction with baseline correction, and BD with baseline correction

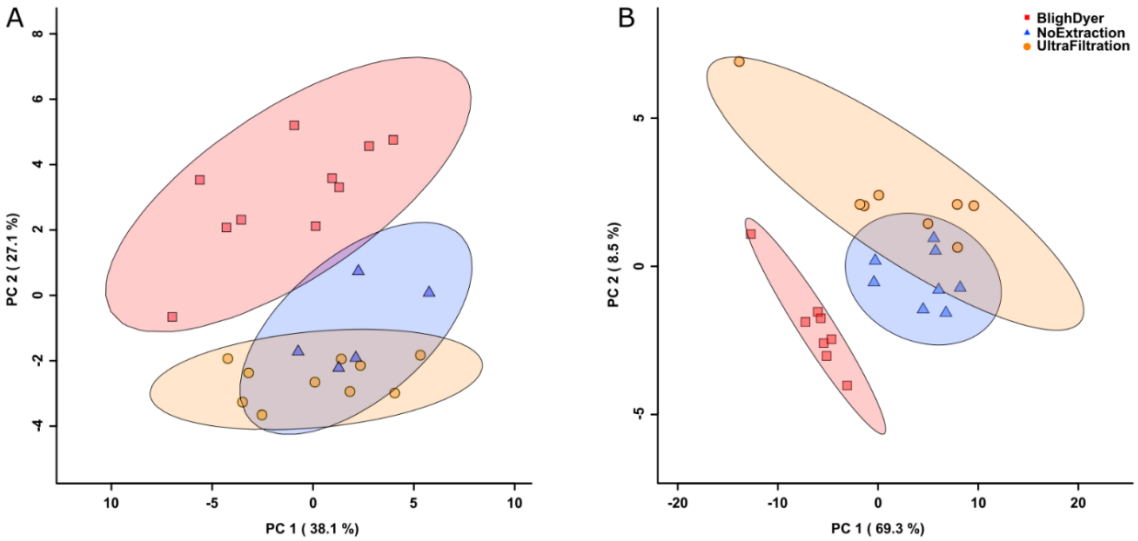

Figure 3-10: Principal component analysis score plots of water-soluble fecal metabolite bins showing unsupervised separation among the three methods evaluated for: **(A)** rats; and **(B)** chickens. Each symbol represents one fecal sample and is plotted with respect to the variability in the metabolome as given by the principal components. The x- and y-axes show principal components one and two, respectively, with brackets indicating percent variance explained by each component. The shaded ellipses represent the 95% confidence intervals

3.6 References

1. Goldsmith, J.R.; Sartor, R.B. The role of diet on intestinal microbiota metabolism: Downstream impacts on host immune function and health, and therapeutic implications. *J. Gastroenterol.* **2014**, *49*, 785-798, doi:10.1007/s00535-014-0953-z.

2. Boulangé, C.L.; Neves, A.L.; Chilloux, J.; Nicholson, J.K.; Dumas, M.-E. Impact of the gut microbiota on inflammation, obesity, and metabolic disease. *Genome Med.* **2016**, *8*, doi:10.1186/s13073-016-0303-2.

3. Bruessow, H. Microbiota and the human nature: Know thyself. *Environ. Microbiol.* **2015**, *17*, 10-15, doi:10.1111/1462-2920.12693.

4. Song, Y.; Garg, S.; Girotra, M.; Maddox, C.; von Rosenvinge, E.C.; Dutta, A.; Dutta, S.; Fricke, W.F. Microbiota dynamics in patients treated with fecal microbiota transplantation for recurrent *Clostridium difficile* infection. *PLoS One* **2013**, *8*, e81330-e81330, doi:10.1371/journal.pone.0081330.

5. Danilova, N.A.; Abdulkhakov, S.R.; Grigoryeva, T.V.; Markelova, M.I.; Vasilyev, I.Y.; Boulygina, E.A.; Ardatskaya, M.D.; Pavlenko, A.V.; Tyakht, A.V.; Odintsova, A.K.; et al. Markers of dysbiosis in patients with ulcerative colitis and Crohn's disease. *Ter. Arkh.* **2019**, *91*, 13-20, doi:10.26442/00403660.2019.04.000211.

6. Casén, C.; Vebø, H.C.; Sekelja, M.; Hegge, F.T.; Karlsson, M.K.; Ciemniejewska, E.; Dzankovic, S.; Frøyland, C.; Nestestog, R.; Engstrand, L.; et al. Deviations in human gut microbiota: A novel diagnostic test for determining dysbiosis in patients with IBS or IBD. *Aliment. Pharmacol. Ther.* **2015**, *42*, 71-83, doi:10.1111/apt.13236.

7. Ochoa-Repáraz, J.; Magori, K.; Kasper, L.H. The chicken or the egg dilemma: Intestinal dysbiosis in multiple sclerosis. *Ann. Transl. Med.* **2017**, *5*, 145-145, doi:10.21037/atm.2017.01.18.

8. Jiang, H.; Ling, Z.; Zhang, Y.; Mao, H.; Ma, Z.; Yin, Y.; Wang, W.; Tang, W.; Tan, Z.; Shi, J.; et al. Altered fecal microbiota composition in patients with major depressive disorder. *Brain. Behav. Immun.* **2015**, *48*, 186-194, doi:10.1016/j.bbi.2015.03.016.

9. Mulle, J.G.; Sharp, W.G.; Cubells, J.F. The gut microbiome: A new frontier in autism research. *Curr. Psychiat. Rep.* **2013**, *15*, doi:10.1007/s11920-012-0337-0.

10. Rowin, J.; Xia, Y.; Jung, B.; Sun, J. Gut inflammation and dysbiosis in human motor neuron disease. *Physiol. Rep.* **2017**, *5*, e13443-n/a, doi:10.14814/phy2.13443.

11. Johnson, J.S.; Spakowicz, D.J.; Hong, B.-Y.; Petersen, L.M.; Demkowicz, P.; Chen, L.; Leopold, S.R.; Hanson, B.M.; Agresta, H.O.; Gerstein, M.; et al. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nat. Commun.* **2019**, *10*, doi:10.1038/s41467-019-13036-1.

12. Langille, M.G.I.; Zaneveld, J.; Caporaso, J.G.; Mcdonald, D.; Knights, D.; Reyes, J.A.; Clemente, J.C.; Burkepile, D.E.; Vega Thurber, R.L.; Knight, R.; et al. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat. Biotechnol.* **2013**, *31*, 814-821, doi:10.1038/nbt.2676.

13. Dethlefsen, L.; Huse, S.; Sogin, M.L.; Relman, D.A. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biol.* **2008**, *6*, e280, doi:10.1371/journal.pbio.0060280.

14. Nicholson, J.K.; Lindon, J.C. Metabonomics. *Nature* **2008**, *455*, 1054-1056, doi:10.1038/4551054a.

15. Nicholson, J.K.; Lindon, J.C.; Holmes, E. 'Metabonomics': Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999**, *29*, 1181-1189, doi:10.1080/004982599238047.

16. Lindon, J.C.; Nicholson, J.K.; Holmes, E.; Everett, J.R. Metabonomics: Metabolic processes studied by NMR spectroscopy of biofluids. *Concept Magnetic Res.* **2000**, *12*, 289-320, doi:10.1002/1099-0534(2000)12:5<289::AID-CMR3>3.0.CO;2-W.

17. Zierer, J.; Jackson, M.A.; Kastenmueller, G.; Mangino, M.; Long, T.; Telenti, A.; Mohney, R.P.; Small, K.S.; Bell, J.T.; Steves, C.J.; et al. The fecal metabolome as a functional readout of the gut microbiome. *Nat. Genet.* **2018**, *50*, 790-795, doi:10.1038/s41588-018-0135-7.

18. Psychogios, N.; Hau, D.D.; Peng, J.; Guo, A.C.; Mandal, R.; Bouatra, S.; Sinelnikov, I.; Krishnamurthy, R.; Eisner, R.; Gautam, B.; et al. The human serum metabolome. *PLoS One* **2011**, *6*, 1- 23, doi:10.1371/journal.

19. Dzeja, P.; Bouatra, S.; Aziat, F.; Mandal, R.; Guo, A.C.; Wilson, M.R.; Knox, C.; Bjorndahl, T.C.; Krishnamurthy, R.; Saleem, F.; et al. The human urine metabolome. *PLoS One* **2013**, *8*, doi:10.1371/journal.pone.0073076.

20. Dame, Z.T.; Aziat, F.; Mandal, R.; Krishnamurthy, R.; Bouatra, S.; Borzouie, S.; Guo, A.C.; Sajed, T.; Deng, L.; Lin, H.; et al. The human saliva metabolome. *Metabolomics* **2015**, *11*, 1864-1883, doi:10.1007/s11306-015-0840-5.

21. Lamichhane, S.; Yde, C.C.; Schmedes, M.S.; Jensen, H.M.; Meier, S.; Bertram, H.C. Strategy for nuclear-magnetic-resonance-based metabolomics of human feces. *Anal. Chem.* **2015**, *87*, 5930-5937, doi:10.1021/acs.analchem.5b00977.

22. Matysik, S.; Le Roy, C.I.; Liebisch, G.; Claus, S.P. Metabolomics of fecal samples: A practical consideration. *Trends Food Sci. Tech.* **2016**, *57*, 244-255, doi:10.1016/j.tifs.2016.05.011.

23. Deda, O.; Gika, H.G.; Theodoridis, G.A. Rat fecal metabolomics-based analysis. *Metabolic Profiling: Methods and Protocols* **2018**, *1738*, 149-157.

24. Deda, O.; Chatziioannou, A.C.; Fasoula, S.; Palachanis, D.; Raikos, N.; Theodoridis, G.A.; Gika, H.G. Sample preparation optimization in fecal metabolic profiling. *J. Chromatogr. B* **2017**, *1047*, 115-123, doi:10.1016/j.jchromb.2016.06.047.

25. Karu, N.; Deng, L.; Slae, M.; Guo, A.C.; Sajed, T.; Huynh, H.; Wine, E.; Wishart, D.S. A review on human fecal metabolomics: Methods, applications and the human fecal metabolome database. *Anal. Chim. Acta.* **2018**, *1030*, 1-24, doi:10.1016/j.aca.2018.05.031.

26. Snytnikova, O.A.; Khlichkina, A.A.; Sagdeev, R.Z.; Tsentalovich, Y.P. Evaluation of sample preparation protocols for quantitative NMR-based metabolomics. *Metabolomics* **2019**, *15*, 84, doi:10.1007/s11306-019-1545-y.

27. Saric, J.; Wang, Y.; Li, J.; Coen, M.; Utzinger, J.; Marchesi, J.R.; Keiser, J.; Veselkov, K.; Lindon, J.C.; Nicholson, J.K.; et al. Species variation in the fecal metabolome gives insight into differential gastrointestinal function. *J. Proteome Res.* **2008**, *7*, 352-360, doi:10.1021/pr070340k.

28. Bezabeh, T.; Somorjai, R.L.; Smith, I.C.P. MR metabolomics of fecal extracts: Applications in the study of bowel diseases. *Magn. Reson. Chem.* **2009**, *47*, S54-S61, doi:10.1002/mrc.2530.

29. Lamichhane, S.; Sundekilde, U.K.; Blædel, T.; Dalsgaard, T.K.; Larsen, L.H.; Dragsted, L.O.; Astrup, A.; Bertram, H.C. Optimizing sampling strategies for NMR-based metabolomics of human feces: Pooled vs. unpooled analyses. *Anal. Methods* **2017**, *9*, 4476-4480, doi:10.1039/c7ay01465a.

30. Jacobs, D.M.; Deltimple, N.; Van Velzen, E.; Van Dorsten, F.A.; Bingham, M.; Vaughan, E.E.; Van Duynhoven, J. ^{1}H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR Biomed.* **2008**, *21*, 615-626, doi:10.1002/nbm.1233.

31. Amiot, A.; Dona, A.C.; Wijeyesekera, A.; Tournigand, C.; Baumgaertner, I.; Lebaleur, Y.; Sobhani, I.; Holmes, E. ^{1}H NMR spectroscopy of fecal extracts enables detection of advanced colorectal neoplasia. *J. Proteome Res.* **2015**, *14*, 3871-3881, doi:10.1021/acs.jproteome.5b00277.

32. Chai, Y.; Wang, J.; Wang, T.; Yang, Y.; Su, J.; Shi, F.; Wang, J.; Zhou, X.; He, B.; Ma, H.; et al. Application of ^{1}H NMR spectroscopy-based metabonomics to

feces of cervical cancer patients with radiation-induced acute intestinal symptoms. *Radiother. Oncol.* **2015**, *117*, 294-301, doi:10.1016/j.radonc.2015.07.037.

33. Gratton, J.; Phetcharaburanin, J.; Mullish, B.H.; Williams, H.R.T.; Thursz, M.; Nicholson, J.K.; Holmes, E.; Marchesi, J.R.; Li, J.V. Optimized sample handling strategy for metabolic profiling of human feces. *Anal. Chem.* **2016**, *88*, 4661-4668, doi:10.1021/acs.analchem.5b04159.

34. Wu, H.; Southam, A.D.; Hines, A.; Viant, M.R. High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Anal. Biochem.* **2008**, *372*, 204-212, doi:10.1016/j.ab.2007.10.002.

35. Cui, M.; Trimigno, A.; Aru, V.; Khakimov, B.; Engelsen, S.B. Human faecal ^{1}H-NMR metabolomics: Evaluation of solvent and sample processing on coverage and reproducibility of signature metabolites. *Anal. Chem.* **2020**, doi:10.1021/acs.analchem.0c00606.

36. Tiziani, S.; Emwas, A.-H.; Lodi, A.; Ludwig, C.; Bunce, C.M.; Viant, M.R.; Günther, U.L. Optimized metabolite extraction from blood serum for ^{1}H nuclear magnetic resonance spectroscopy. *Anal. Biochem.* **2008**, *377*, 16-23, doi:10.1016/j.ab.2008.01.037.

37. Izquierdo-Garcia, J.L.; Villa, P.; Kyriazis, A.; del Puerto-Nevado, L.; Perez-Rial, S.; Rodriguez, I.; Hernandez, N.; Ruiz-Cabello, J. Descriptive review of current NMR-based metabolomic data analysis packages. *Prog. Nucl. Mag. Res. Sp.* **2011**, *59*, 263-270, doi:10.1016/j.pnmrs.2011.02.001.

38. Smolinska, A.; Blanchet, L.; Buydens, L.M.C.; Wijmenga, S.S. NMR and pattern recognition methods in metabolomics: From data acquisition to biomarker discovery: A review. *Anal. Chim. Acta.* **2012**, *750*, 82-97, doi:10.1016/j.aca.2012.05.049.

39. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911-917, doi:10.1139/o59-099.

40. Cordoni, G.; Woodward, M.J.; Wu, H.; Alanazi, M.; Wallis, T.; La Ragione, R.M. Comparative genomics of european avian pathogenic *E. coli* (APEC). *BMC Genomics* **2016**, *17*, 960, doi:10.1186/s12864-016-3289-7.

41. de Graaf, R.A.; Behar, K.L. Quantitative ^{1}H NMR spectroscopy of blood plasma metabolites. *Anal. Chem* **2003**, *75*, 2100-2104, doi:10.1021/ac020782.

42. Paxman, E.J.; Boora, N.S.; Kiss, D.; Laplante, D.P.; King, S.; Montina, T.; Metz, G.A.S. Prenatal maternal stress from a natural disaster alters urinary metabolomic profiles in project ice storm participants. *Sci. Rep.* **2018**, *8*, 12932, doi:10.1038/s41598-018-31230-x.

43. Kiss, D.; Ambeskovic, M.; Montina, T.; Metz, G.A.S. Stress transgenerationally programs metabolic pathways linked to altered mental health. *Cell. Mol. Life Sci.* **2016**, *73*, 4547-4557, doi:10.1007/s00018-016-2272-4.

44. Le Roy, C.I.; Mappley, L.J.; La Ragione, R.M.; Woodward, M.J.; Claus, S.P. NMR-based metabolic characterization of chicken tissues and biofluids: A model for avian research. *Metabolomics* **2016**, *12*, 157-157, doi:10.1007/s11306-016-1105-7.

45. Wishart, D.S.; Feunang, Y.D.; Marcu, A.; Guo, A.C.; Liang, K.; Vázquez-Fresno, R.; Sajed, T.; Johnson, D.; Li, C.; Karu, N.; et al. HMDB 4.0: The human metabolome database for 2018. *Nucleic Acids Res.* **2018**, *46*, D608-D617, doi:10.1093/nar/gkx1089.

46. Meiboom, S.; Gill, D. Modified spin-echo method for measuring nuclear relaxation times. *Rev. Sci. Instrum.* **1958**, *29*, 688-691, doi:10.1063/1.1716296.

47. Gowda, G.A.N.; Gowda, Y.N.; Raftery, D. Expanding the limits of human blood metabolite quantitation using NMR spectroscopy. *Anal. Chem.* **2015**, *87*, 706-715, doi:10.1021/ac503651e.

48. Daykin, C.A.; Foxall, P.J.D.; Connor, S.C.; Lindon, J.C.; Nicholson, J.K. The comparison of plasma deproteinization methods for the detection of low-molecular-weight metabolites by ^{1}H nuclear magnetic resonance spectroscopy. *Anal. Biochem.* **2002**, *304*, 220-230, doi:10.1006/abio.2002.5637.

49. Hazendonk, P.; Wormald, P.; Montina, T. The direct DIVAM experiment: A spin dynamics analysis. *J. Phys. Chem. A* **2008**, *112*, 6262-6274, doi:10.1021/jp711489d.

50. Montina, T.; Hazendonk, P.; Wormald, P.; Iuga, D. The refocused discrimination induced by variable amplitude minipulses (DIVAM) experiment — improved domain selection in semicrystalline fluoropolymers by ^{19}F solid state nuclear magnetic resonance spectroscopy. *Can. J. Chem.* **2011**, *89*, 1065-1075, doi:10.1139/v11-043.

51. Wormald, P.; Ameduri, B.; Harris, R.K.; Hazendonk, P. Fluorine-19 solid state NMR study of vinylidenefluoride polymers using selective relaxation filters. *Solid State Nucl. Magn. Reson.* **2006**, *30*, 114-123, doi:10.1016/j.ssnmr.2006.05.003.

52. Singh, R. Membrane technology and engineering for water purification. In *Introduction to membrane technology* 2nd ed.; Butterworth-Heinemann: 2015; pp. 1-80.

53. Alum, M.F.; Shaw, P.A.; Sweatman, B.C.; Ubhi, B.K.; Haselden, J.N.; Connor, S.C. 4,4-dimethyl-4-silapentane-1-ammonium trifluoroacetate (dsa), a promising universal internal standard for NMR-based metabolic profiling studies of

biofluids, including blood plasma and serum. *Metabolomics* **2008**, *4*, 122-127, doi:10.1007/s11306-008-0103-9.

4 FEATHER PULP: A NOVEL SAMPLE FOR ^{1}H-NMR METABOLOMICS SENSITIVE TO BIOLOGICAL FACTORS IN CHICKENS

4.1 Abstract

Non-invasive biomarkers of stress that are predictive of poultry health are needed. Feather pulp is highly vascularized and represents a potential source of biomarkers that has not been extensively explored. We investigated the feasibility and use of feather pulp for novel biomarker discovery using Nuclear Magnetic Resonance Spectroscopy (NMR)-based metabolomics. To this end, high quality ^{1}H-NMR metabolomic spectra were obtained from chicken feather pulp extracted using either ultrafiltration (UF) or Bligh-Dyer methanol-chloroform (BD) methods. In total, 121 and 160 metabolites were identified using the UF and BD extraction methods, respectively, with 71 of these common to both methods. The metabolome of feather pulp differed in broiler breeders that were 1-, 23-, and 45-weeks-of-age. Moreover, feather pulp was more difficult to obtain from older birds, indicating that age must be considered when targeting feather pulp as a source of biomarkers. The metabolomic profile of feather pulp obtained from 12-day-old broilers administered corticosterone differed significantly from control birds, indicating that the metabolome of feather pulp was sensitive to induced physiological stress. A comparative examination of feather pulp and serum in 12-day-old broilers revealed that the feather pulp metabolome differed from that of serum, but provided more information. The study findings showed that metabolite biomarkers in chicken feather pulp may allow producers to effectively monitor stress, and to objectively develop and evaluate on-farm mitigations, including practices that reduce stress and enhance bird health.

4.2 Introduction

Stress is known to have a substantial impact on bird health, but is also very difficult to quantify [1]. As such, there is an increasing need for reliable and non-invasive biomarkers of stress [2,3]. Ideally, a biomarker should be sensitive and provide an early indication of the stress status of birds before there are any outward signs of distress [4]. For example, physiological stress has been shown to adversely affect chicken production [5,6] as evidenced in previous studies by reduced feed intake [7,8], decreased growth rate and increased abdominal fat [9], and the development of fatty liver disease [10]. Significantly, stress has also been shown to pre-dispose chickens to a variety of diseases incited by pathogens, such as necrotic enteritis [11,12], colibacillosis [13-16], and salmonellosis [17,18], due at least in part to a suppression of the immune system [12,19,20]. For these reasons, an effective biomarker of stress would be beneficial to the chicken industry, as it would allow for early detection and the implementation of mitigation strategies before stress can negatively affect bird health and production.

When birds experience a stressor, it stimulates the hypothalamic-pituitary-adrenal (HPA) axis, which results in varying behavioral, physiological, and metabolic changes that help the birds cope with the stressor [21,22]. In chickens, exposure to a stressor stimulates production of the glucocorticoid hormone, corticosterone (CORT) [21,23], and CORT levels in blood are often used as a biomarker of stress [24,25]. However, there are difficulties associated with CORT as a biomarker in blood. Firstly, the act of collecting blood is a stressor for birds, and the collection process has been shown to result in an increase in the concentration of CORT [26-29]. Secondly, CORT levels are known to fluctuate tremendously throughout the day [5,22,30-35]. Some groups have attempted to identify alternative biomarkers of stress in broiler chickens, primarily targeting

biomarkers that are immune markers or mRNA transcripts measured in blood or tissues [36-38] that require destructive sampling, making them unsuitable for tracking stress in a production setting. There is an imminent need for the discovery of biomarkers in novel substrates (e.g. tissue or biofluid) to effectively measure stress and subsequently bird health on farm.

Several alternative substrates, such as feces and feathers, can be obtained with little to no handling of the birds and provide additional possibilities for biomarker discovery [39-41]. The use of feces for biomarker discovery has garnered some interest [42-45]; however, feces are the accumulated results of both microbial and host metabolism. Thus, the relationship between stress and metabolites in feces is not straightforward nor fully understood [29]. Feathers are much like hair in that they are produced and supported by follicles in the skin [46], and they are not subjected to the same metabolome altering processes as feces. A feather consists of a cylindrical shaft, called a calamus, which starts in the follicle, and a long portion called a rachis. The two are connected by a region called the superior umbilical region [47-49]. The rachis is highly vascularized and creates a dermal core, referred to as feather pulp or feather follicle epithelium, which is surrounded by feather tissue and an outer sheath [25]. Feathers differ from hair in that they have nerves and their own muscles [49]. Relative to blood, feathers have a sampling advantage in that their collection can be less invasive [50] as they can be collected once molted [25,51]. Furthermore, feather pulp is often used for detecting pathogens that incite important diseases such as West Nile virus [52], Marek's disease virus [53], and avian leukosis virus [54]. Ample research measuring CORT in whole feathers has been conducted; however, interpretation of CORT concentrations in feathers can be difficult because the exact mechanism by which CORT is incorporated into feathers is currently

unclear [22,24,34,55], and CORT concentrations within different feathers from the same individual vary [25,26]. The relationship between feather CORT and blood CORT concentrations is not straightforward [23,28,51,56-59], and the relationship between feather CORT and stress is also nebulous [23,59-63].

Metabolomics is the study of endogenous small molecular weight compounds, or metabolites, present in a tissue or biofluid, and allows for the analysis of all the metabolic responses of living systems [64] to external stimuli, such as stressors. ^{1}H-nuclear magnetic resonance (NMR) spectroscopy-based metabolomics is used to characterize the metabolome of various biological tissues and fluids. NMR spectroscopy is a non-destructive and highly reproducible technique [65-67] that does not require lengthy chemical derivatization unlike other instruments used for metabolomic analyses such as liquid chromatography (LC) mass spectrometry (MS) and gas chromatography (GC)-MS [68]. Recent research has explored the use of ^{1}H-NMR to elucidate the metabolomes of different chicken samples including eggs, breast muscle, liver, kidney, and feces for model development [69], as well as to identify biomarkers of health [10,70-73]. However, limited research to date has focused on identifying novel biomarkers of stress that are correlated with bird health, and to our knowledge none have examined metabolomic biomarkers in feather pulp. Given the high degree of vascularization in the feather pulp, we hypothesized that the metabolome of the feather pulp will possess a diverse metabolome that is comparable to the metabolome of blood, and that metabolites will be altered by a physiological stressor, as well as other biological factors, such as age. To test this hypothesis, the following objectives were established: (1) determine if ^{1}H-NMR spectra can be produced from chicken feather pulp and the best metabolite extraction method for NMR-based metabolomic studies; (2) ascertain if the feather pulp

metabolome is affected by bird age; (3) determine if the feather pulp metabolome differs in birds in which stress is induced; and (4) comparatively examine the feather pulp and blood metabolomes.

4.3 Materials and methods

Ethics statement

The studies involving broilers were carried out in strict accordance with the recommendations specified in the Canadian Council on Animal Care Guidelines. The project was reviewed and approved by the Lethbridge Research and Development Centre (LeRDC) Animal Care Committee (Animal Use Protocol Review #2010) before commencement of the research. For analysis of the metabolome of feather pulp obtained from broiler breeder chickens of different ages, birds were euthanized on farm by a producer following industry standards for animal euthanization; researchers had no contact with living broiler breeder chickens.

Broiler breeder birds for age analysis

Ross 308FF broiler breeder chickens were obtained from a broiler breeder farm located near Lethbridge, AB. Chickens from the following three age classes were examined: (1) 1-week-old birds (n = 6); (2) 23-week-old birds (n = 4); and (3) 45-week-old birds (n = 4).

Stress induction in broiler birds

The study was designed as a factorial experiment with two levels of stress treatment arranged as a completely randomized design. The two stress treatments were: (1) control birds provided a basal diet (n = 6); and (2) birds provided the basal diet containing CORT to induce physiological stress (n = 6). Each bird was treated as a replicate, and the

replicates were conducted on three separate occasions to ensure independence (two replicate birds per time).

Ross 308FF broiler chicks were obtained from a local hatchery on the day they hatched (day 0). The chicks originated from a single broiler breeder farm from adults of known health status. Upon arrival at the LeRDC small animal facility, chicks were randomly assigned to the control or CORT treatment, and placed in individually ventilated cages (IVCs) (Techniplast, Montreal, QC) on autoclaved wood shavings (United Farmers of Alberta Co-operative Ltd., Lethbridge, AB) at a stocking density of two birds per IVC. Birds had *ad libitum* access to a non-medicated starter diet (Hi-Pro Feeds, Lethbridge, AB) and water. Birds were maintained on a 12 hr light: 12 hr dark cycle. The ambient temperature in the animal room was 30°C for the first 2 days, 28°C for the next 2 days, and 26°C thereafter. On day 5, chickens assigned to the CORT treatment were provided feed containing 15 mg kg^{-1} CORT, and were maintained on the CORT-amended diet until the end of the experiment (day 12).

Processing of samples obtained from broiler breeders of different ages

Within 5 min of euthanization, 5-10 of the largest feathers from the right wing of each bird were removed; the number of feathers collected per bird depending on its size, with fewer feathers taken from larger birds. A composite feather pulp sample from each bird was obtained by squeezing the calamus of the feathers into a 1.5 mL centrifuge tube [54], and the collected feather pulp was mixed to ensure uniformity. Within ca. 5 min of collection, feather pulp samples were snap frozen in liquid nitrogen, transported to the laboratory on dry ice, and stored at -80°C until processed. In preparation for metabolomic analysis, feather pulp samples were thawed on ice, and samples were divided into two aliquots of approximately 150 mg each. One aliquot was used for ultrafiltration (UF) and

the other for Bligh-Dyer (BD) methanol chloroform extraction allowing for direct comparison of identical metabolomes via the two methods.

Ultrafiltration

Amicon Ultra 0.5 mL centrifugal filters with a molecular weight cut-off of 3 kDa (Millipore Sigma, Oakville, ON) were used. Each filter was washed by adding 500 µL of Millipore water (Merck Millipore) to the filter and centrifuging at 14,000 x g for 5 min. This washing step was repeated ten times in order to ensure that all glycerol in the filter had been removed [74,75]. Metabolomics buffer (0.125 M KH_2PO_4, 0.5 M K_2HPO_4, 0.00375 M NaN_3, and 0.375 M KF; pH 7.4) was added to the feather pulp samples at a 2:1 volume to mass ratio, and samples were homogenized using a Bullet Blender tissue homogenizer (Next Advance, Troy, NY) and 150 mg of 2-mm-diameter zirconium oxide beads (Next Advance, Troy, NY) on setting eight for 5 min. Samples were then centrifuged at 14,000 x g for 5 min, and 365 µL of the supernatant was added to a pre-washed filter along with 135 µL of metabolomics buffer. The filters were then centrifuged at 14,000 x g for 30 min at 4ºC.

Bligh-Dyer methanol chloroform extraction

A 275 µL subsample of the homogenized feather pulp was combined with 687.5 µL of methanol and 343.8 µL of chloroform in a 2 mL tube. The sample was vortexed, placed at -20ºC for 15 min to precipitate proteins, and then centrifuged at 15,300 x g for 15 min at 4ºC. The supernatant was decanted into a new tube containing equal volumes (343.8 µL) of chloroform and deionized water. This mixture was vortexed and then centrifuged at 6,700 x g for 5 min at 4°C. Following centrifugation, 1 mL of the top layer containing the water-soluble metabolites was pipetted into a 1.5 mL tube, and placed in a

nitrogen gas flow box for 5-6 days to dry. Once dry, samples were rehydrated in 360 µL of Millipore water.

Processing of samples obtained from broiler chickens

Birds were anesthetized with isoflurane (5% isoflurane; 1 L O_2 min^{-1}) and blood was collected via intracardiac puncture into serum separator tubes (Mississauga, ON). Under anesthesia, birds were euthanized by cervical dislocation, ten feathers from the end of each wing were removed (20 feathers total), and pulp samples were collected, mixed, frozen, and stored as described above. To obtain serum, blood in separator tubes were left at room temperature for 1 hr, centrifuged at 1,000 x g for 2 min, and the serum was decanted and stored at -80°C. Feather pulp and serum samples were processed via UF as described above with the exception that the homogenization step was not used for serum samples; 150 µL of serum was added directly to a pre-washed filter along with 350 µL of metabolomics buffer and centrifuged as previously described.

Sample preparation for ^{1}H-NMR spectroscopy

A 140 µL aliquot of deuterium oxide containing 0.05% v/v trimethylsilylpropanoic acid (TSP) and 200 µL of metabolomics buffer was added to 360 µL of the samples extracted using the BD method and/or by UF (total volume of 700 µL); the TSP was used as a chemical shift reference for ^{1}H-NMR spectroscopy. The solution was vortexed on high setting, and then centrifuged at 12,000 x g for 5 min at 4°C to pellet any remaining particulate matter. A 550 µL aliquot of the supernatant was placed into a 5 mm NMR tube, and each sample was run on a 700 MHz Bruker Avance III HD spectrometer (Bruker, Milton, ON) for spectral data collection.

NMR data acquisition and processing

Spectra were obtained using the Bruker 1-D NOESY gradient water suppression pulse sequence 'noesygppr1d' with 10 ms mixing time. Each sample was run for 512 scans to a total acquisition size of 128 k, a spectral window of 20.5 ppm, a transmitter offset of 4.7 ppm, and a recycle delay of 4 sec. All measurements were recorded using a Bruker triple resonance room temperature TBO-Z probe. The above acquisition parameters correspond to an approximate data collection time of 1 hr per sample. The Bruker automation program "pulsecal" was used on each sample before data acquisition to guarantee that the 90-degree pulse was calibrated correctly, ensuring quantitative and comparable data across samples [76]. The spectra were zero filled to 256 k, automatically phased, baseline corrected, and line-broadened by 0.3 Hz [77]. Spectra were then exported to MATLAB (MathWorks, Natick, MA) as ASCII files where they underwent Dynamic Adaptive Binning [78], followed by manual inspection and correction. The dataset was then normalized to the total metabolome, excluding the region containing the water peak, and pareto scaled.

Statistical analysis

Metabolomics analysis was performed using MATLAB (Math Works, MA, USA) and the MetaboanalystR R package [79]. Spectral bins were subjected to both univariate and multivariate analysis to determine which bins were significantly altered among independent variables (e.g. bird age, stress *versus* no stress, and feather pulp *versus* serum). MATLAB was also used to calculate the percent difference of the bins. MetaboanalystR was used to carry out the principle component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Metabolites were identified using the Chenomx 8.2 NMR Suite (Chenomx Inc, Edmonton, AB).

4.4 Results and discussion

^{1}H-NMR spectra were obtained from feather pulp

A large number of metabolites were observed in feather pulp following extraction by both the UF and BD methods (**Figure 4-1**). In total, 210 metabolites were identified in the NMR spectra. For samples extracted using the UF and BD methods, 121 and 160 metabolites were identified, respectively, with 71 metabolites common to both methods (**Table A3- 1**, **Table A3- 2**). In total, 50 and 89 metabolites were unique to the UF and BD methods, respectively (**Figure 4-2**). In comparison, the human serum metabolome, which has been explored extensively for biomarker discovery, was only able to quantify 49 previously detected metabolites by NMR [80]. Moreover, only 37 of the metabolites were previously detected in chicken plasma by NMR [69]. As more metabolites were observed in feather pulp extracted using the BD method relative to samples extracted using UF this suggests that the BD method of extraction may be better suited for untargeted metabolomics studies of feather pulp. Relative to UF, the spectra of feather pulp extracted using the BD method contained a larger number of metabolites involved in the citric acid cycle, such as succinate, cis-aconitate, oxaloacetate, and citrate. In addition, more of the metabolites identified with the BD method are involved in the beta-Alanine (L-aspartate, carnosine, beta-alanyl-N(pi)-methyl-L-histidine, uracil, and L-histidine), phenylalanine (L-tyrosine, 2-hydroxyphenylacetate, and L-phenylalanine), and pyruvate (oxaloacetate, pyruvate, (S)-lactate, acetate, and fumarate) metabolism pathways. Conversely, more metabolites involved in the arginine and proline metabolism (guanidinoacetate, creatine, agmatine, hydroxyproline, L-proline, L-glutamate, phosphocreatine, and homocarnosine), ascorbate and aldarate metabolism (ascorbate, myo-Inositol, and UDP-glucose), and galactose metabolism (sucrose, UDP-glucose,

glycerol, D-sorbitol, and myo-inositol) pathways were observed in the spectra of samples extracted by UF. Although the BD extraction method resulted in the detection of the most metabolites from feather pulp, the value of the metabolites as biomarkers is far more important than metabolite number, particularly as a relatively large number of metabolites were detected for both extraction methods. For targeted metabolomic analyses, care should be taken to make sure the extraction method used is best suited for the metabolites and pathways of interest.

Citric acid often experiences selective signal loss when processed using UF [75], which is consistent with the findings of this study in that citric acid was only identified in BD processed samples. Isopropanol, serine, and choline were previously found to be present at higher concentrations in human blood processed using UF as compared to samples processed using BD [81], which is consistent with the findings of the current study of feather pulp. Other NMR studies have shown that UF removes methionine [82], acetate [83], and leucine [74,83] from blood samples, whereas BD does not. Methionine, acetate, and leucine were not identified by either UF or BD in feather pulp in the current study, and other studies have observed that UF does not preferentially retain acetate, alanine, lactate, or valine [84]. Despite some inconsistency within the literature on the relative metabolite extraction merits of the UF and BD methods, UF is often recommended for the extraction of metabolites from many types of samples [74,80] as it provides a good signal-to-noise ratio, exhibits excellent reproducibility without undesirable evaporation of volatile metabolites, and has minimal loss of metabolites due to dissolution in various solvents, or due to hydrolysis caused by the need to neutralize the solution [74]. From a logistics perspective, UF is the quickest of the two extraction methods that we evaluated, with the least amount of manipulation of the sample. A salient

downside of UF is the cost, as the filters can be prohibitively expensive (≈$5 USD/filter). There is also the possibility that the filters can break or become clogged, which would require re-prepping, or possibly, total loss of the sample. Another issue that can arise with UF is incomplete filtration, where proteins filtered out of the sample become concentrated on the membrane surface and inside the pores due to adsorption, and block other metabolites from passing through the filter [85]. Also, during filtration, impermeable macromolecules accumulate on the surface of the membrane, and this can cause both concentration polarization and membrane fouling, which prevents metabolites that are smaller than the molecular weight cut-off from passing through the filter [85].

One potential limitation of the BD extraction method is the occurrence of significant baseline distortions due to the incomplete removal of macromolecules in samples that are high in proteins, fats, and/or fiber such as feces [86]. We did not observe significant baseline distortions for feather pulp samples extracted with the BD method. The BD method is capable of removing some fat and protein in the sample [87,88], and the lack of any significant baseline distortion suggests that the method was able to sufficiently remove problematic macromolecules that were present in feather pulp. The effectiveness of the BD method for extracting feather pulp metabolites from different feather types and feathers from different species remains to be determined. Moreover, as the BD method requires more manipulation of the sample than UF, it can result in a decrease in reproducibility and the possible loss of volatile metabolites during the drying step, as well as due to dissolution in methanol and chloroform [74]. Salient advantages of the BD method are the generation of fractions for analysis of both water-soluble and water-insoluble metabolites [89], it is much less expensive than UF, and it extracted a larger number of metabolites from feather pulp than UF. For targeted metabolomic analyses the

variability caused by the chosen extraction method should be considered to ensure that the targeted metabolites are not adversely affected by the method. As the effectiveness of extraction methods may be differently affected by the substrate extracted, comparative validation of methods should be conducted in advance.

Bird age influences metabolite profiles in feather pulp

To ascertain the impact of age on the chicken feather pulp metabolome, broiler breeder chickens at three ages were compared (i.e. 1-, 23-, and 45-weeks-old). The PCA score plots from both the UF and BD methods show unsupervised separation of metabolite bins among the three age groups examined, with some confidence interval overlap observed for the BD method (**Figure 4-3A** and **4-3C**). For samples extracted using UF, OPLS-DA analysis provided a good model fit for the three age classes of birds ($Q^2 = 0.804$, $p < 0.001$, $R^2 = 0.982$, $p < 0.001$), and supervised group separation of metabolite bins among the age classes (**Figure 4-3B**). For samples extracted using the BD method, OPLS-DA analysis also showed supervised group separation, but a poorer model fit between the three age classes ($Q^2 = 0.673$, $p = 0.026$, $R^2 = 0.980$, $p = 0.342$) (**Figure 4-3D**) relative to UF. Thus, the BD method delivered better unsupervised results, whereas UF provided better supervised results. For metabolomics studies, unsupervised separation is preferred as it is not prone to model overfitting [90], further suggesting that the BD method may be better suited for untargeted metabolomic studies of feather pulp. Regardless of extraction method used, the different metabolomes in feather pulp obtained from broiler breeders of different ages indicates that the feather pulp metabolome is highly sensitive to biological host factors.

The feather pulp metabolome is altered in birds administered corticosterone

To explore the utility of using feather pulp as a substrate for measuring the impacts of physiological stress on the host, CORT was administered to broiler chickens in feed. Previous research has shown that CORT administered to chickens in water or feed induces a defined physiological stress response [10,12,91], including conspicuous impacts on the metabolome of the liver, kidney, and breast muscle of chickens [10,73]. We observed that the feather pulp metabolome was significantly altered in 12-day-old birds that were administered CORT relative to control birds, as indicated by the unsupervised separation observed in the PCA score plot (**Figure 4-4A**).

Several metabolites were significantly altered in feather pulp due to CORT administration (**Table 4-1**). Interestingly, CORT was quantifiable by NMR in the feather pulp of birds that were administered the glucocorticoid, but was not detected in serum. In addition, many of the identified amino acids, including tyrosine, threonine, glycine, and phenylalanine, were down-regulated in the feather pulp of birds administered CORT (**Table 4-1**). Previous work has shown a reduction in feather growth rates [58,92] and feather quality [23,62,63] due to stress. The primary protein in feathers is β-keratin [49,93], which comprises ≈80% of the crude protein of feathers [94]. Thus, the decrease in amino acids that we observed in feather pulp from birds administered CORT could be an important contributor to a reduction in feather growth and quality. Cysteine, which is a precursor to glutathione, was up-regulated in feather pulp of birds administered CORT. Glutathione is a well-known antioxidant that protects cells from oxidative stress [95,96], and it is possible that the observed increase in cysteine levels in CORT treatment birds was caused by increased reactive oxidative species that are commonly associated with the stress response [97]. β-keratin is high in cysteine residues, which form disulfide bridges

providing strength and rigidity [98]. It is thus plausible that the higher concentration of free cysteine that was observed is due to reduced incorporation of this amino acid into keratin, thereby adversely affecting feather growth and quality. Future studies investigating the use of feather pulp as a biomarker of stress and bird health should consider metabolites and metabolomic pathways related to feather biosynthesis.

The feather pulp metabolome is not reflective of the serum metabolome

The metabolome of feather pulp of 12-day-old broiler chickens was comparatively examined to serum to ascertain if feather pulp is a good surrogate for serum. Despite being highly vascularized during growth [26], unsupervised analyses indicated that the metabolome of feather pulp and serum obtained from control birds differed significantly (**Figure 4-4B**). This is contrary to the study hypothesis that the feather pulp metabolome is reflective of the serum metabolome. To examine this further, the metabolome of feather pulp was compared to the metabolome of serum of birds administered CORT. A distinct unsupervised separation between the metabolomes of these two treatments was observed (**Figure 4-4C**). Moreover, the total percentage of variance explained by Principal Component 1 (PC1) increased between feather pulp and serum of birds administered CORT. Significantly, the relative scale of the variance along PC1 was increased by almost 20-fold as compared to the percent variation between control pulp and serum of birds not administered CORT, indicating that instead of making the pulp and serum metabolomes more similar, the administration of CORT made them more dissimilar.

To further examine the effects of stress, the serum metabolome after CORT treatment was compared to control broilers and the observed differences were then compared with those in feather pulp. A distinct unsupervised separation between the control and CORT serum metabolomes was observed (**Figure 4-4D**). In serum, all of the identified amino

acids were down-regulated except for proline (**Table 4-2**). Three of these, tyrosine, threonine, and phenylalanine, were also significantly altered in feather pulp due to CORT administration (**Table 4-1**). It has previously been shown that under conditions of stress collagen is broken down to make more proline available for downstream cell cycle arrest, autophagy, and apoptosis [99], which have been shown to increase under some stress conditions [100]. Proline is also a main substrate required to produce glutamate, which is also involved in the body's response to oxidative stress [101,102]. We observed that hydroxyproline was significantly altered in serum of birds administered CORT, and this metabolite is involved in the synthesis of glycine, which is an essential amino acid for chickens [103]. Glycine is also involved in redox regulation [99].

Although feather pulp was not a good surrogate for serum, the high number of metabolites detected in feather pulp, coupled with superior sampling logistics and the clear separation observed between the control and CORT treatment, indicates that feather pulp may be a good, and possibly superior alternative to serum for biomarker discovery. These results further support feather pulp as a substrate for biomarker discovery in chickens.

Advantages and limitations of feather pulp

We confirmed that feather pulp is an easy sample to obtain as the calamus of a feather can be squeezed between a sample tube and its lid for collection [54]. Another advantage of using feather pulp is that it leaves the rest of the feather for further analysis, as it has been shown that acute stressors can increase the incidence of fault bars, which are malformations perpendicular to the rachis of the feather caused by stressful experiences during feather growth [62]. In the current study we showed that feather pulp contains a diverse metabolome that is sensitive to both biological and experimental

factors. Collectively our data indicates that feather pulp may be an excellent substrate for novel biomarker discovery, including biomarkers of stress. Consistent with this conclusion, we showed that there are more metabolites in the feather pulp metabolome than the serum metabolome. The current study did not however examine if the feather pulp from different feathers from the same bird differed in their ^{1}H-NMR spectra, as all the feather samples analyzed were composites of wing feathers pulled from the same place on each bird. This requires further investigation. A salient disadvantage of feather pulp is that feathers in rest phase have much less pulp due to the shrinking of size of the follicle, and depending on how close the birds are to molting, it is possible to collect feathers that have little to no pulp [104]. In this regard, we observed that the collection of feather pulp was more difficult from feathers obtained from birds at 23 days-of-age, which is the approximate time of the first molt.

4.5 Conclusions

The metabolomics research that has been conducted in chickens to date is often focused on the quality of the chicken meat produced [105-109]. The discovery of biomarkers that are predictive of bird health is in its infancy. We observed that NMR-based metabolomics was able to identify a large number of metabolites in feather pulp. Moreover, we showed that broiler feather pulp is an easily obtained sample that is sensitive to biological and experimental variations within its metabolome. Two different extraction methods were used, and both the UF and BD methods produced excellent spectra with both overlapping and unique metabolites. The choice of extraction method for feather pulp will depend on the goals of the research. In this regard, if a larger number of metabolites are desired, such as the case in an untargeted analysis, the BD extraction

method may be the most appropriate method of extraction. In the case of untargeted analysis, the extraction method must be carefully chosen based on the biochemical pathways of interest. We hypothesized that the feather pulp metabolome would be very similar to the serum metabolome, as feather pulp is highly vascularized during growth. Contrary to our hypothesis, the feather pulp metabolome differed from the serum metabolome, and this difference was maintained in birds administered CORT as a physiological stressor. Although feather pulp was not a good proxy for the metabolome of serum, more metabolites were detected in feather pulp relative to serum. Furthermore, the metabolome of feather pulp differed across both broiler age and in birds experiencing physiological stress. The ease of feather pulp collection coupled with its diverse metabolome that was influenced by biological parameters suggests that feather pulp may be a good target to ascertain the impacts of various factors important for optimizing and monitoring bird health.

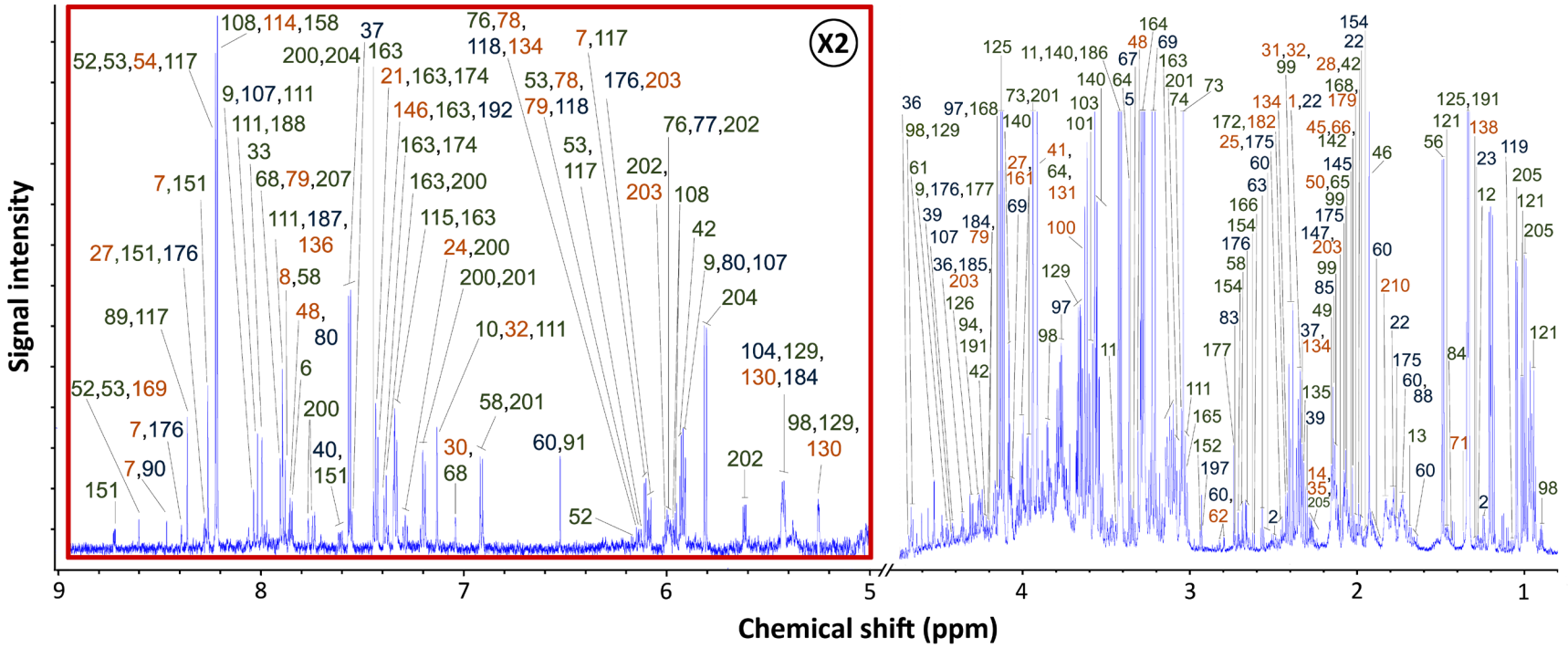

Figure 4-1: Average water-soluble chicken feather pulp metabolome. The numbering in the figure corresponds to **Table A3- 1** and **A3- 2**. Numbers in blue and yellow were only identified using ultrafiltration and Bligh-Dyer extraction, respectively. Numbers in green were found using both extraction methods. The spectrum has been split at the water peak and the vertical scale has been increased to better illustrate the metabolites that are present.

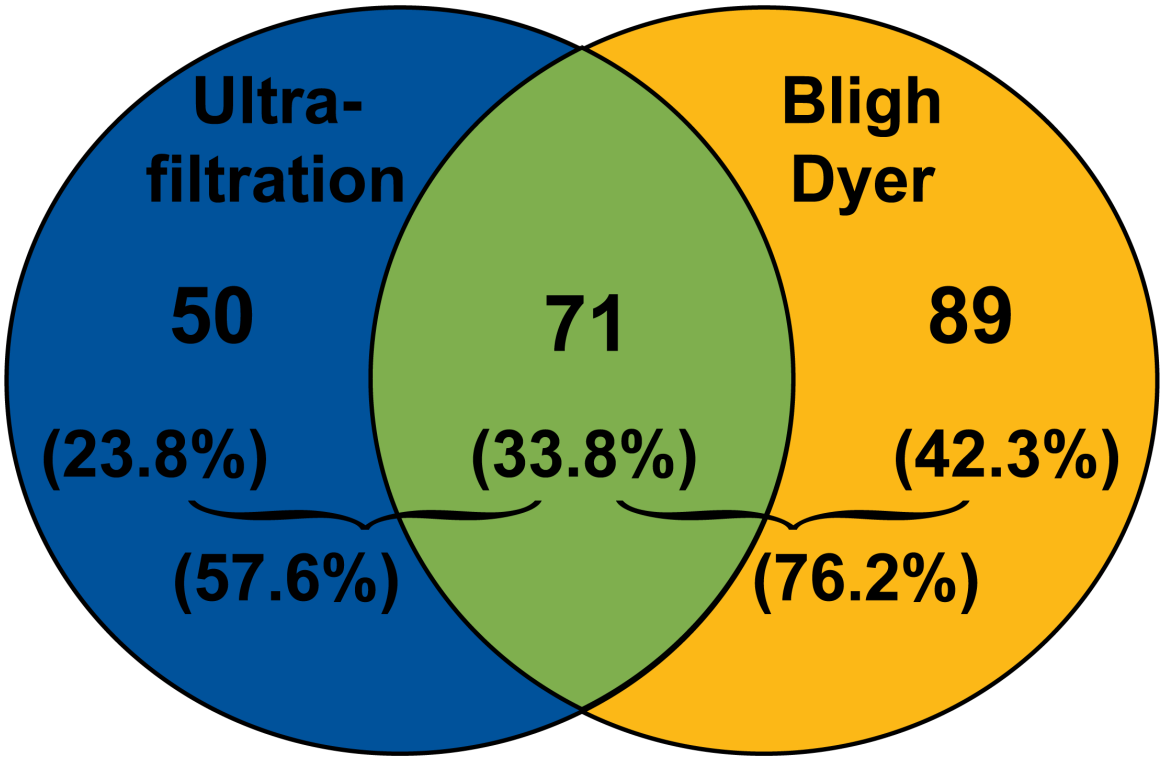

Figure 4-2: Venn Diagram showing the metabolites identified in chicken feather pulp spectra using both the ultrafiltration and Bligh-Dyer methanol-chloroform extraction methods

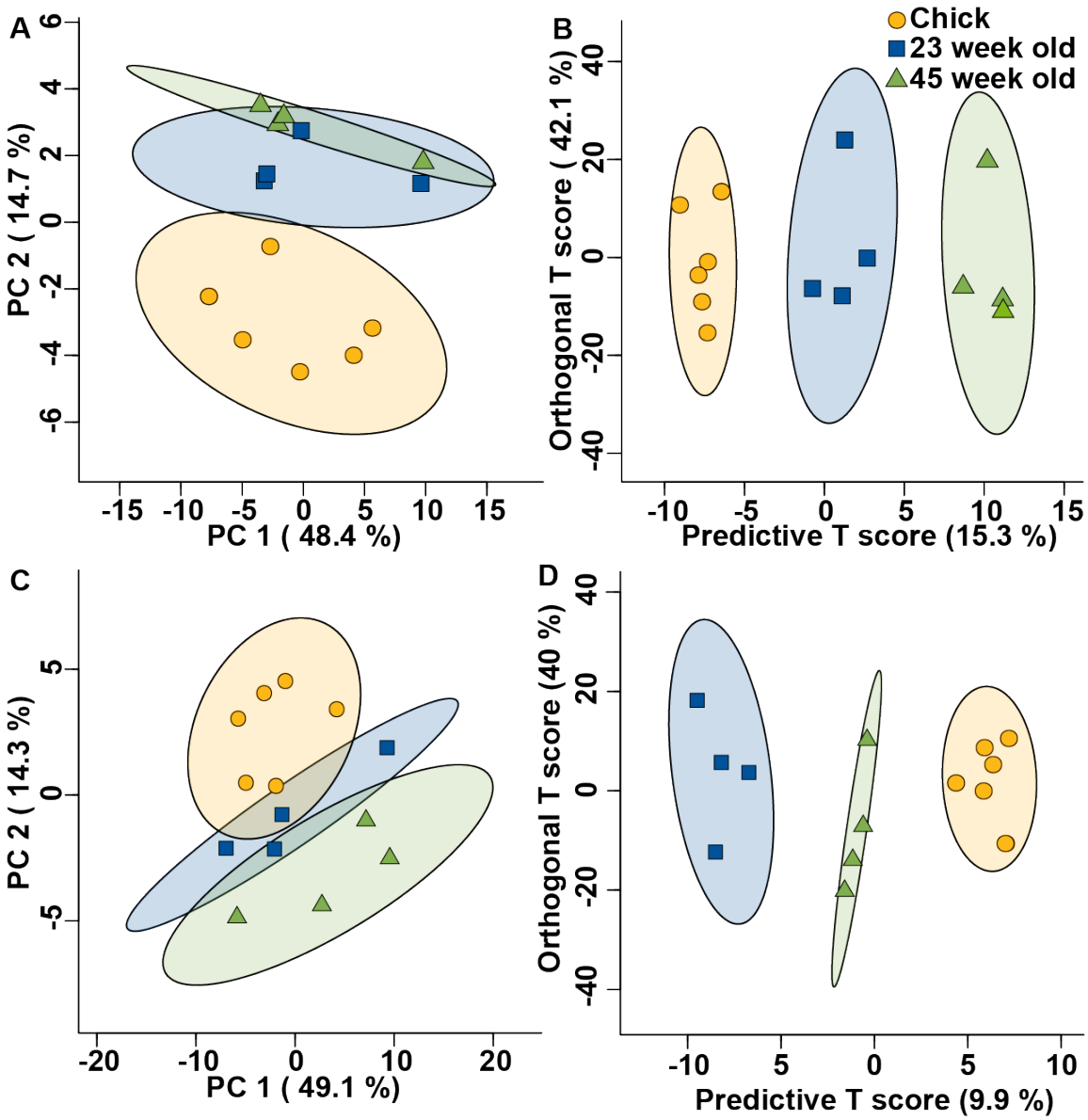

Figure 4-3: P Principal component analysis score plots of feather pulp from chicks, 23-week-old, and 45-week-old birds processed using: **(A)** ultrafiltration; and **(C)** Bligh-Dyer. The x- and y-axes show principal components one and two, respectively, with brackets indicating percent variance explained by each component. Orthogonal partial least squares determinant analysis score plots of feather pulp from chicks, 23-week-old, and 45-week-old birds processed using: **(B)** ultrafiltration $Q^2 = 0.804$, $p < 0.001$, $R^2 = 0.982$, $p < 0.001$; and **(D)** Bligh-Dyer $Q^2 = 0.673$, $p = 0.026$, $R^2 = 0.980$, $p = 0.342$. The x- and y-axis represent the predictive (between group separation) and orthogonal (within group variation) components of the data, respectively. Each shape represents one chicken under study (n=14) plotted using all bins. The shaded ellipses represent the 95% confidence intervals. All figures were plotted using all variables.

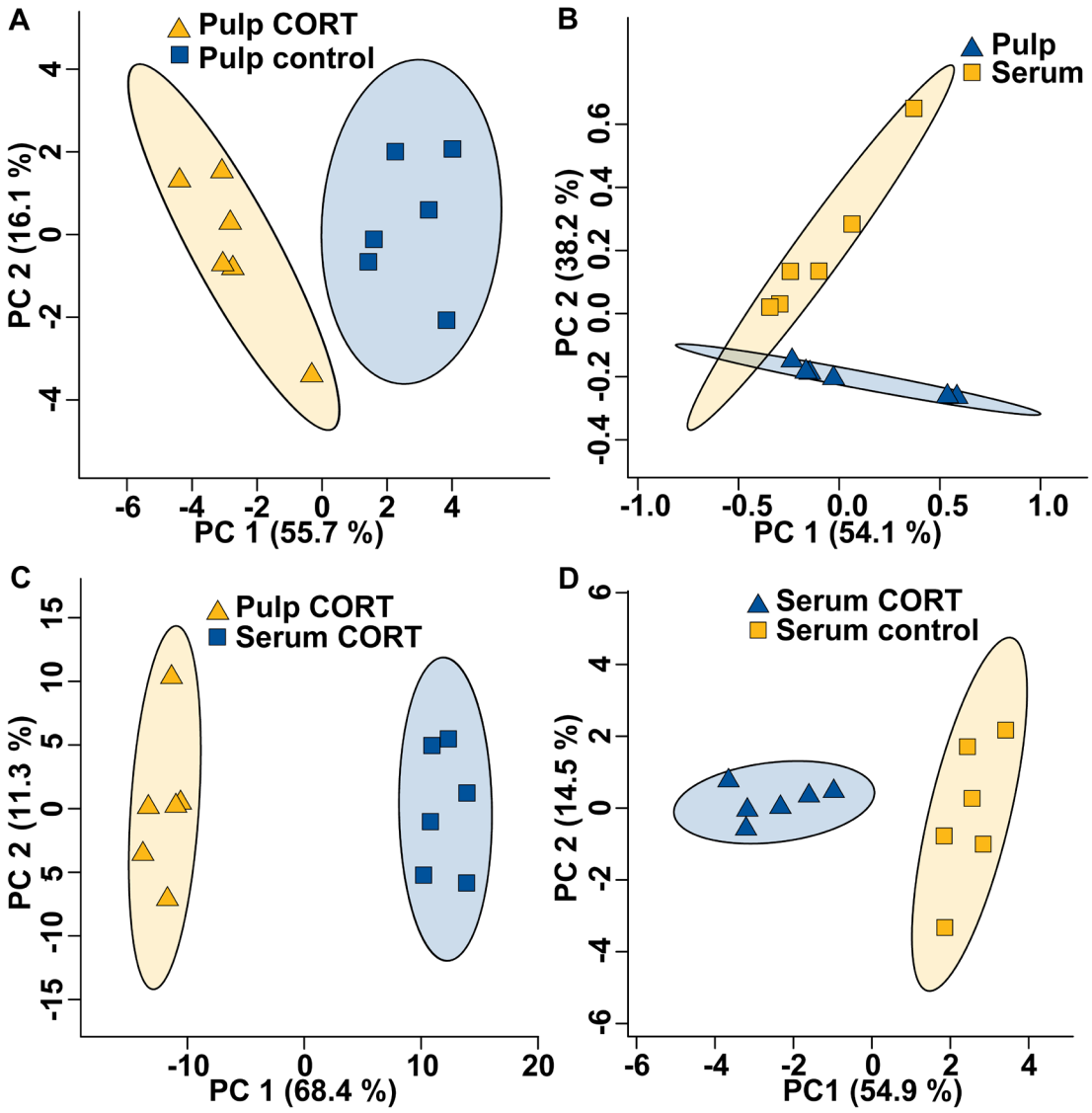

Figure 4-4: Principal component analysis score plots for the following comparisons: (A) feather pulp after 8 d of corticosterone (CORT) administration vs. control feather pulp; (B) control feather pulp vs. control serum; (C) feather pulp with CORT vs. serum with CORT; and (D) serum with CORT vs. control serum. Each marker represents one bird, and the shaded ellipses represents the 95% confidence interval for each group. The x- and y-axes show principal components one and two, respectively, with the number in brackets indicating the percent variance explained by each component. Bins that were determined to be significant via univariate Mann-Whitney were plotted in A and D, whereas all bins were plotted in B and C.

Table 4-1: Significantly altered metabolites between control feather pulp and feather pulp from birds administered corticosterone for 8 days

VIAVC p-value	MW p-value	Metabolite	Percent Difference
6.44E-03	0.002	L-Tyrosine	-63.01
1.61E-03	0.008	Threonine	-23.81
	0.009	2-Hydroxy-2-methylbutyric Acid	-35.72
	0.009	Ethanol	88.85
	0.015	Creatine	43.88
	0.015	Glycine	-25.4
	0.015	L-Cysteine	15.13
	0.015	L-Phenylalanine	-33.45
	0.019	Glutamate	20.1
	0.023	Ribose	-11.78
	0.026	1,3,7 - Trimethyluric Acid	25.92
	0.026	Corticosterone	95.84
	0.026	L-Homoserine	-16.21
	0.026	N1-Acetylspermine	27.93
	0.041	1,9- Dimethyluric Acid	19.08
	0.041	3,7-Dimethyluric Acid	19.01
	0.041	D-Lysine	18.9
	0.041	Myoinositol	-9.22
	0.041	N-Methyl-D-aspartic acid	-22.08

Table 4-2: Significantly altered metabolites between control serum and serum from birds administered corticosterone for eight days

MW p-value	Metabolite	Regulation
0.002	Mannose	17.93
0.004	Citramalic acid	-37.5
0.009	D-Glucose	-23.03
0.01	Traumatic acid	26.78
0.015	4-Aminobutyrate	39.33
0.015	4-Hydroxyproline	-22.82
0.015	D-Alanine	-41.34
0.015	L-Phenylalanine	-27.92
0.018	DL-2-Aminooctanoic acid	21.65
0.02	L-Threonine	-30.7
0.02	L-Tyrosine	-61.48
0.023	2-Hydroxy-2-methylbutyric acid	18.77
0.026	Malonic acid	-56.86
0.026	Proline	18.75
0.027	Citric acid	-21.79

4.6 References

1. Romero, L.M.; Romero, R.C. Corticosterone responses in wild birds: The importance of rapid initial sampling. *The Condor* **2002**, *104*, 129-135, doi:10.1093/condor/104.1.129.

2. Isaksson, C.; Örnborg, J.; Stephensen, E.; Andersson, S. Plasma glutathione and carotenoid coloration as potential biomarkers of environmental stress in Great Tits. *EcoHealth* **2005**, *2*, 138-146, doi:10.1007/s10393-005-3869-5.

3. Bouwer, M.; Ngcamphalala, C.A.; Ganswindt, A.; Mckechnie, A.E. Validation of a non-invasive technique for quantifying a stress-associated biomarker in a southern African hornbill. *J. Ornithol.* **2021**, *162*, 615-619, doi:10.1007/s10336-021-01861-5.

4. Ferreira, N.G.C.; Da Silva, L.P.; Fonseca, C.; Soares, A.M.V.M.; Loureiro, S. Habitats: Can they stress birds? An example of the use of biomarkers as an evaluation tool. *Integr. Environ. Assess. Manag.* **2010**, *6*, 779-780, doi:10.1002/ieam.113.

5. Scanes, C.G. Biology of stress in poultry with emphasis on glucocorticoids and the heterophil to lymphocyte ratio. *Poult. Sci.* **2016**, *95*, 2208-2215, doi:10.3382/ps/pew137.

6. Dai, S.F.; Wang, L.K.; Wen, A.Y.; Wang, L.X.; Jin, G.M. Dietary glutamine supplementation improves growth performance, meat quality and colour stability of broilers under heat stress. *Br. Poult. Sci.* **2009**, *50*, 333-340, doi:10.1080/00071660902806947.

7. Jastrebski, S.F.; Lamont, S.J.; Schmidt, C.J. Chicken hepatic response to chronic heat stress using integrated transcriptome and metabolome analysis. *PLoS One* **2017**, *12*, e0181900, doi:10.1371/journal.pone.0181900.

8. Chowdhury, V.S. Heat stress biomarker amino acids and neuropeptide afford thermotolerance in chicks. *J. Poult. Sci.* **2019**, *56*, 1-11, doi:10.2141/jpsa.0180024.

9. Hayashi, K.; Nagai, Y.; Ohstuka, A.; Tomita, Y. Effects of dietary corticosterone and trilostane on growth and skeletal muscle protein turnover in broiler cockerels. *Br. Poult. Sci.* **1994**, *35*, 789-798, doi:10.1080/00071669408417743.

10. Zaytsoff, S.J.M.; Brown, C.L.J.; Montina, T.; Metz, G.A.S.; Abbott, D.W.; Uwiera, R.R.E.; Inglis, G.D. Corticosterone-mediated physiological stress modulates hepatic lipid metabolism, metabolite profiles, and systemic responses in chickens. *Sci. Rep.* **2019**, *9*, 19225, doi:10.1038/s41598-019-52267-6.

11. Zaytsoff, S.J.M.; Boras, V.F.; Uwiera, R.R.E.; Inglis, G.D. The development of a stress-induced model of necrotic enteritis in broiler chickens using acute dietary corticosterone administration. *Poult. Sci.* **2021**, Submitted.

12. Zaytsoff, S.J.M.; Lyons, S.M.; Garner, A.M.; Uwiera, R.R.E.; Zandberg, W.F.; Abbott, D.W.; Inglis, G.D. Host responses to *Clostridium perfringens* challenge in a chicken model of chronic stress. *Gut Pathog.* **2020**, *12*, doi:10.1186/s13099-020-00362-9.

13. Rodriguez-Siek, K.E.; Giddings, C.W.; Doetkott, C.; Johnson, T.J.; Nolan, L.K. Characterizing the APEC pathotype. *Vet. Res.* **2005**, *36*, 241-256, doi:10.1051/vetres:2004057.

14. Maryvonne Dho-Moulin, J.M.F. Avian pathogenic *Escherichia coli* (APEC). *Vet. Res.* **1999**, *30*, 299-316, doi:hal.archives-ouvertes.fr/hal-00902571.

15. Poulsen, L.L.; Thofner, I.; Bisgaard, M.; Christensen, J.P.; Olsen, R.H.; Christensen, H. Longitudinal study of transmission of *Escherichia coli* from broiler breeders to broilers. *Vet. Microbiol.* **2017**, *207*, 13-18, doi:10.1016/j.vetmic.2017.05.029.

16. Guabiraba-Brito, R.; Schouler, C. Avian colibacillosis: Still many black holes. *FEMS Microbiol. Lett.* **2015**, *362*, 1-8, doi:10.1093/femsle/fnv118.

17. Antunes, P.; Mourão, J.; Campos, J.; Peixe, L. Salmonellosis: The role of poultry meat. *Clin. Microbiol. Infect.* **2016**, *22*, 110-121, doi:10.1016/j.cmi.2015.12.004.

18. Whiley, H.; Ross, K. *Salmonella* and eggs: From production to plate. *Int. J. Environ. Res. Public Health* **2015**, *12*, 2543-2556, doi:10.3390/ijerph120302543.

19. Hangalapura, B.N.; Nieuwland, M.G.B.; Buyse, J.; Kemp, B.; Parmentier, H.K. Effect of duration of cold stress on plasma adrenal and thyroid hormone levels and immune responses in chicken lines divergently selected for antibody responses. *Poult. Sci. Assoc.* **2004**, *83*, 1644-1649, doi:10.1093/ps/83.10.1644.

20. Zhao, F.Q.; Zhang, Z.W.; Qu, J.P.; Yao, H.D.; Li, M.; Li, S.; Xu, S.W. Cold stress induces antioxidants and Hsps in chicken immune organs. *Cell Stress Chaperon.* **2014**, *19*, 635-648, doi:10.1007/s12192-013-0489-9.

21. Cockrem, J.F. Stress, corticosterone responses and avian personalities. *J. Ornithol.* **2007**, *148*, 169-178, doi:10.1007/s10336-007-0175-8.

22. Breuner, C.W.; Orchinik, M. Plasma binding proteins as mediators of corticosteroid action in vertebrates. *J. Endocrinol.* **2002**, *175*, 99-112, doi:10.1677/joe.0.1750099

23. Lattin, C.R.; Reed, J.M.; Desrochers, D.W.; Romero, L.M. Elevated corticosterone in feathers correlates with corticosterone-induced decreased feather

quality: A validation study. *J. Avian Biol.* **2011**, *42*, 247-252, doi:10.1111/j.1600-048x.2010.05310.x.

24. Harris, C.M.; Madliger, C.L.; Love, O.P. An evaluation of feather corticosterone as a biomarker of fitness and an ecologically relevant stressor during breeding in the wild. *Oecologia* **2017**, *183*, 987-996, doi:10.1007/s00442-017-3836-1.

25. Harris, C.M.; Madliger, C.L.; Love, O.P. Temporal overlap and repeatability of feather corticosterone levels: Practical considerations for use as a biomarker. *Conserv. Physiol.* **2016**, *4*, 1-11, doi:10.1093/conphys/cow051.

26. Bortolotti, G.R.; Marchant, T.; Blas, J.; Cabezas, S. Tracking stress: Localization, deposition and stability of corticosterone in feathers. *J. Exp. Biol.* **2009**, *212*, 1477-1482, doi:10.1242/jeb.022152.

27. Romero, L.M.; Reed, J.M. Collecting baseline corticosterone samples in the field: Is under 3 min good enough? *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2005**, *140*, 73-79, doi:10.1016/j.cbpb.2004.11.004.

28. Weimer, S.L.; Wideman, R.F.; Scanes, C.G.; Mauromoustakos, A.; Christensen, K.D.; Vizzier-Thaxton, Y. An evaluation of methods for measuring stress in broiler chickens. *Poult. Sci.* **2018**, *97*, 3381-3389, doi:10.3382/ps/pey204.

29. Mormède, P.; Andanson, S.; Aupérin, B.; Beerda, B.; Guémené, D.; Malmkvist, J.; Manteca, X.; Manteuffel, G.; Prunet, P.; Van Reenen, C.G.; et al. Exploration of the hypothalamic–pituitary–adrenal function as a tool to evaluate animal welfare. *Physiol. Behav.* **2007**, *92*, 317-339, doi:10.1016/j.physbeh.2006.12.003.

30. De Jong, I.C.; Van Voorst, A.S.; Erkens, J.H.F.; Ehlhardt, D.A.; Blokhuis, H.J. Determination of the circadian rhythm in plasma corticosterone and catecholamine concentrations in growing broiler breeders using intravenous cannulation. *Physiol. Behav.* **2001**, *74*, 299-304, doi:10.1016/s0031-9384(01)00562-5.

31. Etches, R.J. Plasma concentrations of progesterone and corticosterone during the ovulation cycle of the hen (*Gallus domesticus*). *Poult. Sci.* **1979**, *58*, 211-216, doi:10.3382/ps.0580211.

32. Beuving, G.; Vonder, G.M. Daily rhythm of corticosterone in laying hens and the influence of egg laying. *J. Reprod. Fertil.* **1977**, *51*, 169.

33. Rosner, W. The functions of corticosteroid-binding globulin and sex hormone-binding globulin: Recent advances. *Endocr. Rev.* **1990**, *11*, 80-91, doi:10.1210/edrv-11-1-80.

34. Romero, L.M.; Cyr, N.E.; Romero, R.C. Corticosterone responses change seasonally in free-living house sparrows (*Passer domesticus*). *Gen. Comp. Endocrinol.* **2006**, *149*, 58-65, doi:10.1016/j.ygcen.2006.05.004.

35. Breuner, C.W.; Orchinik, M. Seasonal regulation of membrane and intracellular corticosteroid receptors in the house sparrow brain. *J. Neuroendocrinol.* **2001**, *13*, 412-420, doi:10.1046/j.1365-2826.2001.00646.x.

36. Chen, J.; Tellez, G.; Richards, J.D.; Escobar, J. Identification of potential biomarkers for gut barrier failure in broiler chickens. *Front. Vet. Sci.* **2015**, *2*, 14-14, doi:10.3389/fvets.2015.00014.

37. Giannuzzi, D.; Biolatti, B.; Longato, E.; Divari, S.; Starvaggi Cucuzza, L.; Pregel, P.; Scaglione, F.E.; Rinaldi, A.; Chiesa, L.M.; Cannizzo, F.T. Application of RNA-sequencing to identify biomarkers in broiler chickens prophylactic administered with antimicrobial agents. *Animal* **2021**, *15*, 1751-7311, doi:10.1016/j.animal.2020.100113.

38. Gou, Z.; Abouelezz, K.F.M.; Fan, Q.; Li, L.; Lin, X.; Wang, Y.; Cui, X.; Ye, J.; Masoud, M.A.; Jiang, S.; et al. Physiological effects of transport duration on stress biomarkers and meat quality of medium-growing Yellow broiler chickens. *Animal* **2021**, *15*, 1751-7311, doi:10.1016/j.animal.2020.100079.

39. Greene, E.S.; Rajaei-Sharifabadi, H.; Dridi, S. Feather HSP70: A novel non-invasive molecular marker for monitoring stress induced by heat exposure in broilers. *Poult. Sci.* **2019**, *98*, 3400-3404, doi:10.3382/ps/pez120.

40. Matysik, S.; Le Roy, C.I.; Liebisch, G.; Claus, S.P. Metabolomics of fecal samples: A practical consideration. *Trends Food Sci. Tech.* **2016**, *57*, 244-255, doi:10.1016/j.tifs.2016.05.011.

41. Will, A.; Wynne-Edwards, K.; Zhou, R.; Kitaysky, A. Of 11 candidate steroids, corticosterone concentration standardized for mass is the most reliable steroid biomarker of nutritional stress across different feather types. *Ecol. Evol.* **2019**, *9*, 11930-11943, doi:10.1002/ece3.5701.

42. Dehnhard, M.; Schreer, A.; Krone, O.; Jewgenow, K.; Krause, M.; Grossmann, R. Measurement of plasma corticosterone and fecal glucocorticoid metabolites in the chicken (*Gallus domesticus*), the great cormorant (*Phalacrocorax carbo*), and the goshawk (*Accipiter gentilis*). *Gen. Comp. Endocrinol.* **2003**, *131*, 345-352, doi:10.1016/s0016-6480(03)00033-9.

43. Rettenbacher, S.; Palme, R.; Hackl, R.; Bamberg, E. Distribution and elimination of radiolabelled corticosterone in chicken broilers. *Wien. Tierarztl. Monatsschr.* **2006**, *93*, 283-287.

44. Rettenbacher, S.; Möstl, E.; Hackl, R.; Ghareeb, K.; Palme, R. Measurement of corticosterone metabolites in chicken droppings. *Br. Poult. Sci.* **2004**, *45*, 704-711, doi:10.1080/00071660400006156.

45. Alm, M.; Holm, L.; Tauson, R.; Wall, H. Corticosterone metabolites in laying hen droppings—effects of fiber enrichment, genotype, and daily variations. *Poult. Sci.* **2014**, *93*, 2615-2621, doi:10.3382/ps.2014-04193.

46. Di-Poï, N.; Milinkovitch, M.C. The anatomical placode in reptile scale morphogenesis indicates shared ancestry among skin appendages in amniotes. *Sci. Adv.* **2016**, *2*, 1-8, doi:10.1126/sciadv.1600708.

47. Maderson, P.F.A.; Hillenius, W.J.; Hiller, U.; Dove, C.C. Towards a comprehensive model of feather regeneration. *J. Morphol.* **2009**, *270*, 1166-1208, doi:10.1002/jmor.10747.

48. Perry, R.J.; Wang, Y.; Cline, G.W.; Rabin-Court, A.; Song, J.D.; Dufour, S.; Zhang, X.M.; Petersen, K.F.; Shulman, G.I. Leptin mediates a glucose-fatty acid cycle to maintain glucose homeostasis in starvation. *Cell* **2018**, *172*, 234-248.e217, doi:10.1016/j.cell.2017.12.001.

49. Stettenheim. The integumentary morphology of modern birds: An overview. *Am. Zool.* **2000**, *40*, 461-477, doi:10.1093/icb/40.4.461.

50. Marsden, J.E.; May, B. Feather pulp: A non-destructive sampling technique for electrophoretic studies of birds. *The Auk* **1984**, *101*, 173-175, doi:10.1093/auk/101.1.173.

51. Bortolotti, G.R.; Marchant, T.A.; Blas, J.; German, T. Corticosterone in feathers is a long-term, integrated measure of avian stress physiology. *Funct. Ecol.* **2008**, *22*, 494-500, doi:10.1111/j.1365-2435.2008.01387.x.

52. Docherty, D.E. *Corvidae* feather pulp and West Nile Virus detection. *Emerg. Infect. Dis.* **2004**, *10*, 907-909, doi:10.3201/eid1005.030825.

53. Cortes, A.L.; Montiel, E.R.; Lemiere, S.; Gimeno, I.M. Comparison of blood and feather pulp samples for the diagnosis of Marek's disease and for monitoring Marek's disease vaccination by real time-PCR. *Avian Dis.* **2011**, *55*, 302-310, doi:10.1637/9578-101510-ResNote.1.

54. Zavala, G.; Jackwood, M.W.; Hilt, D.A. Polymerase chain reaction for detection of avian leukosis virus subgroup J in feather pulp. *Avian Dis.* **2002**, *46*, 971-978, doi:10.1637/0005-2086(2002)046[0971:PCRFDO]2.0.CO;2.

55. Jenni-Eiermann, S.; Helfenstein, F.; Vallat, A.; Glauser, G.; Jenni, L. Corticosterone: Effects on feather quality and deposition into feathers. *Methods Ecol. Evol.* **2015**, *6*, 237-246, doi:10.1111/2041-210x.12314.

56. Fairhurst, G.D.; Marchant, T.A.; Soos, C.; Machin, K.L.; Clark, R.G. Experimental relationships between levels of corticosterone in plasma and feathers in a free-living bird. *J. Exp. Biol.* **2013**, *216*, 4071-4081, doi:10.1242/jeb.091280.

57. Koren, L.; Nakagawa, S.; Burke, T.; Soma, K.K.; Wynne-Edwards, K.E.; Geffen, E. Non-breeding feather concentrations of testosterone, corticosterone and cortisol are associated with subsequent survival in wild house sparrows. *P. Roy. Soc. B-Biol. Sci.* **2012**, *279*, 1560-1566, doi:10.1098/rspb.2011.2062.

58. Berk, S.A.; Mcgettrick, J.R.; Hansen, W.K.; Breuner, C.W. Methodological considerations for measuring glucocorticoid metabolites in feathers. *Conserv. Physiol.* **2016**, *4*, cow020, doi:10.1093/conphys/cow020.

59. Hõrak, P.; Männiste, M.; Meitern, R.; Sild, E.; Saks, L.; Sepp, T. Dexamethasone inhibits corticosterone deposition in feathers of greenfinches. *Gen. Comp. Endocrinol.* **2013**, *191*, 210-214, doi:10.1016/j.ygcen.2013.07.002.

60. Romero, L.M.; Strochlic, D.; Wingfield, J.C. Corticosterone inhibits feather growth: Potential mechanism explaining seasonal down regulation of corticosterone during molt. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2005**, *142*, 65-73, doi:10.1016/j.cbpa.2005.07.014.

61. James, C.; Asher, L.; Herborn, K.; Wiseman, J. The effect of supplementary ultraviolet wavelengths on broiler chicken welfare indicators. *Appl. Anim. Behav. Sci.* **2018**, *209*, 55-64, doi:10.1016/j.applanim.2018.10.002.

62. Arrazola, A.; Torrey, S. The development of fault bars in domestic chickens (*Gallus gallus domesticus*) increases with acute stressors and individual propensity: Implications for animal welfare. *Anim. Welfare* **2019**, *28*, 279-286, doi:10.7120/09627286.28.3.279.

63. Desrochers, D.W.; Reed, J.M.; Awerman, J.; Kluge, J.A.; Wilkinson, J.; Van Griethuijsen, L.I.; Aman, J.; Romero, L.M. Exogenous and endogenous corticosterone alter feather quality. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **2009**, *152*, 46-52, doi:10.1016/j.cbpa.2008.08.034.

64. Karu, N.; Deng, L.; Slae, M.; Guo, A.C.; Sajed, T.; Huynh, H.; Wine, E.; Wishart, D.S. A review on human fecal metabolomics: Methods, applications and the human fecal metabolome database. *Anal. Chim. Acta.* **2018**, *1030*, 1-24, doi:10.1016/j.aca.2018.05.031.

65. Nicholson, J.K.; Lindon, J.C.; Holmes, E. 'Metabonomics': Understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* **1999**, *29*, 1181-1189, doi:10.1080/004982599238047.

66. Markley, J.L.; Bruschweiler, R.; Edison, A.S.; Eghbalnia, H.R.; Powers, R.; Raftery, D.; Wishart, D.S. The future of NMR-based metabolomics. *Curr. Opin. Biotechnol.* **2017**, *43*, 34-40, doi:10.1016/j.copbio.2016.08.001.

67. Wishart, D.S. Quantitative metabolomics using NMR. *Trends Analyt. Chem.* **2008**, *27*, 228-237, doi:10.1016/j.trac.2007.12.001.

68. Emwas, A.-H.M.; Salek, R.M.; Griffin, J.L.; Merzaban, J. NMR-based metabolomics in human disease diagnosis: Applications, limitations, and recommendations. *Metabolomics* **2013**, *9*, 1048-1072, doi:10.1007/s11306-013-0524-y.

69. Le Roy, C.I.; Mappley, L.J.; La Ragione, R.M.; Woodward, M.J.; Claus, S.P. NMR-based metabolic characterization of chicken tissues and biofluids: A model for avian research. *Metabolomics* **2016**, *12*, 157-157, doi:10.1007/s11306-016-1105-7.

70. Abasht, B.; Mutryn, M.F.; Michalek, R.D.; Lee, W.R. Oxidative stress and metabolic perturbations in wooden breast disorder in chickens. *PLoS One* **2016**, *11*, e0153750, doi:10.1371/journal.pone.0153750.

71. Beauclercq, S.; Nadal-Desbarats, L.; Hennequet-Antier, C.; Gabriel, I.; Tesseraud, S.; Calenge, F.; Le Bihan-Duval, E.; Mignon-Grasteau, S. Relationships between digestive efficiency and metabolomic profiles of serum and intestinal contents in chickens. *Sci. Rep.* **2018**, *8*, 6678, doi:10.1038/s41598-018-24978-9.

72. Inglis, G.D.; Wright, B.D.; Sheppard, S.A.; Abbott, D.W.; Oryschak, M.A.; Montina, T. Expeller-pressed canola (*Brassica napus*) meal modulates the structure and function of the cecal microbiota, and alters the metabolome of the pancreas, liver, and breast muscle of broiler chickens. *Animals* **2021**, *11*, 1-22, doi:10.3390/ani11020577.

73. Brown, C.L.J.; Zaytsoff, S.J.M.; Montina, T.; Inglis, G.D. Corticosterone-mediated physiological stress alters liver, kidney, and breast muscle metabolomic profiles in chickens. **2021**, *11*, 3056, doi:doi.org/10.3390/ani11113056.

74. Tiziani, S.; Emwas, A.-H.; Lodi, A.; Ludwig, C.; Bunce, C.M.; Viant, M.R.; Günther, U.L. Optimized metabolite extraction from blood serum for ^{1}H nuclear magnetic resonance spectroscopy. *Anal. Biochem.* **2008**, *377*, 16-23, doi:10.1016/j.ab.2008.01.037.

75. de Graaf, R.A.; Behar, K.L. Quantitative ^{1}H NMR spectroscopy of blood plasma metabolites. *Anal. Chem* **2003**, *75*, 2100-2104, doi:10.1021/ac020782.

76. Paxman, E.J.; Boora, N.S.; Kiss, D.; Laplante, D.P.; King, S.; Montina, T.; Metz, G.A.S. Prenatal maternal stress from a natural disaster alters urinary metabolomic profiles in project ice storm participants. *Sci. Rep.* **2018**, *8*, 12932, doi:10.1038/s41598-018-31230-x.

77. Kiss, D.; Ambeskovic, M.; Montina, T.; Metz, G.A.S. Stress transgenerationally programs metabolic pathways linked to altered mental health. *Cell. Mol. Life Sci.* **2016**, *73*, 4547-4557, doi:10.1007/s00018-016-2272-4.

78. Anderson, P.E.; Mahle, D.A.; Doom, T.E.; Reo, N.V.; Delraso, N.J.; Raymer, M.L. Dynamic adaptive binning: An improved quantification technique for NMR

spectroscopic data. *Metabolomics* **2011**, *7*, 179-190, doi:10.1007/s11306-010-0242-7.

79. Chong, J.; Xia, J. MetaboAnalystR: An R package for flexible and reproducible analysis of metabolomics data. *Bioinformatics* **2018**, *34*, 4313-4314, doi:10.1093/bioinformatics/bty528.

80. Psychogios, N.; Hau, D.D.; Peng, J.; Guo, A.C.; Mandal, R.; Bouatra, S.; Sinelnikov, I.; Krishnamurthy, R.; Eisner, R.; Gautam, B.; et al. The human serum metabolome. *PLoS One* **2011**, *6*, 1- 23, doi:10.1371/journal.

81. McHugh, C.E.; Flott, T.L.; Schooff, C.R.; Smiley, Z.; Puskarich, M.A.; Myers, D.D.; Younger, J.G.; Jones, A.E.; Stringer, K.A. Rapid, reproducible, quantifiable NMR metabolomics: Methanol and methanol: Chloroform precipitation for removal of macromolecules in serum and whole blood. *Metabolites* **2018**, *8*, 93, doi:10.3390/metabo8040093.

82. Daykin, C.A.; Foxall, P.J.D.; Connor, S.C.; Lindon, J.C.; Nicholson, J.K. The comparison of plasma deproteinization methods for the detection of low-molecular-weight metabolites by ^{1}H nuclear magnetic resonance spectroscopy. *Anal. Biochem.* **2002**, *304*, 220-230, doi:10.1006/abio.2002.5637.

83. Snytnikova, O.A.; Khlichkina, A.A.; Sagdeev, R.Z.; Tsentalovich, Y.P. Evaluation of sample preparation protocols for quantitative NMR-based metabolomics. *Metabolomics* **2019**, *15*, 84, doi:10.1007/s11306-019-1545-y.

84. Kriat, M.; Confortgouny, S.; Viondury, J.; Sciaky, M.; Viout, P.; Cozzone, P.J. Quantitation of metabolites in human blood-serum by proton magnetic-resonance spectroscopy. A comparative-study of the use of formate and TSP as concentration standards. *NMR Biomed.* **1992**, *5*, 179-184, doi:10.1002/nbm.1940050404.

85. Singh, R. Membrane technology and engineering for water purification. In *Introduction to membrane technology* 2nd ed.; Butterworth-Heinemann: 2015; pp. 1-80.

86. Brown, C.L.J.; Scott, H.; Mulik, C.; Freund, A.S.; Opyr, M.; Metz, G.A.S.; Inglis, G.D.; Montina, T. Fecal ^{1}H-NMR metabolomics: A comparison of sample preparation methods for NMR and novel *in silico* baseline correction. *Metabolites* **2021**, Submitted.

87. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911-917, doi:10.1139/o59-099.

88. Wu, H.; Southam, A.D.; Hines, A.; Viant, M.R. High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Anal. Biochem.* **2008**, *372*, 204-212, doi:10.1016/j.ab.2007.10.002.

89. Lin, C.Y.; Wu, H.; Tjeerdema, R.S.; Viant, M.R. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics* **2007**, *3*, 55-67, doi:10.1007/s11306-006-0043-1.

90. Szymanska, E.; Saccenti, E.; Smilde, A.K.; Westerhuis, J.A. Double-check: Validation of diagnostic statistics for PLS-DA models in metabolomics studies. *Metabolomics* **2012**, *8*, 3-16, doi:10.1007/s11306-011-0330-3.

91. Post, J.; Rebel, J.M.; ter Huurne, A.A. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiological effects of stress. *Poult. Sci.* **2003**, *82*, 1313-1318, doi:10.1093/ps/82.8.1313.

92. Patterson, A.G.L.; Kitaysky, A.S.; Lyons, D.E.; Roby, D.D. Nutritional stress affects corticosterone deposition in feathers of Caspian tern chicks. *J. Avian Biol.* **2015**, *46*, 18-24, doi:10.1111/jav.00397.

93. Sharma, S.; Gupta, A.; Chik, S.M.S.T.; Kee, C.G.; Mistry, B.M.; Kim, D.H.; Sharma, G. Characterization of keratin microparticles from feather biomass with potent antioxidant and anticancer activities. *Int. J. Biol. Macromol.* **2017**, *104*, 189-196, doi:10.1016/j.ijbiomac.2017.06.015.

94. Tesfaye, T.; Sithole, B.; Ramjugernath, D.; Chunilall, V. Valorisation of chicken feathers: Characterisation of chemical properties. *Waste Manag. Res.* **2017**, *68*, 626-635, doi:10.1016/j.wasman.2017.06.050.

95. Alanazi, A.M.; Mostafa, G.A.E.; Al-Badr, A.A. Glutathione. In *Profiles of drug substances, excipients, and related methodology*; Netherlands, 2015; Volume 40, p. 43.

96. Lu, S.C. Glutathione synthesis. *Biochim. Biophys. Acta* **2013**, *1830*, 3143-3153, doi:10.1016/j.bbagen.2012.09.008.

97. Lin, H.; Decuypere, E.; Buyse, J. Oxidative stress induced by corticosterone administration in broiler chickens (*Gallus gallus domesticus*) - 2. Short-term effect. *Comp. Biochem. Physiol. B* **2004**, *139*, 745-751, doi:10.1016/j.cbpc.2004.09.014.

98. Tesfaye, T.; Sithole, B.; Ramjugernath, D. Valorisation of chicken feathers: A review on recycling and recovery route—current status and future prospects. *Clean Technol. Envir.* **2017**, *19*, 2363-2378, doi:10.1007/s10098-017-1443-9.

99. Phang, J.M.; Liu, W.E.I.; Zabirnyk, O. Proline metabolism and microenvironmental stress. *Annu. Rev. Nutr.* **2010**, *30*, 441-463, doi:10.1146/annurev.nutr.012809.104638.

100. Arimoto, K.; Fukuda, H.; Imajoh-Ohmi, S.; Saito, H.; Takekawa, M. Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. *Nat. Cell Biol.* **2008**, *10*, 1324-1332, doi:10.1038/ncb1791.

101. Shen, J.-c.; Lian, L.-y.; Zhang, Y.; He, Q.-d.; Chen, J.-l.; Zhang, L.-b.; Huang, M.-s.; Liu, M.; Qian, L.-c.; Liu, C.-c.; et al. Dynamic analysis of metabolic response in gastric ulcer (GU) rats with electroacupuncture treatment using ^{1}H NMR-based metabolomics. *Evid. Based Complement. Alternat. Med.* **2019**, *2019*, doi:10.1155/2019/1291427.

102. Dryer, S.E. Glutamate receptors in the kidney. *Nephrol. Dial. Transplant.* **2015**, *30*, 1630-1638, doi:10.1093/ndt/gfv028.

103. Wu, J.; An, Y.; Yao, J.; Wang, Y.; Tang, H. An optimised sample preparation method for NMR-based faecal metabonomic analysis. *The Analyst* **2010**, *135*, 1023, doi:10.1039/b927543f.

104. Chen, C.-F.; Foley, J.; Tang, P.-C.; Li, A.; Jiang, T.X.; Wu, P.; Widelitz, R.B.; Chuong, C.M. Development, regeneration, and evolution of feathers. *Annu. Rev. Anim. Biosci.* **2015**, *3*, 169-195, doi:10.1146/annurev-animal-022513-114127.

105. Aggrey, S.E.; Milfort, M.C.; Fuller, A.L.; Yuan, J.; Rekaya, R. Effect of host genotype and *Eimeria acervulina* infection on the metabolome of meat-type chickens. *PLoS One* **2019**, *14*, e0223417, doi:10.1371/journal.pone.0223417.

106. Beauclercq, S.; Nadal-Desbarats, L.; Hennequet-Antier, C.; Collin, A.; Tesseraud, S.; Bourin, M.; Le Bihan-Duval, E.; Berri, C. Serum and muscle metabolomics for the prediction of ultimate pH, a key factor for chicken-meat quality. *J. Proteome Res.* **2016**, *15*, 1168-1178, doi:10.1021/acs.jproteome.5b01050.

107. Zhang, L.; Yue, H.Y.; Zhang, H.J.; Xu, L.; Wu, S.G.; Yan, H.J.; Gong, Y.S.; Qi, G.H. Transport stress in broilers: I. Blood metabolism, glycolytic potential, and meat quality. *Poult. Sci.* **2009**, *88*, 2033-2041, doi:10.3382/ps.2009-00128.

108. Lytou, A.E.; Nychas, G.-J.E.; Panagou, E.Z. Effect of pomegranate based marinades on the microbiological, chemical and sensory quality of chicken meat: A metabolomics approach. *Int. J. Food Microbiol.* **2018**, *267*, 42-53, doi:10.1016/j.ijfoodmicro.2017.12.023.

109. Xiao, Z.; Luo, Y.; Wang, G.; Ge, C.; Zhou, G.; Zhang, W.; Liao, G. ^{1}H-NMR-based water-soluble low molecular weight compound characterization and fatty acid composition of boiled Wuding chicken during processing. *J. Sci. Food Agric.* **2019**, *99*, 429-435, doi:10.1002/jsfa.9204.

5 GENERAL CONCLUSIONS, CONSIDERATIONS FOR FUTURE RESEARCH, AND FUTURE DIRECTIONS

5.1 General conclusions

Mounting restrictions on antibiotic use in poultry production has resulted in an urgent need to develop non-antibiotic strategies to mitigate diseases and enhance bird production health. Although the mechanisms are currently unresolved, stress is recognized as an important factor in determining bird health and susceptibility to disease. My thesis research applied metabolomics to provide insights into the chicken metabolome with a focus on identifying biomarkers of stress in birds. Currently, many of the biomarkers of stress are from tissue samples that require terminal sampling, which is not a logistically viable option in a chicken production setting. In this regard, the chicken industry would benefit greatly from a knowledge of biomarkers of stress that are correlated with bird health and that can be measured in a non-destructive manner. Blood can be taken non-destructively, but the process is quite stressful to the bird which confounds analyses and actively adds to the stress problem. Samples such as feces and feathers can be collected with minimal handling of the bird, thereby allowing stakeholders to monitor stress without excessively stressing the birds during sample collection. An overarching goal of my thesis research is to pave the way for the identification of novel biomarkers of stress predictive of disease manifestation in broiler chickens, which will provide stakeholders with the tools to quantify stress, and allow producers to objectively develop and evaluate mitigation strategies to enhance bird health.

The metabolomics strategy that I employed involved the use of Nuclear Magnetic Resonance (NMR) spectroscopy. NMR is an ideal starting point for novel biomarker

discovery due to the ability to conduct global and untargeted metabolomic analyses of tissues of small size and/or limited availability. Furthermore, NMR metabolomics is a non-destructive technique facilitating follow-up analyses on individual samples. Another advantage of NMR metabolomics is that it is a high throughput technique requiring minimal sample preparation and data collection is rapid [1-3].

In the first study conducted (presented in chapter 2), corticosterone (CORT), a physiological stressor, was administered to chickens at two different concentrations (10 and 30 mg/L) in drinking water to determine the effects of stress on the metabolomes of three different chicken tissues (i.e. liver, kidney, and breast muscle). Also investigated were the effects of the ethanol carrier used to dissolve the non-polar CORT in the drinking water on the chicken metabolomes. NMR-based metabolomics was demonstrated to detect stress-induced and systemic changes in chickens in a highly sensitive manner. In this regard, I showed that CORT administration significantly altered the kidney, liver, and breast muscle metabolomes. More specifically, CORT administration caused a decrease in kidney glutamate, an increase in glucose and a decrease in amino acids in liver, and increased glucose and decreased branched chain amino acids in breast muscle. Other metabolites identified as significantly altered in breast muscle have been previously linked to decreased meat quality. The high detection sensitivity of CORT-induced changes in the metabolome of all three tissues examined indicated that this method will have value in identifying biomarkers induced by stressors in production settings, such as temperature fluctuations, overcrowding, and social interactions (e.g. isolation). In stress-based studies in chickens, CORT is often administered in water. However, since CORT is non-polar, ethanol is used as a solvent. My research showed that ethanol alone significantly altered the metabolome of the

chicken tissues studied. More specifically, ethanol resulted in a decrease in glycerol and alanine in kidney, a decrease in lactate in liver, and a decrease in glutamine in breast muscle. Thus, alternate strategies of administering CORT are required (e.g. administration in feed). It is noteworthy that although ethanol affected the metabolomes of the liver, kidney, and breast muscle of chickens, the effects of ethanol alone were substantially less than the metabolomic effects of CORT orally administered in ethanol. However, the experimental design that I applied did not allow me to determine if CORT and ethanol together had a synergistic effect.

As indicated previously, feces can be non-destructively obtained from chickens, and it has potential to provide key information on bird health (e.g. function of the enteric microbiota). As a prelude to ascertaining the value of the fecal metabolome to ascertain bird health status, a study was conducted with the goal of optimizing fecal sample preparation for ^{1}H-NMR metabolomics (presented in Chapter 3). Crucially, a consensus method for preparing fecal samples for ^{1}H-NMR metabolomics does not exist. Four different fecal sample metabolite extraction methods were tested, which included ultrafiltration (UF), Bligh-Dyer (BD) methanol chloroform extraction, a modified BD extraction method that favours the extraction of fatty acids (M-BD), and no extraction where the sample was homogenized in buffer only. The M-BD method produced spectra with minimal to no metabolite information in comparison to the other three methods. For this reason, the UF, BD, and no extraction method were further investigated with respect to inter-sample variation in mammalian and avian fecal samples. The UF method provided a consistent baseline across all samples and both species tested; however, the BD and no extraction methods resulted in significant baseline distortions that were caused by the incomplete removal of macromolecules (fats and proteins). These distortions led to

inaccurate quantification of metabolites and need to be corrected. Thus, a novel *in silico* baseline correction method was also tested on the samples that had been processed using the BD and no extraction methods. In the case of mammalian fecal samples, the baseline correction method was able to produce a consistent baseline for the spectra obtained from both the BD and no extraction methods. In the case of avian fecal samples, the baseline correction method was only able to produce a consistent baseline for spectra obtained from the no extraction method. This suggests that future studies should focus on either utilizing the UF method of metabolite extraction or the no extraction method followed by *in-silico* baseline correction. Each of these methods provide a similar amount of metabolite information (260 versus 227 for the UF and no extraction methods, respectively) and have pros and cons. On the one hand, the UF method requires no additional data processing steps, such as baseline correction, but the cost of the filters is high. On the other hand, the no extraction method has a lower cost and a simpler sample preparation, but the application of *in silico* baseline correction adds an additional data processing step. Often the best option will be situation dependent, but the advantages of investigating fecal metabolites cannot be overstated as the ease of collection without having to handle and stress the chickens makes it an excellent target for an assay for biomarkers of stress.

Feathers unlike mammalian hair are highly vascularized (i.e. vascularized feather pulp located at the base of the rachis) (Figure 1-4). To my knowledge using feather pulp to measure biomarkers of stress has not previously been examined. Importantly, feathers like feces represent a logistically available sample source, but may be superior to feces for examining the metabolome of the host. Thus, I examined the metabolome within feather pulp of broiler chickens, and how the metabolome is affected by bird age and

stress incited by CORT administration (presented in Chapter 4). Moreover, I comparatively examined the metabolome of feather pulp and blood to determine whether the metabolome of pulp was reflective of blood. Two different methods of extracting the metabolome of feather pulp were tested (BD and UF), and it was observed using both extraction methods that pulp possesses a diverse metabolome. Although both methods produced ^{1}H-NMR spectra with no acquisition problems, conspicuously more metabolites were observed in pulp extracted with the BD method suggesting that this method is better suited for conducting untargeted metabolomics of feather pulp. An examination of the metabolome of feather pulp revealed that metabolite profiles differed as birds aged, thus suggesting that feather pulp may be a good target to ascertain the impact of a variety of intrinsic and extrinsic factors on the host, including stress. In this regard, the administration of CORT to broiler chickens was demonstrated to impact the metabolome of feather pulp further supporting pulp as a potentially excellent sample type to ascertain the health status of chickens and likely other bird species. Unexpectedly, the metabolome of feather pulp and blood serum differed. Moreover, more and different metabolites were observed in feather pulp than in blood. These results led me to conclude that feather pulp has considerable potential as a novel and non-destructive sample type to sensitively monitor biomarkers of stress in chickens.

5.2 Considerations for future research

My research findings indicate that CORT should be administered in an alternate fashion to water to remove the confounding effects of ethanol on the metabolome of chickens. This could include incorporation of CORT into the feed in future studies. To my knowledge it is currently unknown if the addition of CORT to the feed alters its

palatability, and if this has an impact on how much of the feed containing CORT that chickens consume compared to the feed with no CORT. An examination of the feather pulp metabolome from different feather types (e.g. wing feathers vs body feathers) to ascertain if there are metabolomic differences among feathers is also required. Such information is need to determine if the metabolome differs among feather types, and to inform decisions on the best types of feathers to sample as a function of biological factors (e.g. bird sex and age) with the goal of measuring biomarkers of stress and bird health. The breed of broiler chicken most commonly used by my research team is Ross 308 Fast Feather (FF). This breed of chickens grows feathers too quickly to be feather sexed (an art form in itself); thus, the only way to currently determine sex in our studies is at necropsy or by DNA analysis. Necropsies cannot be performed at the commencement of the study to ensure that an equal number of males and females are in each treatment group. Moreover, Ross 308FF chickens grow rapidly, and the time required to complete DNA-based determination of bird sex represents a significant obstacle to using this technology in practice. Thus, it would be exceptionally useful to identify a metabolite biomarker that can be quickly and easily detected in feces or feathers for sexing of birds.

5.3 Future research

In conjunction with the considerations for future research presented above, my research findings have precipitated the formulation of a variety of follow-up studies. Three such studies include: (i) a comparative examination of the metabolome of chickens administered CORT (i.e. defined model of stress) with the metabolome of chickens exposed to simulated production stressors; (ii) identification of biomarkers of stress that are correlated with chicken health (e.g. disease); and (iii) evaluation/validation of

biomarkers of stress and bird health in a production setting (e.g. correlated with production parameters) (Figure 5-1). The results presented in chapter 2 showed that it is possible to alter the metabolome of chickens in response to stress induced by the administration of CORT. However, it is currently unknown if the stress response generated by CORT is representative of stress impacts on the metabolome of chickens exposed to common production stressors. The next logical step is to compare the stress response created by administering CORT to the stress response generated by important production stressors, such as temperature fluctuations, overcrowding, social interactions (e.g. isolation), and feed and or water disruptions (e.g. inability of a chicken to locate feed and water during brooding). Some study objectives could be to: (1) induce physiological stress by administering CORT; (2) induce stress using simulated production stressors (e.g. thermal and social stress); (3) obtain a variety of samples, and comprehensively identify biomarkers of stress; (4) compare the impacts of CORT versus production stressors. Another logical experiment is to correlate physiological stress, including biomarkers of stress, with disease. There are many bacterial and viral pathogens that can have negative effects on the health of a chicken flock. Some bacterial pathogens that are at the forefront of producers' minds are *Clostridium perfringens* (cause of necrotic enteritis; NE), avian pathogenic *Escherichia coli* (APEC) (cause of colibacillosis; CBAC), and *Salmonella enterica* (cause of salmonellosis), which are responsible for significant bird morbidity and mortality [4,5]. Moreover, *S. enterica* represents a significant zoonotic threat to people [6,7], along with other bacteria, such as *Campylobacter jejuni* [8].

The clinical form of NE, most often seen in 2-5-week-old birds, results in closed eyes and ruffled feathers, severe depression, decreased appetite, diarrhea, followed quickly by death [7,9]. The subclinical form of the disease is chronic and often undetected but leads

to reductions in growth and feed conversions rates. Together, both forms of NE cost the poultry industry an estimated $6 billion USD a year [7]. The etiology of NE is still nebulous, and it is often difficult to recreate in a laboratory setting, but it is known that strains of *C. perfringens* that produce Necrotic enteritis Beta toxin (NetB) [10] in combination with, poorly understood pre-disposing factors, such as changes in the immune system [11], changes in the gut [11], and stress [12], produces NE in all poultry.

It is noteworthy that CBAC is a broad term for a variety of diseases incited by APEC, and includes, but is not limited to, infections of the respiratory tract (e.g. airsacculitis), infections of the reproductive tract (e.g. salpingitis), infections of the skin (e.g. cellulitis), infections of the digestive tract (enteric infections), as well as diseases like swollen head syndrome [13-16]. Salmonellosis is one of the most common food-borne illnesses in the world [17,18] causing mild gastroenteritis to death depending on the severity of the infection [19]. Chickens are known to be a major reservoir of *S. enterica* leading to many of the cases of human salmonellosis [20]. Zoonotic pathogens, such as *S. enterica* and *C. jejuni*, are responsible for considerable morbidity in people, the estimated total cost for human *Salmonella* infections is in excess of 14 billion USD/year in the United States alone [21], but the disease can also impact chicken producers by killing chickens and subclinical forms of the disease lead to lower egg yields and quality [18]. Importantly, all three of these diseases result in billions of dollars in economic losses to the poultry industry annually.

Recent research conducted by other members of the research team has demonstrated that physiological stress has a profound impact on the metabolism of birds and renders them susceptible to NE incited by *C. perfringens* [12,22]. However, the degree to which, and how, normal production stressors affect bird metabolism and the ability of birds to

mount an immune response is poorly understood at present. Moreover, the impact of stress on other important diseases, such as CBAC are currently unknown. Anecdotal evidence suggests that CBAC and NE are diseases of predisposed birds. Regarding CBAC, research is needed to: (i) develop a robust model of CBAC; (ii) elucidate the interactive role that physiological stress has on bird metabolism, the immune system, and the manifestation of CBAC; (iii) identify biomarkers of stress and disease; and (iv) deliver knowledge/tools to the poultry industry to enhance bird health, and mitigate morbidity and mortality caused by CBAC without the use of antibiotics. Following the identification of biomarkers of stress that are correlated with bird health, the next step should be to develop methodologies to accurately and logistically measure the biomarkers; my research has demonstrated that NMR-based metabolomics holds considerable promise in this regard (e.g. metabolome of feather pulp). Subsequent activities should then focus on optimizing assays for on farm applications, and validating them in production settings (e.g. temporally monitoring biomarkers within production flocks as a function of flock health and performance). The acquisition of the above data is crucial to ultimately translate the technology to the marketplace, which will allow stakeholders to monitor the health status of their flocks, and to deploy and evaluate mitigations in an objective manner before disease is manifested.

The goal of my research is to gain insight on the effects of stress on bird metabolism; however, it cannot be overlooked that stress and metabolism are only two small parts of the whole. The overarching goals of the research team are elucidating the systematic effects of stress on chickens. Stress also affects the immune system, behaviour, organ function, and the microbiota (Figure 5-2). How each of these different aspects interact with each other and with pathogens, as well as how they lead to morbidity and mortality

in chickens, is still unknown. The research presented in this thesis provides the initial steps required to address this uncertainty. I contend that the research I have conducted in my M.Sc., and the strategy proposed above for which my research provides foundational information, will provide stakeholders with the ability to monitor and promote bird health in production settings.

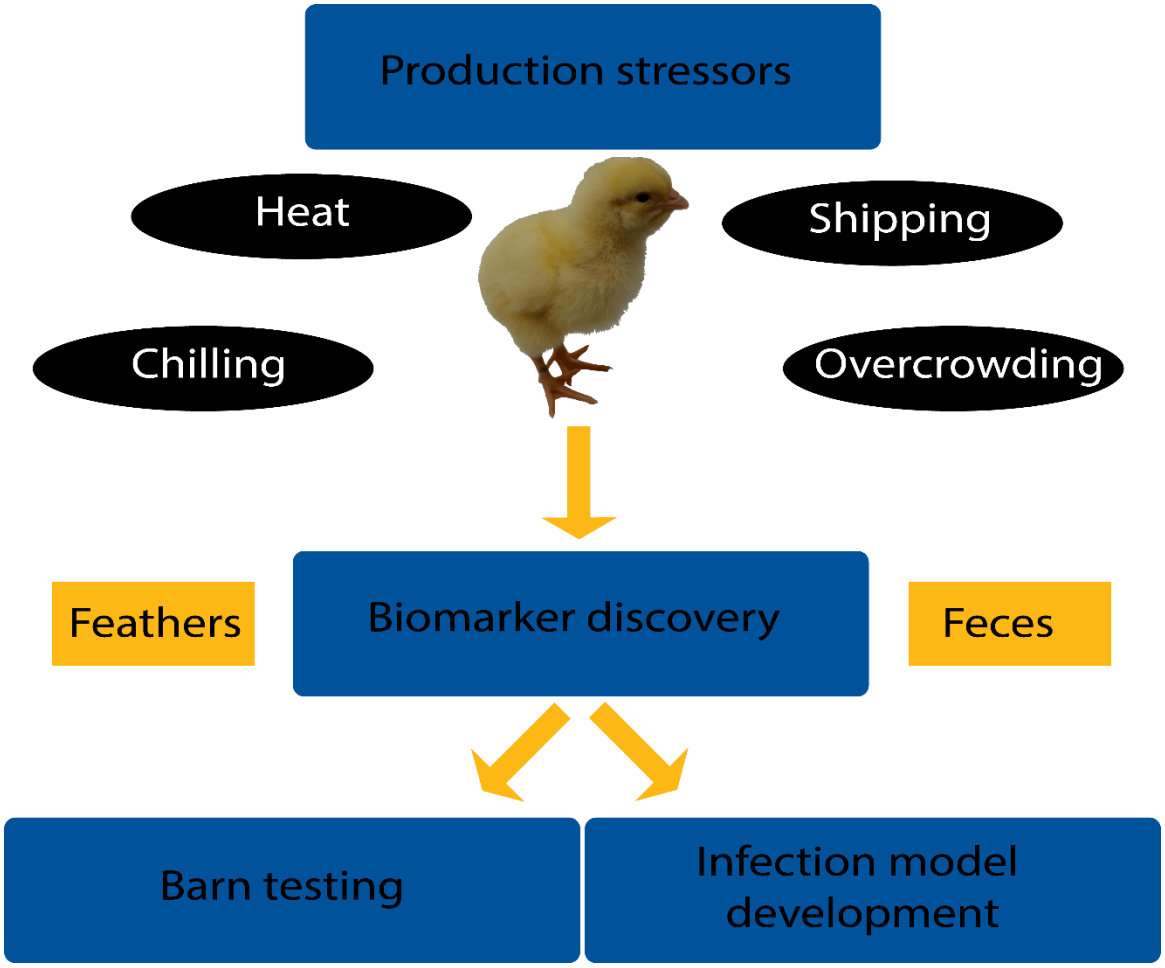

Figure 5-1: Proposed experimental progression and study variables

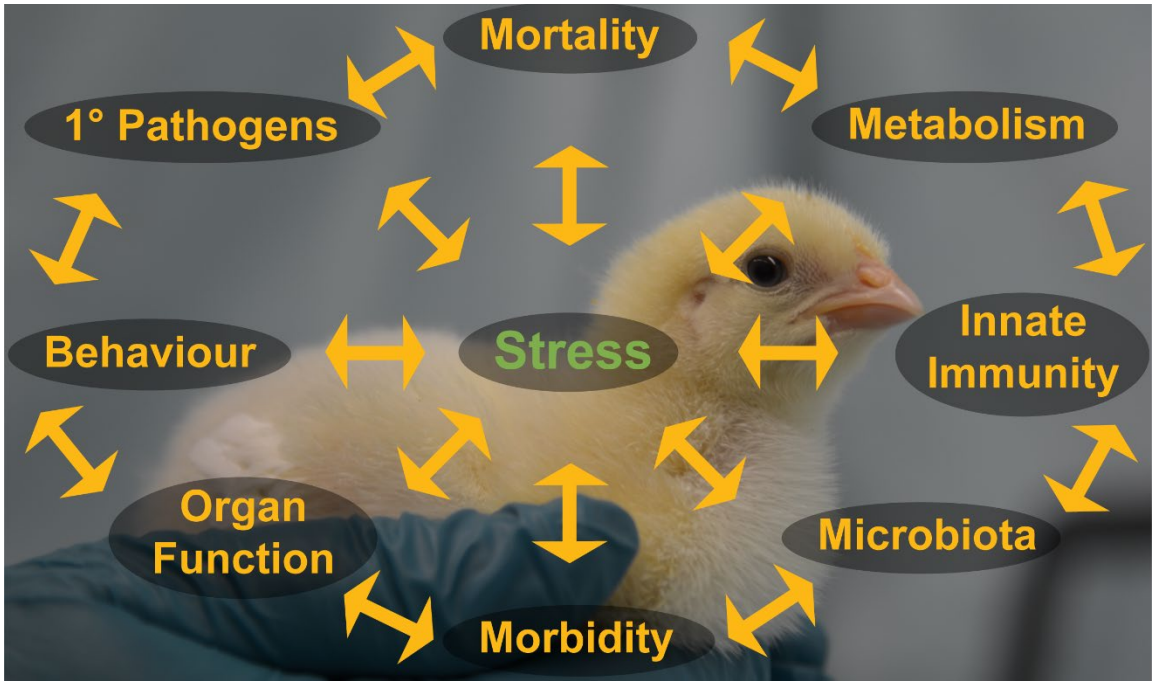

Figure 5-2: Different factors that are affected by stress

5.4 References

1. Psychogios, N.; Hau, D.D.; Peng, J.; Guo, A.C.; Mandal, R.; Bouatra, S.; Sinelnikov, I.; Krishnamurthy, R.; Eisner, R.; Gautam, B.; et al. The human serum metabolome. *PLoS One* **2011**, *6*, 1- 23, doi:10.1371/journal.

2. Dame, Z.T.; Aziat, F.; Mandal, R.; Krishnamurthy, R.; Bouatra, S.; Borzouie, S.; Guo, A.C.; Sajed, T.; Deng, L.; Lin, H.; et al. The human saliva metabolome. *Metabolomics* **2015**, *11*, 1864-1883, doi:10.1007/s11306-015-0840-5.

3. Wishart, D.S.; Lewis, M.J.; Morrissey, J.A.; Flegel, M.D.; Jeroncic, K.; Xiong, Y.; Cheng, D.; Eisner, R.; Gautam, B.; Tzur, D.; et al. The human cerebrospinal fluid metabolome. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2008**, *871*, 164-173, doi:10.1016/j.jchromb.2008.05.001.

4. Jørgensen, S.L.; Kudirkiene, E.; Li, L.; Christensen, J.P.; Olsen, J.E.; Nolan, L.; Olsen, R.H. Chromosomal features of *Escherichia coli* serotype O2:K2, an avian pathogenic *E. coli*. *Stand Genomic Sci.* **2017**, *12*, doi:10.1186/s40793-017-0245-3.

5. Maturana, V.G.; de Pace, F.; Carlos, C.; Pires, M.M.; de Campos, T.A.; Nakazato, G.; Stheling, E.G.; Logue, C.M.; Nolan, L.K.; da Silveira, W.D. Subpathotypes of avian pathogenic Escherichia coli (APEC) exist as defined by their syndromes and virulence traits. *Open Microbiol. J.* **2011**, *5*, 55-64, doi:10.2174/1874285801105010055.

6. Kiu, R.; Hall, L.J. An update on the human and animal enteric pathogen *Clostridium perfringens*. *Emerging Microbes & Infections* **2018**, *7*, 1-15, doi:10.1038/s41426-018-0144-8.

7. Wade, B.; Keyburn, A. The true cost of necrotic enteritis. **2015**.

8. Inglis, G.D.; Boras, V.F.; Webb, A.L.; Suttorp, V.V.; Hodgkinson, P.; Taboada, E.N. Enhanced microbiological surveillance reveals that temporal case clusters contribute to the high rates of campylobacteriosis in a model agroecosystem. *Int. J. Med. Microbiol.* **2019**, *309*, 232-244, doi:10.1016/j.ijmm.2019.04.003.

9. Prescott, J.F.; Parreira, V.R.; Mehdizadeh Gohari, I.; Lepp, D.; Gong, J. The pathogenesis of necrotic enteritis in chickens: What we know and what we need to know: A review. *Avian Pathol.* **2016**, *45*, 288-294, doi:10.1080/03079457.2016.1139688.

10. Shojadoost, B.; Vince, A.R.; Prescott, J.F. The successful experimental induction of necrotic enteritis in chickens by *Clostridium perfringens*: A critical review. *Vet. Res.* **2012**, *43*, 74, doi:10.1186/1297-9716-43-74.

11. Moore, R.J. Necrotic enteritis predisposing factors in broiler chickens. *Avian Pathol.* **2016**, *45*, 275-281, doi:10.1080/03079457.2016.1150587.

12. Zaytsoff, S.J.M.; Lyons, S.M.; Garner, A.M.; Uwiera, R.R.E.; Zandberg, W.F.; Abbott, D.W.; Inglis, G.D. Host responses to *Clostridium perfringens* challenge in a chicken model of chronic stress. *Gut Pathog.* **2020**, *12*, doi:10.1186/s13099-020-00362-9.

13. Rodriguez-Siek, K.E.; Giddings, C.W.; Doetkott, C.; Johnson, T.J.; Nolan, L.K. Characterizing the APEC pathotype. *Vet. Res.* **2005**, *36*, 241-256, doi:10.1051/vetres:2004057.

14. Maryvonne Dho-Moulin, J.M.F. Avian pathogenic *Escherichia coli* (APEC). *Vet. Res.* **1999**, *30*, 299-316, doi:hal.archives-ouvertes.fr/hal-00902571.

15. Poulsen, L.L.; Thofner, I.; Bisgaard, M.; Christensen, J.P.; Olsen, R.H.; Christensen, H. Longitudinal study of transmission of *Escherichia coli* from broiler breeders to broilers. *Vet. Microbiol.* **2017**, *207*, 13-18, doi:10.1016/j.vetmic.2017.05.029.

16. Guabiraba-Brito, R.; Schouler, C. Avian colibacillosis: Still many black holes. *FEMS Microbiol. Lett.* **2015**, *362*, 1-8, doi:10.1093/femsle/fnv118.

17. Antunes, P.; Mourão, J.; Campos, J.; Peixe, L. Salmonellosis: The role of poultry meat. *Clin. Microbiol. Infect.* **2016**, *22*, 110-121, doi:10.1016/j.cmi.2015.12.004.

18. Whiley, H.; Ross, K. *Salmonella* and eggs: From production to plate. *Int. J. Environ. Res. Public Health* **2015**, *12*, 2543-2556, doi:10.3390/ijerph120302543.

19. Chappell, L.; Kaiser, P.; Barrow, P.; Jones, M.A.; Johnston, C.; Wigley, P. The immunobiology of avian systemic salmonellosis. *Vet. Immunol. Immunopathol.* **2009**, *128*, 53-59, doi:10.1016/j.vetimm.2008.10.295.

20. Wellawa, D.H.; Allan, B.; White, A.P.; Köster, W. Iron-uptake systems of chicken-associated *Salmonella* serovars and their role in colonizing the avian host. *Microorganisms (Basel)* **2020**, *8*, 1203, doi:10.3390/microorganisms8081203.

21. Cosby, D.E.; Cox, N.A.; Harrison, M.A.; Wilson, J.L.; Buhr, R.J.; Fedorka-Cray, P.J. *Salmonella* and antimicrobial resistance in broilers: A review. *Journal of Applied Poultry Research* **2015**, *24*, 408-426, doi:10.3382/japr/pfv038.

22. Huang, T.; Gao, B.; Chen, W.-L.; Xiang, R.; Yuan, M.-G.; Xu, Z.-H.; Peng, X.-Y. Temporal effects of high fishmeal diet on gut microbiota and immune response in *Clostridium perfringens*-challenged chickens. *Front. Microbiol.* **2018**, *9*, 2754-2754, doi:10.3389/fmicb.2018.02754.

6 Appendix 1: Supplemental tables for Chapter 2

Table A1- 1: p-values of metabolites found to be significantly altered in kidneys of birds administered 10 mg L^{-1} corticosterone (CORT) and 30 mg L^{-1} CORT as compared to ethanol alone as determined by a paired t-test and/or the variable importance analysis based on random variable combination analysis

Treatment	Metabolite	Chemical shift (ppm)	T-test p-value	VIAVC p-value	Regulation
10 mg CORT	S-Adenosylhomocysteine 1	4.45	0.0019		13.90
	Fumaric Acid	6.53	0.002	4.450E-60	23.13
	Nicotinate/Nicotinic acid	8.27	0.0039		28.74
	Phenylalanine 1	3.11	0.0057		10.30
	sn-Glycero-3-phosphocholine 1	3.88	0.0073		12.84
	Proline 1	3.36	0.0078		16.50
	Lactate 1	1.32	0.0078		-31.41
	Malate 1	4.30	0.0115		11.85
	Glutamate 1	2.15	0.0117		-15.04
	Glutamate 2	2.11	0.0117		-7.59
	Lactate 2	1.35	0.0117		-23.43
	Malate 2	4.30	0.0129		21.82
	N-Acetylaspartate 1	4.38	0.0137		20.79
	UNIDENTIFIED 1	3.87	0.015		11.11
	AMP 1	6.15	0.0178		38.70
	3-Methyladenine 1 and S-Adenosylhomocysteine 2	8.28	0.0193		22.84
	UDP-N-Acetylglucosamine	6.00	0.0195		19.19
	Lactate 3	4.13	0.0195		-20.31
	Lactate 4	4.12	0.0195		-18.84
	Glutamate 3	2.12	0.0195		-9.05
	Isoleucine 1	0.96	0.0195		-10.19
	Isoleucine 2	0.95	0.0195		-11.45
	sn-Glycero-3-phosphocholine 2	3.70	0.0213		16.11
	Inosine 1	8.37	0.022	2.055E-07	45.32
	N6-Acetyl-L-lysine	3.20	0.0228		15.06
	N-Acetylaspartate 2	4.39	0.0248		14.45
	O-Phosphocholine	3.59	0.0254		7.67
	1,3-Dimethyluric Acid	3.45	0.0254		16.00
	Citicoline 1	4.29	0.0255		20.24
	Glucose 1	3.84	0.0269		18.88
	Choline 1	4.07	0.0273		13.16

4-Hydroxyproline 1	3.48	0.0281		13.43
Xylulose	4.37	0.0282		21.54
AMP 2 and Carnosine 1	4.50	0.0287		26.57
S-Adenosylhomocysteine 3	2.99	0.0305		8.48
Glutamate 4	2.06	0.0309		-6.13
AMP 3 and Carnosine 2	4.51	0.0311		29.46
S-Adenosylhomocysteine 4	8.38	0.0314		41.10
UMP and Citicoline 2	4.42	0.0322		20.47
Proline 2 and 1,5-Anhydrosorbitol 1	3.36	0.0323		11.98
UNIDENTIFIED 2	4.24	0.0325		9.62
Uridine 1	4.22	0.034		13.19
sn-Glycero-3-phosphocholine 3	3.69	0.0347		14.16
sn-Glycero-3-phosphocholine 4	3.89	0.0348		13.36
(R)-3-Hydroxybutyric acid	1.21	0.0351		16.83
AMP 4	8.60	0.0354	1.494E-16	31.76
Glutamate 5	2.13	0.0354		-10.01
Uridine 2	4.23	0.0362		19.41
Agmatine and Carnosine 3	3.07	0.039		7.09
Glutamate 6	2.34	0.0391		-11.71
Glutamate 7	2.07	0.0391		-8.36
Proline 3 and Glucose 2	3.39	0.0394		14.34
Choline 2	4.06	0.0401		18.50
Citicoline 3	4.28	0.0408		18.04
Citicoline 4	4.33	0.0411		14.94
3-Mercaptopyruvic acid	2.87	0.0413		21.55
3-Methyladenine 2	3.98	0.0424		9.33
1,5-Anhydrosorbitol 2	3.44	0.0438		8.65
Inosine 2	6.10	0.0443	7.267E-09	45.66
Phenylalanine 2	3.12	0.0457		4.85
Inosinic Acid	8.57	0.047		44.15
Pyruvate	2.39	0.047		10.26
N-Acetylaspartate 3 and Citicoline 5	4.41	0.0492		27.92
Malate 3	2.66	0.0514	7.272E-47	12.97
Inosine 3	6.11	0.0568	1.078E-19	51.44
Malate 4	2.38	0.0615	1.668E-44	6.35
UNIDENTIFIED 3	7.57	0.2198	1.749E-04	31.36
N-Methylhydantoin	2.93	0.3445	6.001E-34	10.16
Aspartate	2.81	0.4076	1.320E-23	4.55
4-Hydroxyproline 2 and Isovalerylglycine	2.17	0.5372	1.172E-42	2.20

	Carnitine	2.45	0.6014	5.047E-38	-3.14
30 mg CORT	Acetic acid	1.93	0.0004		-23.13
	4-Hydroxyproline 1	3.48	0.0009		39.50
	UNIDENTIFIED 1	1.78	0.0018		-18.88
	Glutamate 1	2.13	0.0033		-20.46
	1,5-Anhydrosorbitol	3.44	0.0036		33.31
	Fumaric Acid	6.53	0.0036		29.42
	Glucose-6-phosphate	3.99	0.0039		-19.09
	Glutamate 2	2.15	0.0039		-32.83
	Glucose 1	3.84	0.0052		34.01
	L-Lysine	3.75	0.0053		15.20
	UNIDENTIFIED 2	4.00	0.007		-20.85
	Choline 1	3.53	0.0078		17.54
	Glutamate 3	2.33	0.0116		-14.21
	Glutamate 4	2.12	0.0117		-17.53
	UNIDENTIFIED 3	3.98	0.0117		-14.13
	UNIDENTIFIED 4	0.97	0.0117		-18.44
	Glutamate 5	2.06	0.0118		-13.58
	Choline 2	3.52	0.0195		29.51
	Glutamate 6	2.11	0.0195		-13.66
	Phosphorylcholine 1	4.17	0.0207		-19.51
	Glucose 2	3.71	0.0229		24.33
	UNIDENTIFIED 5	3.27	0.023		-35.03
	Serine	4.01	0.0246		-15.08
	UNIDENTIFIED 6	2.71	0.026		-15.43
	Isoleucine 1	0.95	0.0273		-25.52
	Malate	4.31	0.0273		12.99
	Dimethylamine and Sarcosine	2.74	0.0291		-10.96
	Beta-Alanine	3.18	0.0308		-12.60
	Glutamate 7	2.05	0.032		-17.57
	sn-Glycero-3-phosphocholine 2 and Isoleucine 2	3.69	0.0339		7.90
	Argininosuccinic acid	1.92	0.0391		-7.81
	Glycerol 1	3.67	0.0391		9.35
	Glycine	3.57	0.0391		-25.30
	Isoleucine 3	0.94	0.0391		-16.92
	Pyroglutamic Acid 1	2.42	0.0391	4.908E-15	-24.88
	sn-Glycero-3-phosphocholine 1	3.69	0.0391		20.40
	Glucose 3	3.49	0.0412		22.87
	Creatine	3.94	0.0422		-11.89
	D-Glucuronic acid	3.75	0.0423		18.74
	Malate	4.30	0.0443		15.87

	Creatinine and Creatine Phosphate	3.04	0.0445		-23.67
	Phosphorylcholine 2	3.60	0.0471		-10.37
	Glycerol 2	3.65	0.0491		8.66
	Choline 3	4.07	0.0663	2.548E-09	21.64
	Myoinositol	3.28	0.0813	1.064E-21	-19.12
	UNIDENTIFIED 7	4.20	0.7344	4.809E-10	0.31
	N-Methylhydantoin	2.93	0.748	1.860E-17	10.99
	Pyroglutamic Acid 2 and 3-Phenylpropionate	2.49	0.8203	2.616E-21	-2.85
	UNIDENTIFIED 8	2.43	0.8867	4.816E-13	-1.03
	4-Hydroxyproline 2 and Isovalerylglycine	2.17	0.9102	1.221E-11	-5.71

Table A1- 2: p-values of metabolites found to be significantly altered in livers of birds administered 10 mg L^{-1} corticosterone (CORT) and 30 mg L^{-1} CORT as compared to ethanol alone as determined by a paired t-test and/or the variable importance analysis based on random variable combination analysis

Treatment	Metabolite	Chemical shift (ppm)	T-test p-value	VIAVC p-value	Regulation
10 mg CORT	UNIDENTIFIED 1	8.02	0.0004		-36.78
	UNIDENTIFIED 2	3.17	0.0005		-24.67
	Mannose	5.20	0.0011		46.89
	4-Hydroxyproline 1	3.37	0.0019	0.00166	-19.53
	Inosine 1	4.45	0.002		-24.91
	Tryptophan 1	7.55	0.0021		-36.39
	Coenzyme A	0.76	0.0028		-27.10
	UNIDENTIFIED 3	4.46	0.003		-27.16
	UMP 1	4.42	0.0031		-26.20
	Phenylalanine 1	7.35	0.0033		-37.46
	Quinone	6.81	0.0036		-41.61
	Tyrosine 1	6.92	0.0037		-43.42
	UNIDENTIFIED 4	4.41	0.0037		-25.20
	Inosine 2	4.45	0.0038		-20.24
	UNIDENTIFIED 5	0.87	0.0038		-32.91
	(R)-3-Hydroxybutyric Acid 1	1.22	0.0039		-45.42
	Glycerophosphocholine 1	3.69	0.0039		28.82
	N-Carbamoylaspartate 1	2.47	0.0039		-35.46
	N-Methylhydantoin	4.10	0.0039		-22.30
	UNIDENTIFIED 6	2.48	0.0039		-32.42
	UNIDENTIFIED 7	2.32	0.0039		-27.16
	UNIDENTIFIED 8	1.19	0.0039		-26.02
	Valine 1	2.30	0.0039		-33.74
	L-Threonine 1	4.28	0.0046		-23.04
	Tryptophan 2	7.21	0.0046		-34.30
	Myoinositol 1	3.63	0.0047		-31.65
	Phenylalanine 2	7.33	0.0049		-35.37
	Valine 2	2.29	0.0051		-27.60
	O-Phosphocholine 1	3.59	0.0054		17.05
	Phenylalanine 3	7.45	0.0055		-31.08
	Tyrosine 2	7.19	0.0055		-36.15
	Tryptophan 3	7.75	0.0057		-29.20
	Myoinositol 2	4.08	0.0062		-37.47
	Gamma-Glutamylcysteine 1	2.19	0.0063		-16.40
	Tyrosine 3	7.20	0.0065		-37.41

UNIDENTIFIED 9	3.00	0.0065	-16.43
Uridine 1	7.88	0.0071	-35.83
UNIDENTIFIED 10	4.46	0.0072	-26.05
Isoleucine 1	0.94	0.0073	-28.97
Tryptophan 4	7.29	0.0073	-34.32
Isoleucine 2	0.93	0.0074	-29.89
Beta-Alanine 1	3.17	0.0075	-24.23
Gamma-Glutamylcysteine 2	2.58	0.0075	-14.88
Flavin Mononucleotide	7.81	0.0076	-36.70
(R)-3-Hydroxybutyric Acid 2	2.42	0.0078	-26.88
(R)-3-Hydroxybutyric Acid 3	1.20	0.0078	-52.53
Glycerophosphocholine 2	3.69	0.0078	28.34
Isoleucine 3	1.26	0.0078	-20.34
N-Acetylglutamate 1	2.23	0.0078	-20.19
Phenylalanine 4	3.16	0.0078	-19.48
Tyrosine 4	3.08	0.0078	-18.98
Tyrosine 5	3.07	0.0078	-20.91
UNIDENTIFIED 11	4.49	0.0078	-23.03
UNIDENTIFIED 12	2.32	0.0078	-22.57
UNIDENTIFIED 13	2.31	0.0078	-28.06
Proline 1	3.34	0.008	18.45
Gluconate	4.13	0.0081	21.36
Phenylalanine 5	7.44	0.0083	-32.78
Phenylalanine 6	7.37	0.0083	-28.40
4-Hydroxyproline 2	2.16	0.0086	-17.81
Inosine 3	4.44	0.0087	-21.19
Allantoin	5.40	0.0089	-30.92
UNIDENTIFIED 14	0.95	0.009	-30.19
Glutamate 1	2.11	0.0091	-25.80
UMP 2	4.43	0.0092	-24.52
1-Methyladenosine	6.09	0.0094	-14.62
UNIDENTIFIED 15	4.25	0.0094	-22.29
Norspermidine 1	1.69	0.0095	-27.66
O-Phosphocholine 2	3.24	0.0095	27.37
Xanthine 1	7.93	0.0095	-30.11
Gamma-Glutamylcysteine 3	2.17	0.0097	-16.27
Myoinositol 3	3.54	0.0098	-8.45
Glucose 1	3.42	0.01	11.83
Phenylalanine 7	7.38	0.0101	-30.68
UNIDENTIFIED 16	3.36	0.0101	12.00
Myoinositol 4	3.64	0.0104	-22.60
Tyrosine 6	6.91	0.0104	-33.50
UNIDENTIFIED 17	4.40	0.0105	-19.78

UNIDENTIFIED 18	2.15	0.0107	-22.45
UNIDENTIFIED 19	5.99	0.0109	18.45
Valine 3	2.28	0.0113	-26.72
Pantothenic acid 1	0.92	0.0114	-22.00
Inosine 4	6.08	0.0115	-22.16
Glucose 2	3.89	0.0117	12.65
L-Threonine 2	4.27	0.0117	-20.98
Niacinamide 1	7.60	0.0117	-19.54
Tyrosine 7	3.09	0.0117	-16.70
UNIDENTIFIED 20	4.47	0.0117	-25.14
Uridine 2	7.89	0.0117	-34.37
Uridine 3	5.93	0.0117	-37.72
Uridine 4	5.92	0.0117	-37.22
Xanthine 2	7.91	0.0118	-18.70
Tryptophan 5	7.73	0.0121	-32.14
Myoinositol 10	3.30	0.0122	-29.32
L-Threonine 3	4.25	0.013	-20.82
Betaine and Trimethylamine N-Oxide	3.27	0.0131	-32.81
UNIDENTIFIED 21	0.91	0.0136	-27.43
Ribose	5.40	0.0138	-22.85
UNIDENTIFIED 22	7.57	0.0139	-23.37
UNIDENTIFIED 23	0.98	0.0139	-27.71
Myoinositol 5	3.56	0.0141	-9.29
UNIDENTIFIED 24	7.92	0.0143	-18.63
UNIDENTIFIED 25	1.70	0.0143	-27.47
Norspermidine 2	1.68	0.0145	-22.91
Gamma-Glutamylcysteine 4	2.59	0.0146	-15.30
Methionine	2.14	0.0146	-28.84
Glucose 3	3.43	0.0147	12.70
Citric Acid 1	2.53	0.0149	-15.65
Glutamate 2	2.07	0.0149	-18.61
Beta-Alanine 2 and Isocitrate 1 and Argininosuccinic acid 1	2.56	0.015	-12.19
Uridine 5	5.91	0.015	-31.84
Valine 4	2.28	0.015	-26.66
Myoinositol 6	3.56	0.0152	-9.56
Proline 2	3.35	0.0152	17.50
Myoinositol 7	3.31	0.0153	-27.01
Gamma-Glutamylcysteine 5	2.99	0.0154	-12.54
UNIDENTIFIED 26	2.49	0.0154	-18.18
Myoinositol 8	3.61	0.0159	-22.07
UNIDENTIFIED 27	1.42	0.0161	-21.41
(R)-3-Hydroxybutyric Acid 4	2.43	0.0163	-28.10

	UNIDENTIFIED 28	0.96	0.0163		-29.47
	Glucose 4	3.39	0.0164		11.93
	UNIDENTIFIED 29	4.57	0.0164		-30.89
	1-Methyladenine	8.31	0.0166		-15.59
	Dimethylamine	2.50	0.017		-16.02
	Pantothenic acid 2	0.89	0.0176		-25.76
	Phenylalanine 8	7.42	0.0177		-26.17
	Phenylalanine 9	7.40	0.0178		-25.88
	7-Methyladenine 1	8.11	0.018		-24.15
	Inosine triphosphate	8.50	0.0181		-18.01
	N-Carbamoylaspartate 2	2.46	0.0181		-37.36
	UNIDENTIFIED 30	1.64	0.0182		-17.96
	UDP-N-Acetylglucosamine 1	5.98	0.0186		18.87
	Valine 5	2.27	0.0186		-24.88
	Valine 6	2.27	0.0191		-24.55
	Gamma-Glutamylcysteine 6	2.22	0.0195		-16.39
	Gamma-Glutamylcysteine 7	2.21	0.0195		-15.16
	Gamma-Glutamylcysteine 8	2.20	0.0195		-15.18
	Glucose 5	3.71	0.0195	0.00312	17.22
	Isoleucine 4	1.26	0.0195		-15.96
	N-Acetylglutamate 2	2.24	0.0195		-19.70
	Niacinamide 2	7.62	0.0195		-20.02
	Nicotinurate 1	7.61	0.0195		-16.33
	Tryptophan 6	7.28	0.0195		-28.97
	Tryptophan 7	7.30	0.0195		-30.56
	Tyrosine 8	3.06	0.0195		-11.85
	UDP-N-Acetylglucosamine 2	5.98	0.0195		22.09
	UNIDENTIFIED 31	4.56	0.0195		-28.46
	UNIDENTIFIED 32	4.49	0.0195		-20.11
	UNIDENTIFIED 33	4.48	0.0195		-22.41
	UNIDENTIFIED 34	1.18	0.0195		-18.12
	Isoleucine 5	1.02	0.0196		-26.51
	Fucose	4.55	0.0203		-21.26
	Citric Acid 2	2.57	0.0204		-12.16
	Argininosuccinic acid 2	2.54	0.0205		-12.79
	Selenomethionine 1	2.64	0.0207		-26.55
	Adenosine Monophosphate 1	8.60	0.021	0.00275	41.94
	Proline 3	3.35	0.0211		19.15
	UNIDENTIFIED 35	4.14	0.0217		15.45
	Adenosine Monophosphate 2 and Cytidine Monophosphate 1	6.15	0.0221		34.84
	Selenomethionine 2	2.65	0.0226		-26.70
	Nicotinurate 2	8.72	0.0234		-14.76

	L-Threonine 4	4.26	0.0238	-18.65
	Norspermidine 3	1.67	0.024	-18.87
	Glycerophosphocholine 3	3.68	0.0247	18.22
	UNIDENTIFIED 36	1.63	0.0261	-15.65
	UNIDENTIFIED 37	1.15	0.0261	-18.95
	Adenine	8.22	0.0264	-23.43
	UMP 3	8.09	0.0264	-49.31
	Tryptophan 8	7.56	0.0265	-23.45
	UNIDENTIFIED 38	1.43	0.0266	-19.37
	Thiamine pyrophosphate	8.05	0.027	-16.90
	Fructose	4.03	0.0273	15.22
	Glucose 10	3.53	0.0273	6.84
	Glucose 11	3.45	0.0273	13.62
	Glucose 12	3.80	0.0273	8.44
	Glucose 6	5.24	0.0273	21.35
	Glucose 7	3.86	0.0273	10.57
	Glucose 8	3.84	0.0273	13.22
	Glucose 9	3.83	0.0273	12.71
	Nicotinurate 3	8.95	0.0273	-15.33
	Nicotinurate 4	8.71	0.0273	-14.90
	S-Adenosylhomocysteine 1	8.39	0.0273	-26.52
	Uridine 6	4.36	0.0273	-25.18
	Valine 7	2.26	0.0275	-21.66
	Valine 8	0.99	0.0282	-23.44
	Inosine 5 and Hypoxanthine	8.23	0.0302	-23.67
	Uridine 7	5.94	0.0304	-29.41
	Argininosuccinic acid 3 and Isocitrate 2	2.52	0.0312	-12.97
	S-Adenosylhomocysteine 1 and Inosine 5 and Adenosine	8.36	0.0323	-9.50
	Isocitrate 3	2.51	0.0327	-13.26
	Myoinositol 9	3.55	0.0336	-8.70
	Methionine Sulfoxide	2.77	0.034	-20.13
	UNIDENTIFIED 39	1.66	0.0347	-17.11
	7-Methyladenine 2	8.19	0.0358	-13.83
	Adenosine Monophosphate 3 and Cytidine Monophosphate 2	6.14	0.0365	29.75
	Isoleucine 6	1.01	0.0366	-24.80
	Valine 9	1.04	0.037	-23.31
	Imidazole	8.32	0.0373	6.19
	UNIDENTIFIED 40	3.85	0.0374	10.16
	Glucose 13	3.78	0.0385	3.73
	Glutamate 3	2.13	0.0389	-21.32

	(R)-3-Hydroxybutyric Acid 5	2.39	0.0391		-22.09
	Beta-Alanine 3	3.18	0.0391		-16.47
	Glucose 14	3.84	0.0391		14.28
	Glucose 15	3.76	0.0391		11.64
	Glucose 16	3.73	0.0391		13.64
	Glucose 17	3.72	0.0391		15.32
	Glycogen	5.41	0.0391		45.07
	L-Threonine 5	4.25	0.0391		-17.25
	N-Acetylglutamate 3	2.25	0.0391		-19.22
	Nicotinurate 5	8.73	0.0391		-15.74
	Nicotinurate 6	7.62	0.0391		-15.76
	Phenylalanine 10	3.15	0.0391		-20.66
	Phenylalanine 11	3.14	0.0391		-17.66
	Tyrosine 9 and Beta-Alanine 4	3.19	0.0391		-16.04
	UNIDENTIFIED 41	4.52	0.0391		-16.81
	Uridine 8	4.24	0.0391		-21.65
	Uridine 9	4.23	0.0391		-18.24
	UNIDENTIFIED 42	0.90	0.0394		-22.91
	Pi-Methylhistidine	7.16	0.0397		-24.13
	UNIDENTIFIED 43	2.42	0.0422		-25.33
	Methylmalonate 1	1.24	0.0427		-16.16
	Methylmalonate 2	1.23	0.0432		-15.63
	UDP-N-Acetylglucosamine 3	7.95	0.0434		8.70
	UNIDENTIFIED 44	3.62	0.0437		-13.62
	Valine 10	1.05	0.045		-23.13
	UNIDENTIFIED 45	1.65	0.0451		-16.78
	Anserine	7.15	0.0454		-20.47
	Gamma-Glutamylcysteine 9	2.98	0.0461		-10.03
	Glutamate 4	2.10	0.0472		-16.12
	UNIDENTIFIED 46	1.65	0.0481		-15.62
	Glycerol	3.81	0.0488		5.69
	3-Methyladenine	8.29	0.0492		20.31
	Thiamine	8.06	0.0493		-17.94
	3-Mercaptopyruvic Acid	2.86	0.0547	0.00208	11.19
	Threonate	4.02	0.2451	0.00039	6.81
30 mg CORT	Betaine and Trimethylamine N-Oxide	3.274811	0.00001	1.40E-03	-55.02
	Coenzyme A	0.762045	0.00001		-43.08
	Glycerophosphocholine 1	3.69339025	0.00001		48.05
	Mannose	5.19833	0.00001		75.00
	UNIDENTIFIED 1	3.8724865	0.00001	9.85E-04	26.44
	UNIDENTIFIED 2	3.6352585	0.00001		21.92
	UNIDENTIFIED 3	3.16603875	0.00001		-36.08

Glycerophosphocholine 2 and Maltose	3.67813075	0.0001		39.49
Glycerophosphocholine 3	3.68501725	0.0002	7.60E-04	50.20
Quinone	6.81344075	0.0002		-55.24
1-Methylguanosine	8.01996375	0.0003		-35.27
Glycerophosphocholine 4	3.2250335	0.0004		42.15
Inosine 1	4.451543	0.0004		-27.68
Tryptophan 1	7.55075	0.0004		-38.80
O-Phosphocholine 1	3.5931865	0.0005		31.26
Nicotinurate 1	8.253198	0.0008		-22.60
Tryptophan 2	7.733	0.0009		-26.08
Allantoin 1	5.3965	0.0011		-33.81
Tryptophan 3	7.28869675	0.0012		-32.83
Tryptophan 4	7.214888	0.0012		-33.75
Valine 1	2.289244	0.0012		-27.33
Phenylalanine 1	7.447597	0.0013		-36.26
Nicotinurate 2	8.7221	0.0015		-26.86
Tyrosine 1	7.1892	0.0015		-36.82
Myoinositol 1	4.0815	0.0016		-34.35
O-Phosphocholine 2	3.236193	0.0016		45.15
4-Hydroxyproline	3.3730335	0.0019		-25.92
UNIDENTIFIED 4	4.248287	0.002		-21.32
Beta-Alanine 1	3.17114475	0.0022		-30.05
UNIDENTIFIED 5	3.95	0.0022		26.25
Glucose 1	3.7765	0.0023		4.89
UNIDENTIFIED 6	3.848027	0.0023		10.55
Gluconate	4.127	0.0024		29.28
Tyrosine 2	7.204088	0.0024		-35.70
Valine 2	2.2769985	0.0024		-24.99
Isoleucine 1	0.9422585	0.0027		-33.68
Histamine 1	7.93006425	0.0028		-44.51
Inosine 2	4.4453885	0.0028		-22.06
Inosine 3	6.08357225	0.0029		-31.71
Tryptophan 5	7.277904	0.003		-27.31
7-Methyladenine 1	8.1092455	0.0031		-26.10
Inosine triphosphate	8.5015	0.0031		-24.98
Myoinositol 2	3.6284505	0.0031		-24.92
Myoinositol 3	3.312	0.0032		-24.39
Tryptophan 6	7.56175	0.0033		-27.83
UDP-N-Acetylglucosamine 1	5.97683275	0.0034		41.80
Valine 3	2.281673	0.0034		-25.11
Tyrosine 3	6.9070865	0.0038		-36.27
(R)-3-Hydroxybutyric Acid 1	1.2171995	0.0039		-35.05

Glucose 2	3.8908375	0.0039	11.25
Glucose 3	3.85905	0.0039	12.93
Glucose 4	3.830115	0.0039	11.62
Glucose 5	3.70511225	0.0039	15.95
Glucose 6 and Glycerophosphocholine 5	3.2476595	0.0039	20.84
Glycogen 1	5.436698	0.0039	41.55
Glycogen 2	5.4231095	0.0039	58.92
Glycogen 3	5.4149115	0.0039	51.66
NADP+	9.303	0.0039	40.69
Nicotinurate 3	8.9535	0.0039	-23.92
Nicotinurate 4	8.7258	0.0039	-27.84
Nicotinurate 5	8.71475	0.0039	-25.78
Nicotinurate 6	7.624	0.0039	-24.39
Nicotinurate 7	7.615	0.0039	-25.20
Nicotinurate 8	7.609	0.0039	-25.09
Nicotinurate 9	7.601	0.0039	-27.14
Phenylalanine 2	7.43567725	0.0039	-42.46
Phenylalanine 3	7.3454015	0.0039	-49.18
Phenylalanine 4	7.3313645	0.0039	-49.59
Phenylalanine 5	3.1581545	0.0039	-26.59
Phenylalanine 6	3.1452235	0.0039	-26.75
Phenylalanine 7	3.136225	0.0039	-24.29
S-Adenosylhomocysteine	8.3945	0.0039	-23.91
Selenomethionine 1	2.63583075	0.0039	-34.74
Tyrosine 4	6.9235865	0.0039	-35.95
Tyrosine 5	3.079	0.0039	-20.60
Tyrosine 6	3.07036725	0.0039	-22.32
UNIDENTIFIED 7	3.181	0.0039	-22.75
UNIDENTIFIED 8	3.1744625	0.0039	-20.60
UNIDENTIFIED 9	3.1517965	0.0039	-26.52
UNIDENTIFIED 10	2.3206385	0.0039	-24.36
UNIDENTIFIED 11	2.3084785	0.0039	-26.53
Valine 4	2.2992225	0.0039	-31.43
UNIDENTIFIED 12	0.95341	0.004	-34.29
Tryptophan 7	7.7455	0.0041	-22.86
Nicotinurate 10	8.27271725	0.0042	-16.74
Threonine 1	4.2810135	0.0043	-20.05
Valine 5	2.26676675	0.0044	-24.26
Phosphocreatine	3.958	0.0045	25.44
Isoleucine 2	0.9329915	0.0048	-31.49
O-Phosphocholine 3	3.606269	0.0048	16.80
UNIDENTIFIED 13	4.4564145	0.0051	-27.19

UNIDENTIFIED 14	3.967	0.0059	21.58
Uridine 1	7.8785	0.0066	-29.06
Myoinositol 4	3.641821	0.0067	-20.15
UNIDENTIFIED 15	0.964489	0.007	-33.19
Myoinositol 5	3.6147985	0.0072	-15.87
(R)-3-Hydroxybutyric Acid 2	2.426871	0.0077	-26.07
Beta-Alanine 2	3.1915	0.0078	-21.22
Glucose 7	5.2418	0.0078	13.95
Glucose 8	3.843958	0.0078	10.86
Glucose 9	3.837573	0.0078	10.78
Glutamate 1	2.107325	0.0078	-31.24
Glutamate 2	2.06925	0.0078	-23.25
Glycine	3.5715	0.0078	-15.30
Nicotinurate 11	8.73345	0.0078	-20.45
Norspermidine 1	1.68882425	0.0078	-27.78
Phenylalanine 8	7.41981125	0.0078	-35.88
Phenylalanine 9	7.3996215	0.0078	-36.29
Phenylalanine 10	7.3839405	0.0078	-42.74
Phenylalanine 11	7.37366075	0.0078	-34.63
Isoleucine 3	1.02094425	0.0079	-28.43
UNIDENTIFIED 16	0.976129	0.008	-30.51
Isoleucine 4	1.00844425	0.0084	-28.38
Valine 6	2.271912	0.0086	-20.44
UNIDENTIFIED 17	2.3156385	0.0088	-22.14
Flavin Mononucleotide	7.8095	0.0089	-35.37
Glucose 10	3.430133	0.0091	9.80
Inosine 4 and Hypoxanthine	8.2305	0.0097	-37.39
Lactate 1	4.1205	0.0099	26.44
Acetylcholine	2.142	0.0102	-30.99
Adenosine monophosphate 1	8.598	0.0108	45.52
Uridine 2	5.9215235	0.011	-30.78
Glutamate 3	2.1007245	0.0113	-20.24
Ribose	5.402	0.0113	-24.18
(R)-3-Hydroxybutyric Acid 3	2.41629	0.0117	-24.98
(R)-3-Hydroxybutyric Acid 4	1.2005	0.0117	-39.53
Gamma-Glutamylcysteine 1	2.21658675	0.0117	-16.64
Gamma-Glutamylcysteine 2	2.19575175	0.0117	-14.98
Glucose 11	3.797585	0.0117	8.74
Glucose 12	3.7555	0.0117	8.17
Selenomethionine 2	2.647295	0.0117	-33.69
Tryptophan 8	7.29929275	0.0118	-28.82
UNIDENTIFIED 18	0.8680155	0.0118	-29.99
Glycerophosphocholine 6	4.3325	0.0121	39.67

Valine 7	0.9887275	0.0121	-25.56
Adenosine	8.1415	0.0122	29.65
Adenine	8.2165	0.0128	-33.95
Glycerol 1	3.8075	0.0141	9.76
Myoinositol 6	3.557273	0.0146	-11.58
Norspermidine 2	1.67632325	0.0153	-23.13
UMP 1	4.42397	0.0154	-26.08
Uridine 3	5.9120235	0.0155	-24.23
Threonine 2	4.27273825	0.0161	-17.57
UNIDENTIFIED 19	4.4624675	0.0161	-24.73
Myoinositol 7	3.541	0.0164	-11.55
Inosine 5	4.4383065	0.0165	-18.87
UDP-glucose	5.989744	0.0169	32.96
Pi-Methylhistidine	7.1644	0.017	-23.31
Glucose 13	3.4151865	0.018	11.87
Uridine 4	5.9298575	0.0183	-29.03
Methionine Sulfoxide	2.7659685	0.0184	-23.30
UNIDENTIFIED 20	7.9168785	0.0187	-20.02
Valine 8	1.037221	0.0187	-25.19
Glycerol 2	3.81235	0.0188	8.99
Threonine 3	4.254254	0.0191	-17.16
Gamma-Glutamylcysteine 3	2.2056115	0.0195	-14.49
Glucose 14	3.446852	0.0195	9.57
N-Acetylglutamate 1	2.228325	0.0195	-17.97
N-Methylhydantoin 1	4.099	0.0195	-20.63
UDP-N-Acetylglucosamine 2	5.96753825	0.0195	32.60
UNIDENTIFIED 21	7.573	0.0195	-18.25
UNIDENTIFIED 22	4.48502425	0.0195	-23.62
UNIDENTIFIED 23	4.47825525	0.0195	-22.29
UNIDENTIFIED 24	4.36566	0.0195	-19.62
UNIDENTIFIED 25	1.702597	0.0195	-28.60
Uridine 5	4.3613245	0.0195	-24.69
Uridine 6	4.2407	0.0195	-19.58
Valine 9	0.9993275	0.0212	-22.16
UNIDENTIFIED 26	4.41	0.0218	-22.82
Valine 10	2.25968475	0.022	-17.86
Adenosine monophosphate 2	6.152	0.0224	43.18
Lactate 2	4.1421505	0.0227	11.07
Valine 11	1.051221	0.0236	-25.81
UNIDENTIFIED 27	4.404	0.0251	-19.36
Myoinositol 8	3.2992265	0.0255	-21.20
Allantoin 2	5.39	0.0256	-20.86
7-Methyladenine 2	8.18565	0.0257	-13.35

Choline	4.0672235	0.0261	-17.16
Myoinositol 9	3.5485	0.0264	-10.81
(R)-3-Hydroxybutyric Acid 5	2.4213375	0.0273	-26.68
Glucose 15	3.74596475	0.0273	7.27
Tyrosine 7	3.0902355	0.0273	-15.01
UNIDENTIFIED 28	3.9405	0.0273	23.54
Uridine 7	4.23414275	0.0273	-15.68
Adenosine Triphosphate	8.547	0.0295	39.03
Lactate and Gluconate	4.1321445	0.0299	18.66
Threonine 4	4.26354675	0.0307	-16.72
Glucose 16	3.7865425	0.0325	5.65
Imidazole	8.32305	0.0342	14.44
N-Methylhydantoin 2	2.929548	0.0358	-23.91
Pantothenic acid	0.9227405	0.0366	-20.80
Myoinositol 10	3.563	0.0372	-9.69
Sucrose	4.0632235	0.0381	-13.70
O-Phosphocholine 4	3.5996425	0.0388	14.67
Glucose 17	3.7282915	0.0391	8.49
Glucose 18	3.718924	0.0391	8.99
Glucose 19	3.462855	0.0391	8.62
Glucose 20	4.6792105	0.0391	7.88
N-Acetylglutamate 2	2.23953475	0.0391	-15.84
N-Carbamoylaspartate	2.47906925	0.0391	-27.81
Tyrosine 8	3.2005	0.0391	-16.18
UDP-N-Acetylglucosamine 3	5.9837385	0.0391	35.01
UNIDENTIFIED 29	4.470371	0.0391	-23.05
UMP 2 and Cytidine Monophosphate	8.09032775	0.0405	-49.99
Carnosine and Histamine 2	7.149295	0.0414	-22.55
Norspermidine 3	1.66812625	0.0417	-18.63
Glucose 21	3.39272	0.042	6.38
UNIDENTIFIED 30	0.911374	0.0429	-22.58
UMP 3	4.430931	0.0433	-20.99
Proline	3.336	0.0437	15.76
Glucose 22	4.6503985	0.0445	8.27
7-Methyladenine 3	4.0585	0.045	-12.64
UDP-N-Acetylglucosamine 4	7.9454045	0.0467	22.49
Glutamate 4	2.059403	0.0486	-15.62
Uridine 8	7.8935	0.0492	-21.80

Table A1- 3: p-values of metabolites found to be significantly altered in breast muscle of birds administered 10 mg L^{-1} corticosterone (CORT) and 30 mg L^{-1} CORT as compared to ethanol alone as determined by a paired t-test and/or the variable importance analysis based on random variable combination analysis

Treatment	Metabolite	Chemical shift (ppm)	T-test p-value	VIAVC p-value	Regulation
10 mg CORT	Glucose 1	3.90	0.001		22.54
	Glucose 2	3.84	0.001	1.61E-07	19.00
	Glycine and Glycerol 1	3.57	0.0022		28.44
	UNIDENTIFIED 1	8.26	0.0023		-69.84
	Glucose 3	3.73	0.0026		19.55
	Glucose 4	3.75	0.0042		17.89
	Glucose-6-phosphate 1 and Nicotinurate	4.00	0.0055		33.76
	Glucose-6-phosphate 2	4.02	0.0061		12.99
	Inosine 1 and Glucose-6-Phosphate 3	3.89	0.0064		43.44
	Glucose 5 and Glucose-6-Phosphate 4	3.52	0.0076		48.08
	Inosine 2 and Glucose-6-Phosphate 5	3.87	0.008		38.83
	Glucose 6 and Myoinositol 1	3.53	0.0098		53.29
	Glucose 7 and Glucose-6-Phosphate 6	5.26	0.0103		43.54
	Glucose 8 and Glucose-6-Phosphate 7 and Myoinositol 2	3.55	0.0124		38.38
	Glucose 9 and Glucose-6-Phosphate 8	3.51	0.0128		28.27
	UNIDENTIFIED 2	4.01	0.0136		21.87
	Glucose 10	3.72	0.0178		19.16
	Glucose 11 and Carnitine	3.43	0.0195		15.09
	UNIDENTIFIED 3	4.01	0.0203		33.85
	Glucose 12 and Glycerol 2	3.56	0.0204		27.44
	1,5-Anhydrosorbitol 1	3.98	0.022		30.14
	Creatine phosphate	3.03	0.0254		-16.49
	UNIDENTIFIED 4	3.96	0.0273		57.96
	1,5-Anhydrosorbitol 2	3.97	0.0283		29.97
	UNIDENTIFIED 5	3.82	0.0286		24.57
	Glucose-6-phosphate 9	3.29	0.0356		32.09
	Creatinine	4.07	0.0377		13.26
	Glucose-6-phosphate 10	5.25	0.039		28.39
	Anserine 1	2.71	0.04		-14.48
	Betaine	3.90	0.0443		18.91

	UNIDENTIFIED 6	3.86	0.0447		17.93
	N-Methylhydantoin	2.93	0.1061	1.02E-32	-25.42
	UNIDENTIFIED 7	8.57	0.1289	4.80E-07	11.44
	Glucose 13	3.71	0.2629	5.48E-08	16.68
	Glucose 14	3.83	0.3878	9.75E-20	15.37
	Glucose 15	3.69	0.4453	8.14E-21	11.71
	UNIDENTIFIED 8	3.06	0.6912	1.18E-13	2.26
	S-Adenosylhomocysteine 1	2.17	0.6916	4.16E-11	-6.19
	Anserine 2	2.67	0.8203	7.23E-13	-1.26
	S-Adenosylhomocysteine 2	2.19	0.8461	1.33E-14	-2.61
30 mg CORT	Glucose 1	3.43	0.00001		43.97
	Glucose 2 and Glucose-6-Phosphate 1	5.26	0.00001		77.26
	Glucose 3 and Glucose-6-Phosphate 2	3.51	0.00001		56.99
	Glucose 4 and Glucose-6-Phosphate 3 and Myoinositol 1	3.55	0.00001		72.65
	Glucose 5 and Glycerol 1	3.56	0.00001		55.13
	Glucose-6-phosphate 4	3.72	0.00001		41.01
	Histamine 1	3.03	0.00001		-28.33
	Inosine 1 and Glucose-6-Phosphate 1	3.89	0.00001		76.84
	Glucose-6-phosphate 2	4.02	0.0001		19.55
	Histamine 2	3.02	0.0001		-47.40
	Glucose-6-phosphate 3	3.29	0.0003		54.51
	Glucose 6	3.41	0.0004		29.60
	Glucose 7 and Glucose-6-Phosphate 4	5.25	0.0004		57.35
	Glucose-6-phosphate 5	4.01	0.0005		36.30
	Glucose 8	3.82	0.0006		34.76
	Glucose 9 and Glycerol 2	3.56	0.0006		42.46
	Glutathione 1	3.00	0.0006		-69.36
	Anserine 1	3.07	0.0007		-29.23
	Glucose 10	3.26	0.0007		23.34
	Anserine 2	3.09	0.001		-41.91
	Glucose 11 and Betaine 1	3.91	0.0012		33.48
	Citric acid	2.64	0.0016		-44.84
	1,5-Anhydrosorbitol 1	3.98	0.0017		45.55
	Acetic acid and Acetylphosphate	1.93	0.0022		-43.83
	Carnosine 1	8.08	0.0023		-78.86
	N-Methylhydantoin	2.93	0.0023		-61.90
	Proline 1	2.35	0.0023		-39.43
	1,5-Anhydrosorbitol 2	3.97	0.0027		42.68
	Proline 2	2.34	0.003		-44.44

UNIDENTIFIED 2	4.01	0.003	52.87
Glucose 12	3.90	0.0039	46.98
Glucose 13	3.85	0.0039	22.86
Glucose 14	3.84	0.0039	36.36
Glucose 15	3.75	0.0039	38.47
Glucose 16 and Glucose-6-Phosphate 6	3.52	0.0039	89.14
Glucose 17 and Myoinositol 2	3.53	0.0039	89.57
Glucose-6-phosphate 7	3.73	0.0039	56.38
Glucose-6-phosphate 8 and Nicotinurate 1	4.00	0.0039	62.74
Glycine and Glycerol 3	3.57	0.0039	61.33
Inosine 2 and Glucose-6-Phosphate 9	3.87	0.0039	59.35
Carnitine 1	2.44	0.0041	-54.11
Methylmalonate 1	1.24	0.0042	-43.16
Carnitine 2	2.43	0.0044	-62.29
Acetylphosphate and S-Adenosylhomocysteine 1 and Glutamine 1	2.12	0.0046	-31.69
Carnitine 3	2.47	0.0046	-55.08
Carnitine 4	2.46	0.0047	-57.82
Methylmalonate 2	1.25	0.0047	-41.57
ATP 1, ADP 1, AMP 1, IMP 1	6.13	0.0048	-80.51
Glutamine 2	2.16	0.0048	-68.52
Niacinamide 1	8.95	0.0054	-59.53
Glutamine 3	2.16	0.0056	-65.38
Argininosuccinic Acid 1 and Glycylproline 1 and Ornithine 1	1.94	0.006	-95.85
Glucose 18	3.25	0.0062	17.83
Lactate	4.15	0.0062	11.92
Anserine 3 and Carnosine 1	2.66	0.0068	-8.73
Acetylcholine	2.15	0.0072	-32.75
Niacinamide 2	8.72	0.0072	-82.11
Tryptophan 1	7.73	0.0072	-103.36
Niacinamide 3	7.61	0.0074	-93.38
Uridine 1	5.91	0.0074	-118.62
GTP or GDP 1 and Uridine 2	5.92	0.0075	-118.27
Proline 3 and Malic acid	2.36	0.0075	-44.50
Niacinamide 4	7.61	0.0076	-94.71
Niacinamide 5	7.60	0.0076	-93.65
UNIDENTIFED 3	6.02	0.0077	-96.88
Adenine 1	8.22	0.0078	-87.13
AMP 2 and ATP 2	8.24	0.0078	-80.34

GTP or GDP 2 and Uridine 3	5.93	0.0078	-116.02
Histamine 3	7.09	0.0078	-88.48
Niacinamide 6	8.73	0.0078	-74.97
S-Adenosylhomocysteine 2	4.46	0.0078	-47.05
Tryptophan 2	7.56	0.0078	-109.84
Tyrosine 1	7.21	0.0078	-76.81
UNIDENTIFED 4	2.99	0.0078	-82.69
UNIDENTIFIED 5	8.27	0.0081	-43.24
Niacinamide 7	7.62	0.0084	-90.78
Tryptophan 3	7.74	0.0088	-105.59
4-Pyridoxic Acid	7.55	0.0091	-109.17
Hypoxanthine	8.17	0.0091	-66.64
Phenylalanine 1	7.45	0.0091	-97.35
Aspartic Acid 1	2.82	0.0095	-84.27
1,5-Anhydrosorbitol 3	3.99	0.01	35.49
UNIDENTIFED 6	7.88	0.0101	-112.06
AMP 3	8.60	0.0102	-94.48
Betaine 2	3.90	0.0102	24.28
S-Adenosylhomocysteine 3 and Proline 4	2.07	0.0102	-40.28
N-Acetylmannosamine 1 and S-Adenosylhomocysteine 4 and Proline 5	2.06	0.0103	-48.99
Nicotinurate 2	4.00	0.0103	35.59
S-Adenosylhomocysteine 5 and Proline 6	2.09	0.0105	-74.65
Tryptophan 4	7.57	0.0105	-114.96
UNIDENTIFED 7	7.49	0.0105	-108.97
UNIDENTIFIED 8	7.49	0.0105	-109.20
S-Adenosylhomocysteine 6	6.10	0.0106	-39.68
UNIDENTIFED 9	8.26	0.0107	-46.65
UNIDENTIFED 10	6.34	0.0107	-115.62
UNIDENTIFIED 11	8.02	0.0112	-110.98
Carnosine 2	8.18	0.0113	-71.52
1-Methyladenine	8.28	0.0117	-46.50
Argininosuccinic Acid 2 and Glycylproline 2 and Ornithine 2	1.95	0.0117	-86.80
Argininosuccinic Acid 3 and Glycylproline 3 and Ornithine 3	1.94	0.0117	-89.24
Argininosuccinic Acid 4 and Glycylproline 4 and Ornithine 4	1.92	0.0117	-72.04
Carnitine 5	2.48	0.0117	-62.88
Dimethylglycine	2.94	0.0117	-49.62

Glutamine 4 and S-Adenosylhomocysteine 7	2.14	0.0117	-63.42
Glutamine 5 and S-Adenosylhomocysteine 8	2.13	0.0117	-63.40
Glutathione 2	3.00	0.0117	-77.46
Glutathione 3	2.99	0.0117	-84.29
Glutathione 4	2.98	0.0117	-75.23
Glutathione 5	2.95	0.0117	-76.94
Guanidinoacetate	3.80	0.0117	20.29
IMP 2	8.23	0.0117	-87.69
Malonate	3.14	0.0117	-33.54
Ornithine 5	1.91	0.0117	-70.12
Phenylalanine 1	3.16	0.0117	-46.52
1-Methylhistidine 1	7.01	0.0117	-108.70
S-Adenosylhomocysteine 9	2.17	0.0117	-56.16
Tyrosine 2	7.19	0.0117	-73.90
Tyrosine 3	6.92	0.0117	-84.36
Tyrosine 4	6.91	0.0117	-87.34
UMP 1	6.01	0.0117	-111.73
UMP 2	5.99	0.0117	-97.27
UMP 3	5.98	0.0117	-104.68
τ-Methylhistidine 1	3.17	0.0117	-51.86
Glucose-6-phosphate 10	4.03	0.0118	14.69
UNIDENTIFED 12	2.21	0.0119	-75.81
UNIDENTIFED 13	6.03	0.0119	-89.81
Proline 7 and Glycylproline 1	2.04	0.0121	-57.53
UNIDENTIFIED 14	7.89	0.0122	-113.08
S-Adenosylhomocysteine 10	4.45	0.0124	-32.49
UNIDENTIFED 15	7.37	0.0124	-104.61
1-Methylhistidine 2	7.70	0.0125	-110.84
Aspartic Acid 2	2.82	0.0125	-78.08
Aspartic Acid 3	2.80	0.0125	-66.09
UNIDENTIFED 16	6.10	0.0125	-81.15
UNIDENTIFIED 17	7.27	0.0126	-77.51
S-Adenosylhomocysteine 11	6.11	0.0131	-45.45
Valine 1	2.26	0.0135	-90.65
Phenylalanine 2	7.33	0.0137	-77.21
Riboflavin 1	7.38	0.0138	-92.38
Valine 2	2.27	0.0138	-85.03
UNIDENTIFED 18	4.67	0.0139	29.68
Beta-Alanine 1	3.17	0.0141	-54.48
UNIDENTIFED 19	8.83	0.0145	-78.94

UNIDENTIFIED 20	2.25	0.0145	-72.22
Phenylalanine 3	7.35	0.0146	-70.65
Aspartic Acid 4	2.79	0.0147	-62.02
Carnosine 3	7.15	0.015	-39.52
Valine 3	2.27	0.015	-86.26
Valine 1	2.26	0.0151	-87.22
UNIDENTIFED 21	8.82	0.0152	-80.15
UNIDENTIFED 22	2.20	0.0153	-69.84
Valine 4	2.28	0.0153	-88.46
Riboflavin 2	7.39	0.0154	-95.96
Valine 5	2.28	0.0154	-84.55
O-Phosphocholine	3.24	0.0156	-14.97
1-Methyladenosine 1	8.42	0.0158	-57.07
Imidazole 1	7.23	0.0162	-100.91
UNIDENTIFIED 23	3.86	0.0175	25.13
Valine 2	2.29	0.0176	-71.13
Glycylproline 2 and Proline 8	1.97	0.0184	-55.63
Glycylproline 3 and Proline 9	2.01	0.0189	-56.17
Glycylproline 4 and Proline 10	2.03	0.0191	-49.00
Glycylproline 5 and Proline 11	2.02	0.0192	-46.46
Mannose-6-Phosphate	5.21	0.0194	82.74
1-Methyladenosine 2	8.15	0.0195	-74.27
Adenine 2	8.19	0.0195	-93.21
ADP 2	8.53	0.0195	-99.53
Alpha-ketoisovaleric acid 1	1.15	0.0195	-87.04
Alpha-ketoisovaleric acid 2	1.14	0.0195	-86.74
Argininosuccinic Acid 5	1.89	0.0195	-67.89
Argininosuccinic Acid 6 and Glycylproline 6	1.95	0.0195	-74.71
Argininosuccinic Acid 7 and Glycylproline 7 and Ornithine 6	1.93	0.0195	-83.89
Argininosuccinic Acid 8 and Ornithine 7	1.90	0.0195	-75.22
ATP 3	8.53	0.0195	-84.34
Carnitine 6	2.42	0.0195	-62.19
Ethanol 1	1.19	0.0195	-96.84
Ethanol 2	1.18	0.0195	-96.35
Ethylmalonic acid	0.92	0.0195	-88.73
Fumaric Acid	6.53	0.0195	-105.01
Glutamine 6	2.15	0.0195	-72.33
Glutathione 6	2.96	0.0195	-81.40
Glutathione 7	2.94	0.0195	-87.36

Glycylproline 8 and Proline 12	1.96	0.0195	-65.06
GMP 1	5.95	0.0195	-109.02
GMP 2	5.94	0.0195	-112.16
Imidazole 2	7.29	0.0195	-89.19
Isoleucine	0.93	0.0195	-46.58
Pantothenic Acid	0.89	0.0195	-83.48
Phenylalanine 4	7.44	0.0195	-71.40
Phenylalanine 5	7.42	0.0195	-79.43
Proline 13	3.36	0.0195	-59.94
Proline 14	2.33	0.0195	-58.53
S-Adenosylhomocysteine 12 and Proline 15	2.10	0.0195	-74.59
Trimethylamine	2.90	0.0195	-102.38
UMP 4	8.11	0.0195	-97.56
UMP 5	8.10	0.0195	-101.07
UNIDENTIFED 1	1.90	0.0195	-84.43
UNIDENTIFED 24	7.50	0.0195	-108.72
UNIDENTIFED 25	5.63	0.0195	-133.76
UNIDENTIFIED 26	5.62	0.0195	-128.54
UNIDENTIFED 27	2.91	0.0195	-86.08
UNIDENTIFED 28	2.89	0.0195	-93.94
UNIDENTIFIED 29	2.54	0.0195	-92.98
UNIDENTIFED 30	8.21	0.0195	-101.25
UNIDENTIFED 31	7.48	0.0195	-106.98
UNIDENTIFIED 32	5.62	0.0195	-128.41
UNIDENTIFED 33	5.61	0.0195	-131.71
UNIDENTIFED 34	2.91	0.0195	-89.52
UNIDENTIFIED 35	2.88	0.0195	-91.59
UNIDENTIFED 36	2.24	0.0195	-67.57
τ-Methylhistidine 2	6.99	0.0195	-83.84
N-Acetylgalactosamine 2 and S-Adenosylhomocysteine 13 and Proline 16	2.05	0.02	-27.57
Proline 17 and Malic acid	2.37	0.0208	-40.15
Glucose 19 and Glucose-6-phosphate 11	3.50	0.0217	20.82
Glycylproline 9 and Proline 18	2.00	0.0229	-55.26
Anserine 4	2.71	0.023	-16.05
Inosine 3	4.28	0.0257	-49.17
UNIDENTIFED 37	9.34	0.0258	-62.58
Inosine 4	4.44	0.0264	-39.88
Inosine 5	4.29	0.0264	-36.25

Beta-Alanine 2 and N-Nitrosodimethylamine and Anserine 5 and Carnosine 4	3.19	0.0266	-9.64
S-Adenosylhomocysteine 14 and Proline 19	2.08	0.0267	-37.03
1-Methyladenosine 3	8.46	0.0273	-62.84
3-Hydroxyisobutyrate	1.08	0.0273	-85.52
Adenosine Phosphosulfate	8.51	0.0273	-75.78
Alanine 1	3.77	0.0273	-16.96
Methionine	2.19	0.0273	-45.02
Phenylalanine 2	3.15	0.0273	-45.74
UNIDENTIFIED 38	7.17	0.0273	-50.92
Glycylproline 10 and Proline 20	1.98	0.0285	-46.57
Purine	9.11	0.0311	-57.96
Glycylproline 11 and Proline 21	1.99	0.0316	-46.28
S-Adenosylhomocysteine 15	8.37	0.0324	-24.85
UNIDENTIFED 39	4.27	0.0335	-48.15
sn-Glycero-3-phosphocholine 1	3.64	0.0365	-52.67
UNIDENTIFED 40	1.52	0.0373	-59.10
Inosine 6	4.28	0.039	-32.39
3-Hydroxybutyric acid 1	1.21	0.0391	-84.07
3-Hydroxybutyric acid 2 and Ethanol 3	1.20	0.0391	-78.79
Lactic acid 1	1.43	0.0391	-28.25
Lactic acid 2	1.42	0.0391	-30.27
UNIDENTIFIED 41	3.96	0.0391	44.06
UNIDENTIFED 42	2.23	0.0391	-77.23
UNIDENTIFED 43	2.81	0.0391	-65.80
Inosine 7	4.30	0.0395	-40.02
Beta-Alanine 3	2.56	0.0397	-61.35
Threonine	3.60	0.04	28.46
sn-Glycero-3-phosphocholine 2	3.68	0.0426	-60.89
Beta-Alanine 4	2.58	0.0448	-65.34
Alanine 2	1.48	0.0455	-18.07
Carnosine 5 and Beta-Alanine 5	3.18	0.0481	-14.24
Glucose 1-Phosphate 1	5.47	0.1641	-94.16
Glucose 1-Phosphate 2	5.46	0.1641	-89.79
UNIDENTIFIED 44	8.57	0.8203	6.69

7 Appendix 2: Supplemental tables for Chapter 3

Table A2- 1: Average concentrations (mM) of all metabolites identified in rat feces that were processed by ultrafiltration, no extraction without baseline correction, Bligh-Dyer (BD) extraction without baseline correction, no extraction with baseline correction, and BD with baseline correction. Metabolites were identified and quantified using Chenomx. The numbers identified in presented in brackets correspond to the standard deviation and percent occurrence of each metabolite, respectively

Compound Name	No extraction Average	Bligh-Dyer Average	Ultra-filtration Average	No extraction BC Average	Bligh-Dyer BC Average
Sebacic acid	0.078 (0.041/100)	0.039 (0.011/90)	0.080 (0.033/100)	0.058 (0.029/75)	0.036 (0.014/80)
Nervonic acid	2.734 (0.989/100)	0.713 (0.025/20)	1.681 (0.391/70)	3.007 (0.582/100)	1.386 (0.565/90)
5-Hydroxy-L-tryptophan	0.100 (0.006/50)	0.042 (0.018/80)	0.083 (0.034/100)	0.017 (0.000/25)	0.032 (0.008/70)
Methyl isobutyl ketone	1.837 (0.645/100)	0.445 (0.117/70)	1.171 (0.639/100)	1.013 (0.462/100)	0.350 (0.139/50)
Erythrose	5.015 (2.183/50)	0.539 (0.179/20)	2.611 (1.437/40)	2.772 (0.000/25)	1.051 (0.330/30)
p-Cresol	0.086 (0.005/50)	0.015 (0.008/40)	0.054 (0.013/60)	0.041 (0.008/100)	0.012 (0.001/20)
Urocanic acid	0.058 (0.025/100)	0.019 (0.005/70)	0.049 (0.020/90)	0.026 (0.013/75)	0.012 (0.003/60)
m-Coumaric acid	0.055 (0.000/25)	0.015 (0.003/70)	0.035 (0.009/60)	0.014 (0.002/50)	0.012 (0.004/80)
Acetoin	0.169 (0.036/75)	0.040 (0.006/40)	0.183 (0.053/30)	0.158 (0.046/75)	0.042 (0.006/30)
Acetylglycine	2.917 (0.342/100)	0.777 (0.276/100)	1.661 (0.542/100)	1.393 (0.000/25)	0.543 (0.173/90)
Homovanillic acid	0.078 (0.044/50)	0.013 (0.006/80)	0.028 (0.021/40)	0.033 (0.013/75)	0.012 (0.004/100)
Sucrose	0.039 (0.033/50)	0.010 (0.005/30)	0.041 (0.014/90)	0.030 (0.037/100)	0.028 (0.013/90)
Deoxycytidine	0.016 (0.000/25)	0.012 (0.002/70)	0.018 (0.006/80)	0.015 (0.000/25)	0.013 (0.002/40)
L-Valine	0.101 (0.034/100)	0.033 (0.012/100)	0.138 (0.061/90)	0.070 (0.034/100)	0.031 (0.012/100)
L-Leucine	0.123 (0.000/25)	0.062 (0.000/10)	0.096 (0.025/90)	0.110 (0.017/50)	0.041 (0.000/10)
N-Acetyl-L-methionine	0.037 (0.010/100)	0.013 (0.006/70)	0.035 (0.006/60)	0.031 (0.003/100)	0.029 (0.058/100)
N-Acetyl-L-aspartic acid	0.230 (0.169/75)	0.119 (0.021/20)	0.179 (0.107/30)	0.079 (0.037/100)	0.202 (0.461/100)
4,5-Dihydroorotic acid	0.028 (0.015/50)	0.062 (0.000/10)	0.154 (0.149/30)	0.059 (0.035/50)	0.022 (0.012/20)
Normetanephrine	0.004 (0.000/25)	0.005 (0.000/20)	0.007 (0.000/10)	0.006 (0.002/75)	0.004 (0.001/50)

Thymine	0.050 (0.000/25)	0.028 (0.010/100)	0.075 (0.013/60)	0.048 (0.001/50)	0.019 (0.005/90)
Phenylacetic acid	0.112 (0.010/50)	0.036 (0.012/80)	0.101 (0.019/80)	0.063 (0.014/75)	0.032 (0.008/90)
Propionic acid	2.543 (0.247/100)	1.005 (0.177/100)	1.793 (0.436/100)	2.544 (0.246/100)	1.039 (0.159/90)
1,3-Dimethyluric acid	0.042 (0.010/100)	0.009 (0.004/80)	0.022 (0.009/100)	0.008 (0.005/100)	0.002 (0.001/80)
Undecanedioic acid	0.054 (0.030/100)	0.028 (0.009/100)	0.055 (0.023/100)	0.042 (0.031/100)	0.020 (0.014/80)
L-Phenylalanine	0.115 (0.044/100)	0.030 (0.009/70)	0.113 (0.046/100)	0.110 (0.056/100)	0.033 (0.008/70)
p-Hydroxyphenylacetic acid	0.056 (0.000/25)	0.014 (0.007/90)	0.035 (0.011/60)	0.018 (0.007/75)	0.020 (0.012/80)
Acetic acid	11.815 (1.319/100)	4.602 (0.774/100)	8.367 (1.864/100)	11.802 (1.312/100)	4.652 (0.798/90)
3,4-Dihydroxybenzeneacetic acid	0.025 (0.007/75)	0.034 (0.013/30)	0.088 (0.035/70)	0.031 (0.013/100)	0.033 (0.014/60)
Ethanol	0.521 (0.445/75)	0.194 (0.100/90)	0.565 (0.306/100)	0.531 (0.433/75)	0.189 (0.094/90)
Pyridoxal	0.022 (0.010/50)	0.003 (0.003/80)	0.015 (0.002/20)	0.002 (0.000/25)	0.002 (0.000/80)
Histamine	0.052 (0.000/25)	0.006 (0.000/10)	0.022 (0.000/10)	0.016 (0.003/100)	0.011 (0.002/30)
Indoxyl sulfate	1.167 (0.503/100)	0.400 (0.144/80)	0.676 (0.240/100)	0.261 (0.000/25)	0.231 (0.062/90)
3-Cresotinic acid	0.208 (0.087/100)	0.113 (0.058/70)	0.108 (0.016/40)	0.116 (0.005/50)	0.093 (0.037/80)
p-Cresol sulfate	0.010 (0.002/100)	0.004 (0.001/90)	0.007 (0.002/100)	0.006 (0.002/100)	0.008 (0.011/90)
L-Glutamic acid	0.428 (0.172/75)	0.169 (0.050/70)	0.347 (0.073/30)	0.307 (0.042/50)	0.098 (0.025/30)
Uracil	0.226 (0.000/25)	0.248 (0.058/100)	0.470 (0.205/90)	0.212 (0.057/75)	0.243 (0.066/100)
Methylimidazoleacetic acid	0.752 (0.425/100)	0.200 (0.109/100)	0.350 (0.180/100)	0.079 (0.038/100)	0.126 (0.070/90)
D-Xylulose	4.564 (1.992/100)	2.447 (0.778/20)	7.173 (4.898/100)	4.342 (2.146/100)	2.520 (1.140/60)
Betaine	0.134 (0.066/50)	0.049 (0.015/20)	0.132 (0.049/60)	0.053 (0.016/100)	0.017 (0.009/80)
Galacturonic acid	1.147 (0.310/100)	0.167 (0.057/100)	1.000 (0.251/100)	1.381 (0.319/100)	0.300 (0.043/90)
D-Galactose	0.140 (0.037/50)	0.023 (0.016/50)	0.105 (0.014/60)	0.219 (0.067/100)	0.151 (0.000/10)
D-Maltose	0.186 (0.147/75)	0.050 (0.035/50)	0.085 (0.061/80)	0.188 (0.171/50)	0.079 (0.028/70)
Phenol	0.034 (0.000/25)	0.088 (0.030/90)	0.092 (0.016/30)	0.096 (0.047/100)	0.077 (0.020/100)
5-Thymidylic acid	0.083 (0.000/25)	0.020 (0.005/80)	0.067 (0.013/70)	0.034 (0.007/75)	0.021 (0.007/90)
Allantoin	0.037 (0.015/100)	0.011 (0.004/60)	0.034 (0.011/100)	0.049 (0.013/100)	0.038 (0.013/60)
Serotonin	0.026 (0.000/25)	0.031 (0.013/90)	0.054 (0.022/70)	0.020 (0.002/50)	0.018 (0.008/80)

L-Aspartic acid	0.169 (0.068/75)	- (-/70)	0.152 (0.081/90)	0.105 (0.030/100)	0.020 (0.011/30)
N-Acetylneuraminic acid	1.129 (0.188/100)	0.333 (0.106/100)	0.887 (0.210/100)	0.117 (0.053/100)	0.112 (0.059/100)
FAD	2.311 (0.614/100)	0.409 (0.214/90)	1.082 (0.514/90)	0.180 (0.000/25)	0.284 (0.057/90)
D-Xylose	0.121 (0.024/50)	0.036 (0.013/30)	0.081 (0.026/60)	0.070 (0.047/100)	0.018 (0.005/70)
5-Hydroxyindoleacetic acid	0.080 (0.043/50)	0.015 (0.010/80)	0.046 (0.017/90)	0.014 (0.002/75)	0.010 (0.008/90)
Thiamine	0.028 (0.017/75)	0.004 (0.003/70)	0.011 (0.005/70)	0.004 (0.002/75)	0.003 (0.001/60)
Raffinose	0.634 (0.122/75)	0.121 (0.055/80)	0.239 (0.262/70)	0.378 (0.231/100)	0.285 (0.211/90)
L-Histidine	0.111 (0.028/50)	0.027 (0.009/80)	0.077 (0.005/30)	0.013 (0.002/50)	0.016 (0.008/40)
Malic acid	0.751 (0.254/100)	0.132 (0.078/100)	0.413 (0.166/100)	0.142 (0.074/100)	0.442 (1.226/100)
Homoveratric acid	0.147 (0.051/100)	0.026 (0.014/50)	0.086 (0.013/80)	0.022 (0.001/50)	0.131 (0.313/100)
7-Methylxanthine	0.747 (0.137/75)	0.236 (0.078/90)	1.155 (0.971/90)	0.922 (0.775/100)	0.227 (0.115/90)
Glycerophosphocholine	0.191 (0.000/25)	0.039 (0.000/10)	0.161 (0.046/60)	0.157 (0.065/100)	0.013 (0.005/70)
Erythritol	0.554 (0.326/75)	0.221 (0.054/40)	0.583 (0.178/100)	0.264 (0.054/100)	0.151 (0.069/30)
Butyric acid	3.848 (1.948/100)	1.324 (0.467/100)	2.551 (1.217/100)	3.734 (2.067/100)	1.305 (0.457/90)
4-Pyridoxic acid	0.033 (0.005/75)	0.007 (0.001/100)	0.022 (0.012/100)	0.016 (0.007/100)	0.007 (0.001/90)
1-Methylhistidine	0.029 (0.000/25)	0.023 (0.014/100)	0.056 (0.017/100)	0.013 (0.002/100)	0.019 (0.007/100)
Mevalonolactone	0.027 (0.000/25)	0.010 (0.002/50)	0.021 (0.004/60)	0.019 (0.007/100)	0.012 (0.016/100)
Levoglucosan	0.118 (0.019/50)	0.018 (0.007/50)	0.092 (0.002/20)	0.157 (0.030/100)	0.074 (0.022/50)
3-Hydroxymethylglutaric acid	0.166 (0.027/75)	0.068 (0.024/90)	0.094 (0.058/90)	0.132 (0.022/100)	0.035 (0.023/90)
3-Methylxanthine	5.395 (1.241/75)	1.411 (0.457/80)	5.416 (0.907/70)	2.046 (1.035/100)	0.691 (0.397/80)
3-Methylhistidine	0.140 (0.047/75)	0.025 (0.017/90)	0.061 (0.023/20)	0.011 (0.005/100)	0.013 (0.012/100)
2-Heptanone	1.176 (0.243/75)	0.313 (0.092/80)	0.583 (0.255/60)	0.084 (0.000/25)	0.131 (0.026/30)
Sumiki's acid	0.036 (0.018/75)	0.004 (0.002/80)	0.012 (0.013/80)	0.004 (0.001/50)	0.004 (0.002/50)
Flavin Mononucleotide	2.821 (0.000/25)	0.659 (0.335/90)	1.637 (0.411/60)	0.378 (0.143/75)	0.294 (0.113/100)
1-Methylnicotinamide	0.004 (0.001/75)	0.002 (0.000/80)	0.003 (0.001/80)	0.003 (0.000/25)	0.002 (0.000/90)
L-Cystine	0.111 (0.000/25)	0.078 (0.043/40)	0.213 (0.177/20)	0.069 (0.026/50)	0.018 (0.000/10)
N-Formyl-L-methionine	0.320 (0.116/75)	0.045 (0.014/50)	0.213 (0.069/100)	0.108 (0.067/100)	0.048 (0.012/70)
3-Hydroxymandelic acid	0.448 (0.015/50)	0.086 (0.033/80)	0.372 (0.077/90)	0.334 (0.058/100)	0.097 (0.047/70)

Capric acid	0.504 (0.079/50)	0.301 (0.113/60)	0.448 (0.149/40)	0.294 (0.000/25)	0.301 (0.124/20)
L-Threonine	0.155 (0.046/75)	0.041 (0.000/10)	0.133 (0.040/100)	0.253 (0.000/50)	0.048 (0.042/30)
Beta-Alanine	0.094 (0.023/50)	0.036 (0.020/80)	0.070 (0.017/50)	0.070 (0.000/25)	0.024 (0.011/30)
Alpha-D-Glucose	6.985 (2.362/75)	0.460 (0.288/50)	0.576 (0.638/90)	2.502 (0.997/75)	0.719 (0.405/70)
Desaminotyrosine	0.014 (0.000/25)	0.004 (0.002/20)	0.019 (0.006/70)	0.014 (0.000/25)	0.004 (0.001/50)
Xanthine	0.325 (0.184/75)	0.124 (0.040/100)	0.176 (0.126/100)	0.279 (0.140/100)	0.153 (0.065/90)
Indoleacetic acid	0.021 (0.019/75)	0.012 (0.010/80)	0.022 (0.024/60)	0.009 (0.000/25)	0.006 (0.001/30)
Glycine	0.179 (0.043/50)	0.019 (0.008/90)	0.124 (0.056/90)	0.159 (0.047/75)	0.023 (0.013/90)
3-Hydroxyisovaleric acid	0.072 (0.059/50)	0.074 (0.019/90)	0.156 (0.041/80)	0.157 (0.050/75)	0.038 (0.026/90)
Putrescine	0.067 (0.000/50)	0.039 (0.000/10)	0.097 (0.000/10)	0.027 (0.000/25)	0.014 (0.007/60)
Succinic acid	0.214 (0.115/100)	0.154 (0.076/100)	0.199 (0.083/100)	0.211 (0.117/100)	0.165 (0.072/90)
Riboflavin	0.441 (0.090/100)	0.113 (0.058/100)	0.329 (0.132/100)	0.227 (0.062/100)	0.074 (0.030/90)
N-Acetyl-L-alanine	0.055 (0.000/25)	0.032 (0.015/40)	0.054 (0.013/20)	0.024 (0.000/25)	0.013 (0.021/70)
N-Acetylserotonin	0.049 (0.005/75)	0.016 (0.011/60)	0.049 (0.009/80)	0.006 (0.000/25)	0.007 (0.006/80)
Pantothenic acid	0.106 (0.069/100)	0.073 (0.039/100)	0.091 (0.053/100)	0.076 (0.038/100)	0.062 (0.019/100)
m-Cresol	0.050 (0.000/25)	0.017 (0.012/40)	0.046 (0.008/50)	0.044 (0.001/50)	0.008 (0.002/70)
Valeric acid	3.050 (0.000/25)	1.560 (0.206/40)	4.654 (2.348/40)	1.741 (0.000/25)	0.448 (0.438/20)
Glyceraldehyde	0.227 (0.000/25)	0.159 (0.032/90)	0.177 (0.026/60)	0.226 (0.000/25)	0.157 (0.044/80)
Gamma-Butyrolactone	0.055 (0.000/25)	0.015 (0.002/30)	0.033 (0.000/10)	0.028 (0.006/50)	0.020 (0.009/20)
Cyclic AMP	0.013 (0.000/25)	0.004 (0.001/60)	0.013 (0.003/50)	0.012 (0.004/75)	0.004 (0.000/10)
N-Acetylmannosamine	1.348 (0.000/25)	0.142 (0.155/90)	0.427 (0.297/60)	0.940 (0.178/50)	0.075 (0.069/80)
Argininosuccinic acid	1.099 (0.181/50)	0.343 (0.141/70)	0.979 (0.191/60)	0.351 (0.189/100)	0.236 (0.063/90)
Citramalic acid	1.462 (0.000/25)	0.907 (0.318/70)	0.810 (0.321/50)	0.248 (0.231/100)	0.181 (0.069/90)
Ureidosuccinic acid	1.304 (0.616/100)	0.183 (0.096/40)	2.014 (0.368/60)	0.473 (0.217/75)	0.142 (0.107/20)
2-Furoylglycine	0.887 (0.572/75)	0.123 (0.040/60)	0.403 (0.241/40)	0.142 (0.056/75)	0.107 (0.028/50)
Isovalerylglycine	0.181 (0.053/50)	0.038 (0.010/60)	0.118 (0.041/80)	0.116 (0.011/75)	0.035 (0.009/60)
Xanthosine	0.011 (0.000/25)	0.011 (0.005/50)	0.031 (0.017/70)	0.033 (0.018/75)	0.014 (0.011/60)
Hydrocinnamic acid	0.091 (0.016/100)	0.035 (0.011/90)	0.074 (0.013/90)	0.076 (0.017/100)	0.037 (0.010/90)

Gulonic acid	3.278 (1.476/50)	0.857 (0.232/60)	2.693 (0.426/40)	2.162 (0.400/50)	0.755 (0.118/90)
Biotin	2.040 (0.679/100)	0.594 (0.304/100)	0.927 (0.465/100)	0.184 (0.062/50)	0.129 (0.069/90)
5-Methylcytidine	0.046 (0.000/25)	0.051 (0.021/70)	0.095 (0.027/20)	0.035 (0.007/100)	0.033 (0.012/90)
3-Hydroxyphenylacetic acid	0.099 (0.030/100)	0.024 (0.010/70)	0.045 (0.021/90)	0.053 (0.001/50)	0.024 (0.007/80)
L-3-Phenyllactic acid	0.057 (0.014/50)	0.011 (0.007/60)	0.040 (0.006/30)	0.037 (0.000/25)	0.018 (0.015/60)
1-Methyladenosine	0.158 (0.077/75)	0.015 (0.005/70)	0.058 (0.037/80)	0.020 (0.003/75)	0.037 (0.022/80)
4-Hydroxyproline	0.130 (0.070/100)	0.033 (0.016/90)	0.100 (0.031/100)	0.020 (0.000/25)	0.016 (0.005/80)
Picolinic acid	0.059 (0.035/50)	0.015 (0.004/40)	0.035 (0.025/40)	0.019 (0.000/25)	0.016 (0.005/20)
L-Alanine	0.338 (0.152/75)	0.159 (0.054/100)	0.261 (0.088/100)	0.340 (0.134/100)	0.118 (0.033/90)
L-Arabinose	0.220 (0.064/100)	0.050 (0.011/50)	0.164 (0.028/90)	0.663 (0.132/50)	0.147 (0.053/50)
Formic acid	0.028 (0.001/50)	0.088 (0.027/80)	0.034 (0.017/100)	0.026 (0.004/75)	0.091 (0.026/70)
Mevalonic acid	0.220 (0.058/50)	0.053 (0.018/20)	0.121 (0.021/30)	0.129 (0.045/75)	0.036 (0.017/90)
Indolelactic acid	0.016 (0.002/75)	0.022 (0.014/70)	0.026 (0.022/90)	0.010 (0.000/25)	0.020 (0.008/40)
Methylsuccinic acid	0.063 (0.020/50)	0.007 (0.000/20)	0.043 (0.019/50)	0.040 (0.000/25)	0.009 (0.009/50)
Glucose 6-phosphate	0.264 (0.166/75)	0.094 (0.030/80)	0.163 (0.128/100)	0.081 (0.016/75)	0.054 (0.008/50)
4-Methylcatechol	0.131 (0.000/25)	0.012 (0.014/90)	0.047 (0.023/90)	0.031 (0.004/100)	0.009 (0.006/70)
Dimethylsulfide	2.028 (0.451/75)	0.233 (0.050/30)	1.324 (0.426/100)	0.666 (0.241/100)	0.207 (0.020/60)
Nicotinic acid	0.399 (0.157/100)	0.160 (0.027/80)	0.331 (0.090/100)	0.402 (0.153/100)	0.164 (0.026/70)
Gluconic acid	0.986 (0.311/75)	0.167 (0.070/100)	0.669 (0.312/100)	0.888 (0.000/25)	0.155 (0.059/90)
N-Butyrylglycine	0.243 (0.016/50)	0.040 (0.000/10)	0.257 (0.061/90)	0.067 (0.000/25)	0.048 (0.000/10)
Theophylline	0.006 (0.000/25)	0.005 (0.003/30)	0.021 (0.009/40)	0.003 (0.001/100)	0.196 (0.432/60)
Gabapentin	0.248 (0.172/100)	0.019 (0.000/10)	0.158 (0.071/70)	0.036 (0.023/100)	0.025 (0.007/50)
L-Lactic acid	0.151 (0.052/50)	0.040 (0.019/30)	0.105 (0.027/40)	0.137 (0.000/25)	0.093 (0.031/20)
Hydroxyacetone	0.151 (0.083/100)	0.034 (0.018/90)	0.094 (0.030/80)	0.173 (0.058/75)	0.060 (0.021/90)
2-Isopropylmalic acid	4.792 (2.549/50)	1.785 (1.351/60)	4.081 (1.655/90)	0.396 (0.097/100)	0.357 (0.243/100)
N-Phenylacetylphenylalanine	0.031 (0.010/50)	0.026 (0.008/90)	0.052 (0.000/10)	0.016 (0.005/50)	0.016 (0.003/60)
Hydroxyphenyllactic acid	0.287 (0.056/75)	0.079 (0.023/70)	0.182 (0.032/70)	0.172 (0.000/25)	0.072 (0.028/80)

Cytidine monophosphate	0.016 (0.003/75)	0.012 (0.002/50)	0.021 (0.004/80)	0.022 (0.008/50)	0.014 (0.004/60)
N-Acetyl-L-tyrosine	0.594 (0.191/50)	0.171 (0.055/70)	0.369 (0.089/60)	0.104 (0.000/25)	0.124 (0.042/80)
Butanone	0.196 (0.028/75)	0.037 (0.020/60)	0.064 (0.044/70)	0.065 (0.000/25)	0.020 (0.005/40)
D-Glucose	0.121 (0.107/50)	0.057 (0.030/90)	0.197 (0.155/100)	0.330 (0.073/100)	0.065 (0.014/60)
Paraxanthine	- (-/0)	0.003 (0.001/100)	0.003 (0.002/80)	0.004 (0.001/100)	0.005 (0.008/100)
Isoferulic acid	- (-/0)	0.013 (0.005/40)	0.022 (0.010/70)	0.006 (0.002/50)	0.035 (0.040/30)
Cytidine	- (-/0)	0.013 (0.002/50)	0.019 (0.000/10)	0.046 (0.000/25)	0.019 (0.008/60)
Ortho-Hydroxyphenylacetic acid	- (-/0)	0.078 (0.022/40)	0.214 (0.035/30)	0.082 (0.014/50)	0.048 (0.012/70)
4-Hydroxyphenylpyruvic acid	- (-/0)	0.002 (0.000/10)	0.007 (0.001/30)	0.002 (0.000/25)	0.009 (0.000/10)
Rhamnose	- (-/0)	0.018 (0.019/80)	0.016 (0.000/10)	0.107 (0.055/50)	0.056 (0.016/50)
Alpha-Hydroxyisobutyric acid	- (-/0)	0.011 (0.000/10)	0.015 (0.005/30)	0.050 (0.014/75)	0.014 (0.006/40)
p-Aminobenzoic acid	- (-/0)	0.007 (0.002/30)	0.012 (0.000/10)	0.017 (0.000/25)	0.007 (0.001/20)
2-Hydroxybutyric acid	- (-/0)	0.105 (0.000/10)	0.575 (0.477/40)	0.353 (0.000/25)	0.163 (0.020/20)
Beta-N-Acetylglucosamine	- (-/0)	0.170 (0.072/80)	0.475 (0.267/80)	0.358 (0.211/100)	0.158 (0.099/90)
Tartaric acid	- (-/0)	0.075 (0.026/60)	0.238 (0.000/10)	0.152 (0.000/25)	0.092 (0.007/20)
Hydroxyoctanoic acid	- (-/0)	0.081 (0.020/70)	0.194 (0.074/30)	0.186 (0.000/25)	0.078 (0.017/60)
L-Methionine	- (-/0)	0.009 (0.006/20)	0.040 (0.006/40)	0.027 (0.007/75)	0.002 (0.000/10)
S-Adenosylhomocysteine	- (-/0)	0.016 (0.002/60)	0.062 (0.023/90)	0.047 (0.000/25)	0.029 (0.000/10)
L-Acetylcarnitine	- (-/0)	0.049 (0.014/70)	0.114 (0.019/20)	0.052 (0.000/25)	0.023 (0.022/80)
N-Acetylgalactosamine	- (-/0)	0.095 (0.000/10)	0.582 (0.144/40)	0.095 (0.000/25)	0.159 (0.058/50)
Methylcysteine	- (-/0)	0.183 (0.208/100)	0.766 (0.716/60)	0.494 (0.157/75)	0.066 (0.053/90)
Trigonelline	- (-/0)	0.004 (0.001/90)	0.005 (0.000/10)	0.005 (0.000/25)	0.006 (0.003/50)
1,3,7-Trimethyluric acid	0.376 (0.000/25)	- (-/0)	0.049 (0.023/50)	0.037 (0.029/100)	0.037 (0.041/50)
Homogentisic acid	0.391 (0.000/25)	- (-/0)	0.183 (0.032/50)	0.082 (0.000/25)	0.073 (0.018/70)
D-Threitol	0.450 (0.000/25)	- (-/0)	0.306 (0.000/10)	0.259 (0.025/50)	0.061 (0.044/20)
Glycyl-glycine	0.115 (0.000/25)	- (-/0)	0.080 (0.016/30)	0.078 (0.019/100)	0.012 (0.002/30)
2-Aminoisobutyric acid	0.059 (0.000/25)	- (-/0)	0.016 (0.006/30)	0.008 (0.004/100)	0.029 (0.063/80)

Methylamine	0.159 (0.000/25)	- (-/0)	0.114 (0.065/80)	0.146 (0.031/100)	0.011 (0.008/70)
Adenosine monophosphate	0.031 (0.009/50)	- (-/0)	0.010 (0.000/10)	0.006 (0.000/25)	0.007 (0.001/30)
Pyruvic acid	0.046 (0.000/25)	- (-/0)	0.049 (0.005/20)	0.020 (0.015/100)	0.011 (0.004/40)
Guanidinosuccinic acid	0.030 (0.000/25)	- (-/0)	0.010 (0.000/10)	0.020 (0.000/25)	0.024 (0.010/30)
Cysteine-S-sulfate	0.587 (0.335/50)	- (-/0)	0.715 (0.000/10)	0.716 (0.000/25)	0.038 (0.030/20)
dCMP	0.166 (0.000/25)	- (-/0)	0.129 (0.000/10)	0.169 (0.000/25)	0.020 (0.000/10)
2-Hydroxy-3-methylbutyric acid	3.252 (0.000/25)	- (-/0)	1.575 (0.000/10)	2.796 (1.273/50)	0.935 (0.215/20)
3-Methyl-2-oxovaleric acid	1.134 (0.404/100)	- (-/0)	0.500 (0.319/70)	0.965 (0.267/50)	0.004 (0.000/10)
Methanol	0.206 (0.042/100)	- (-/0)	0.665 (0.143/100)	0.068 (0.112/100)	0.001 (0.000/30)
Trimethylamine N-oxide	0.006 (0.000/25)	- (-/0)	0.011 (0.010/20)	0.003 (0.001/100)	0.001 (0.001/40)
Methylguanidine	0.031 (0.000/25)	- (-/0)	0.134 (0.075/50)	0.041 (0.014/75)	0.019 (0.009/40)
Citric acid	0.043 (0.000/25)	- (-/0)	0.057 (0.012/80)	0.008 (0.004/75)	0.007 (0.003/70)
Alpha-ketoisovaleric acid	0.030 (0.008/75)	- (-/0)	0.039 (0.017/80)	0.023 (0.000/25)	0.098 (0.000/10)
Trehalose	0.090 (0.040/75)	- (-/0)	0.093 (0.059/50)	0.177 (0.018/50)	- (-/0)
Epicatechin	0.120 (0.040/50)	0.060 (0.032/50)	- (-/0)	0.104 (0.057/50)	0.111 (0.062/80)
Ribothymidine	0.048 (0.000/25)	0.009 (0.003/50)	- (-/0)	0.014 (0.007/75)	0.117 (0.250/70)
Uridine 5'-monophosphate	0.030 (0.000/25)	0.021 (0.000/10)	- (-/0)	0.032 (0.000/25)	0.020 (0.000/10)
Phosphorylcholine	0.343 (0.000/25)	0.028 (0.000/10)	- (-/0)	0.028 (0.000/25)	0.006 (0.000/10)
Sarcosine	0.023 (0.000/25)	0.011 (0.000/10)	- (-/0)	0.009 (0.005/100)	0.007 (0.003/90)
Hypoxanthine	0.040 (0.000/25)	0.048 (0.022/20)	- (-/0)	0.007 (0.000/25)	0.033 (0.024/30)
1-Methylguanosine	0.031 (0.000/25)	0.037 (0.000/10)	- (-/0)	0.016 (0.006/50)	0.021 (0.011/40)
Indole	0.128 (0.000/25)	0.025 (0.005/70)	0.050 (0.000/10)	- (-/0)	0.029 (0.013/60)
Pyrimidine	0.048 (0.000/25)	0.042 (0.000/10)	0.042 (0.006/30)	- (-/0)	0.040 (0.000/10)
Ureidopropionic acid	0.170 (0.000/25)	0.060 (0.000/10)	0.147 (0.000/10)	- (-/0)	0.029 (0.009/60)
N-Acetylglutamine	0.091 (0.035/75)	0.032 (0.013/30)	0.076 (0.043/20)	- (-/0)	0.005 (0.000/10)
Sedoheptulose	0.327 (0.009/75)	0.066 (0.017/50)	0.232 (0.040/20)	- (-/0)	0.052 (0.009/50)
Tryptamine	0.086 (0.044/75)	0.031 (0.009/80)	0.049 (0.027/90)	- (-/0)	0.016 (0.008/90)
L-Kynurenine	1.569 (0.641/100)	0.372 (0.215/90)	0.970 (0.299/100)	- (-/0)	0.321 (0.116/60)

Hexadecanedioic acid	0.435 (0.170/75)	0.095 (0.034/100)	0.855 (0.359/80)	- (-/0)	0.026 (0.000/10)
3-Methylindole	0.023 (0.015/100)	0.010 (0.004/100)	0.029 (0.008/100)	- (-/0)	0.012 (0.005/50)
L-Dopa	0.033 (0.000/25)	0.024 (0.008/60)	0.014 (0.000/10)	- (-/0)	0.025 (0.010/50)
o-Cresol	0.149 (0.124/75)	0.127 (0.032/50)	0.143 (0.130/70)	- (-/0)	0.063 (0.037/100)
5'-Methylthioadenosine	0.003 (0.000/25)	0.002 (0.001/40)	0.002 (0.000/10)	- (-/0)	0.053 (0.086/40)
N6-Acetyl-L-lysine	2.009 (0.000/25)	0.484 (0.293/50)	1.148 (0.160/40)	- (-/0)	0.089 (0.024/40)
Deoxyuridine	0.057 (0.028/75)	0.016 (0.004/50)	0.041 (0.016/80)	- (-/0)	0.019 (0.006/50)
D-Mannose	0.697 (0.245/100)	0.126 (0.067/100)	0.387 (0.298/80)	- (-/0)	0.124 (0.014/80)
Mannose 6-phosphate	0.076 (0.035/50)	0.066 (0.025/70)	0.171 (0.068/80)	- (-/0)	0.424 (0.399/20)
Cinnamic acid	0.230 (0.097/100)	0.042 (0.015/50)	0.123 (0.056/100)	- (-/0)	0.026 (0.009/50)
Taurodeoxycholic acid	0.034 (0.000/25)	0.018 (0.009/90)	0.030 (0.025/100)	- (-/0)	0.019 (0.002/30)
Kynurenic acid	0.039 (0.016/75)	0.013 (0.003/40)	0.031 (0.004/50)	- (-/0)	0.014 (0.004/60)
2-Oxohexane	0.584 (0.076/75)	0.170 (0.130/50)	0.472 (0.259/80)	- (-/0)	0.095 (0.035/50)
Caffeic acid	0.084 (0.024/50)	0.021 (0.002/30)	0.075 (0.026/40)	- (-/0)	0.016 (0.001/40)
4-Hydroxybenzoic acid	0.005 (0.000/25)	0.008 (0.005/20)	0.021 (0.004/40)	- (-/0)	0.014 (0.000/10)
Hydrochlorothiazide	0.663 (0.179/50)	0.169 (0.062/70)	0.230 (0.160/20)	- (-/0)	0.113 (0.029/40)
L-Tryptophan	0.044 (0.021/100)	0.024 (0.012/100)	0.044 (0.016/100)	- (-/0)	0.016 (0.007/80)
D-Glucuronic acid	0.096 (0.006/50)	0.019 (0.002/50)	0.075 (0.045/100)	- (-/0)	0.045 (0.021/20)
Deoxyinosine	0.628 (0.000/25)	0.232 (0.038/30)	0.312 (0.000/10)	- (-/0)	0.145 (0.077/50)
2-Aminobenzoic acid	0.210 (0.000/25)	0.183 (0.041/70)	0.408 (0.102/50)	- (-/0)	0.145 (0.030/50)
Fumaric acid	0.013 (0.009/100)	0.002 (0.000/20)	0.014 (0.009/90)	0.029 (0.012/100)	- (-/0)
Uridine	0.017 (0.000/25)	0.004 (0.000/10)	0.032 (0.000/20)	0.028 (0.000/25)	- (-/0)
D-Ribose 5-phosphate	1.802 (0.000/25)	0.956 (0.000/10)	1.871 (0.712/50)	1.806 (0.188/50)	- (-/0)
Gallic acid	- (-/0)	0.015 (0.008/50)	- (-/0)	0.015 (0.001/50)	0.021 (0.009/80)
Folic acid	0.015 (0.004/50)	- (-/0)	0.016 (0.005/70)	- (-/0)	0.015 (0.000/10)
Suberic acid	- (-/0)	0.087 (0.011/30)	0.266 (0.014/20)	- (-/0)	0.116 (0.000/10)
Guanidoacetic acid	- (-/0)	0.199 (0.027/20)	- (-/0)	0.735 (0.000/25)	0.165 (0.034/70)
Thymidine	0.056 (0.000/25)	0.012 (0.003/20)	- (-/0)	- (-/0)	0.006 (0.003/70)

Decanoylcarnitine	- (-/0)	- (-/0)	0.063 (0.002/20)	0.034 (0.008/75)	0.021 (0.005/70)
Aminocaproic acid	- (-/0)	0.227 (0.051/50)	0.696 (0.200/20)	- (-/0)	0.126 (0.044/40)
L-Dihydroorotic acid	1.380 (0.000/25)	0.225 (0.082/20)	0.909 (0.808/20)	- (-/0)	- (-/0)
Methionine sulfoxide	- (-/0)	0.007 (0.003/30)	0.003 (0.002/20)	- (-/0)	0.009 (0.002/90)
Biocytin	- (-/0)	0.200 (0.077/30)	0.749 (0.000/10)	- (-/0)	0.066 (0.028/50)
Propyl alcohol	- (-/0)	0.115 (0.000/10)	0.646 (0.312/20)	- (-/0)	0.165 (0.014/50)
Salicyluric acid	- (-/0)	0.030 (0.000/10)	0.026 (0.008/20)	- (-/0)	0.015 (0.007/30)
3,7-Dimethyluric acid	0.252 (0.092/75)	- (-/0)	- (-/0)	0.558 (0.179/100)	0.048 (0.035/20)
Deoxycholic acid glycine conjugate	- (-/0)	0.006 (0.003/50)	0.016 (0.009/70)	- (-/0)	0.010 (0.005/20)
Pelargonic acid	- (-/0)	0.055 (0.011/20)	0.091 (0.006/20)	- (-/0)	0.057 (0.004/20)
Glyoxylic acid	- (-/0)	0.002 (0.000/10)	- (-/0)	0.049 (0.006/75)	0.072 (0.012/90)
3-Methyladipic acid	0.534 (0.060/75)	- (-/0)	0.692 (0.178/50)	0.305 (0.000/25)	- (-/0)
Geraniol	- (-/0)	0.190 (0.012/20)	- (-/0)	0.112 (0.042/75)	0.032 (0.010/90)
Alpha-Lactose	- (-/0)	0.038 (0.019/30)	0.212 (0.008/20)	0.199 (0.000/25)	- (-/0)
Propanal	- (-/0)	0.007 (0.002/60)	0.009 (0.000/10)	- (-/0)	0.092 (0.000/10)
1-Butanol	- (-/0)	0.017 (0.000/10)	0.064 (0.006/20)	- (-/0)	0.025 (0.007/60)
Chenodeoxycholic acid	- (-/0)	0.097 (0.049/90)	0.201 (0.167/90)	- (-/0)	0.085 (0.026/80)
1-Methyluric acid	0.457 (0.000/25)	- (-/0)	- (-/0)	0.213 (0.128/75)	0.383 (0.000/10)
N-Methylhydantoin	- (-/0)	0.061 (0.000/10)	- (-/0)	0.040 (0.006/75)	0.044 (0.021/80)
Xanthurenic acid	- (-/0)	0.009 (0.003/60)	0.016 (0.008/20)	- (-/0)	0.010 (0.002/80)
Maltotriose	- (-/0)	0.076 (0.015/20)	0.193 (0.028/50)	- (-/0)	0.307 (0.092/80)
3-Aminoisobutanoic acid	- (-/0)	0.596 (0.000/10)	- (-/0)	0.330 (0.089/75)	0.303 (0.000/10)
Traumatic acid	0.027 (0.010/100)	0.007 (0.000/10)	0.021 (0.006/50)	- (-/0)	- (-/0)
Carnosine	- (-/0)	0.016 (0.000/10)	- (-/0)	0.016 (0.004/50)	0.024 (0.013/20)
Caproic acid	- (-/0)	0.165 (0.048/20)	0.275 (0.114/30)	- (-/0)	0.104 (0.015/20)
Anserine	- (-/0)	0.028 (0.000/10)	- (-/0)	0.068 (0.045/100)	0.069 (0.018/30)
Niacinamide	0.014 (0.004/100)	- (-/0)	0.020 (0.015/30)	- (-/0)	0.147 (0.138/20)
Pyridoxine	- (-/0)	0.003 (0.000/10)	- (-/0)	0.007 (0.000/25)	0.006 (0.002/50)

Glycerol	0.639 (0.000/25)	0.280 (0.000/10)	1.744 (0.344/30)	- (-/0)	- (-/0)
L-Norleucine	- (-/0)	2.509 (0.361/20)	5.294 (0.014/20)	- (-/0)	1.647 (0.444/30)
Ethylmethylacetic acid	- (-/0)	- (-/0)	0.279 (0.118/50)	0.116 (0.015/50)	0.019 (0.000/10)
Quinolinic acid	- (-/0)	0.012 (0.002/40)	0.014 (0.003/30)	- (-/0)	0.015 (0.003/50)
Quinic acid	1.043 (0.000/25)	- (-/0)	- (-/0)	0.144 (0.065/75)	0.095 (0.009/20)
L-Isoleucine	- (-/0)	0.061 (0.014/70)	0.156 (0.053/90)	- (-/0)	0.054 (0.014/80)
4-Heptanone	- (-/0)	0.249 (0.058/50)	0.493 (0.039/30)	- (-/0)	0.005 (0.000/10)
Glycylproline	0.151 (0.000/25)	0.146 (0.042/90)	- (-/0)	- (-/0)	0.091 (0.036/90)
Choline	- (-/0)	0.019 (0.005/30)	- (-/0)	0.017 (0.000/25)	0.007 (0.005/30)
Phenyllactic acid	- (-/0)	0.022 (0.000/10)	0.080 (0.000/10)	0.051 (0.000/25)	- (-/0)
Inosinic acid	- (-/0)	0.004 (0.000/10)	- (-/0)	0.005 (0.000/25)	0.027 (0.023/20)
Methylglutaric acid	- (-/0)	0.140 (0.000/10)	0.305 (0.000/10)	- (-/0)	0.008 (0.000/10)
L-Tyrosine	- (-/0)	0.328 (0.103/20)	- (-/0)	0.843 (0.000/25)	0.358 (0.151/20)
3,4-Dihydroxyhydrocinnamic acid	0.392 (0.000/25)	- (-/0)	- (-/0)	0.141 (0.000/25)	0.098 (0.018/30)
5-Hydroxylysine	0.091 (0.000/25)	0.032 (0.011/60)	0.075 (0.051/80)	- (-/0)	- (-/0)
myo-Inositol	0.200 (0.006/50)	- (-/0)	- (-/0)	0.077 (0.000/25)	0.027 (0.004/20)
Tyramine	- (-/0)	0.003 (0.000/10)	- (-/0)	0.008 (0.000/25)	0.002 (0.000/10)
Gentisic acid	- (-/0)	0.014 (0.000/10)	0.011 (0.000/10)	- (-/0)	0.019 (0.007/20)
N-Acetyl-L-phenylalanine	- (-/0)	0.026 (0.000/10)	0.054 (0.010/30)	- (-/0)	0.022 (0.006/20)
Ribitol	0.550 (0.001/50)	- (-/0)	0.367 (0.050/40)	0.307 (0.000/25)	- (-/0)
4-Aminohippuric acid	- (-/0)	0.007 (0.000/10)	0.012 (0.005/20)	- (-/0)	0.004 (0.000/10)
L-Cysteine	0.086 (0.000/25)	0.174 (0.000/10)	0.323 (0.000/10)	- (-/0)	- (-/0)
Pseudouridine	- (-/0)	0.006 (0.002/40)	- (-/0)	0.003 (0.000/25)	0.004 (0.001/30)
Acetoacetic acid	- (-/0)	- (-/0)	0.154 (0.000/10)	0.095 (0.030/100)	0.032 (0.007/30)
1-Methyladenine	- (-/0)	0.010 (0.002/30)	- (-/0)	0.011 (0.002/75)	0.010 (0.005/20)
Dopamine	0.098 (0.082/50)	0.021 (0.007/50)	- (-/0)	- (-/0)	0.016 (0.003/30)
Pyridoxamine	0.220 (0.000/25)	- (-/0)	- (-/0)	0.068 (0.023/50)	0.065 (0.042/60)

Urea	- (-/0)	0.147 (0.020/30)	0.237 (0.010/20)	- (-/0)	0.423 (0.079/30)
4-Guanidinobutanoic acid	- (-/0)	0.315 (0.000/10)	- (-/0)	- (-/0)	0.041 (0.007/20)
Adenosine	- (-/0)	- (-/0)	- (-/0)	0.006 (0.002/50)	0.003 (0.000/10)
L-Lysine	- (-/0)	0.024 (0.006/40)	- (-/0)	- (-/0)	0.045 (0.036/80)
N-Acetylputrescine	0.009 (0.000/25)	- (-/0)	- (-/0)	- (-/0)	0.019 (0.018/40)
Mannitol	- (-/0)	0.294 (0.081/40)	- (-/0)	- (-/0)	0.350 (0.000/10)
Acetylcysteine	- (-/0)	- (-/0)	- (-/0)	0.073 (0.015/50)	0.043 (0.013/60)
Ethanolamine	- (-/0)	- (-/0)	- (-/0)	0.014 (0.006/50)	0.008 (0.000/10)
Creatine	- (-/0)	0.012 (0.002/60)	- (-/0)	- (-/0)	0.008 (0.003/70)
1,11-Undecanedicarboxylic acid	0.218 (0.028/50)	- (-/0)	- (-/0)	- (-/0)	0.064 (0.000/10)
Vanillylmandelic acid	- (-/0)	- (-/0)	- (-/0)	0.095 (0.016/75)	0.039 (0.023/70)
L-Asparagine	- (-/0)	- (-/0)	0.105 (0.000/10)	- (-/0)	0.031 (0.000/10)
Galactonic acid	- (-/0)	0.350 (0.157/20)	- (-/0)	- (-/0)	0.673 (0.091/20)
L-Allothreonine	- (-/0)	0.142 (0.000/10)	- (-/0)	- (-/0)	0.049 (0.002/20)
Glycocholic acid	- (-/0)	0.009 (0.006/30)	0.014 (0.010/40)	- (-/0)	- (-/0)
Octanal	- (-/0)	1.623 (0.000/10)	- (-/0)	- (-/0)	1.602 (0.000/10)
Leucinic acid	- (-/0)	- (-/0)	0.172 (0.000/10)	- (-/0)	0.018 (0.000/10)
Hippuric acid	0.019 (0.000/25)	- (-/0)	0.014 (0.000/10)	- (-/0)	- (-/0)
Isopropyl alcohol	- (-/0)	0.017 (0.000/10)	0.029 (0.000/10)	- (-/0)	- (-/0)
3-Hydroxybutyric acid	- (-/0)	- (-/0)	0.795 (0.000/10)	- (-/0)	0.416 (0.000/10)
Undecanoic acid	- (-/0)	0.057 (0.000/10)	- (-/0)	- (-/0)	0.384 (0.000/10)
Symmetric dimethylarginine	0.008 (0.000/25)	- (-/0)	- (-/0)	0.007 (0.000/25)	- (-/0)
2-Piperidinone	- (-/0)	0.043 (0.000/10)	0.060 (0.000/10)	- (-/0)	- (-/0)
Agmatine	- (-/0)	- (-/0)	- (-/0)	0.382 (0.000/25)	0.245 (0.000/10)
2-Furoic acid	0.051 (0.000/25)	- (-/0)	- (-/0)	- (-/0)	0.009 (0.000/20)
Deoxyguanosine	- (-/0)	0.025 (0.003/30)	- (-/0)	- (-/0)	0.026 (0.003/40)
L-Alloisoleucine	- (-/0)	0.063 (0.000/10)	- (-/0)	- (-/0)	0.005 (0.000/10)

D-Alpha-aminobutyric acid	- (-/0)	- (-/0)	0.176 (0.141/20)	- (-/0)	0.054 (0.000/10)
Oxoglutaric acid	- (-/0)	- (-/0)	0.215 (0.000/10)	- (-/0)	0.092 (0.000/10)
Protocatechuic acid	- (-/0)	0.016 (0.000/10)	- (-/0)	0.014 (0.002/50)	- (-/0)
Butanal	- (-/0)	0.614 (0.000/10)	- (-/0)	0.525 (0.000/25)	- (-/0)
Dihydrouracil	- (-/0)	0.066 (0.000/10)	- (-/0)	- (-/0)	0.003 (0.000/10)
6-Hydroxynicotinic acid	- (-/0)	- (-/0)	0.064 (0.000/10)	- (-/0)	0.039 (0.000/10)
Taurine	- (-/0)	- (-/0)	- (-/0)	0.039 (0.000/25)	0.006 (0.000/10)
Saccharopine	- (-/0)	- (-/0)	- (-/0)	0.728 (0.000/25)	0.481 (0.371/20)
Citrulline	- (-/0)	- (-/0)	- (-/0)	0.046 (0.000/25)	0.061 (0.000/10)
Maleic acid	- (-/0)	0.001 (0.000/10)	- (-/0)	- (-/0)	0.439 (0.000/10)
O-Phosphoethanolamine	- (-/0)	- (-/0)	- (-/0)	0.108 (0.000/25)	0.015 (0.000/10)
2-Hydroxycaproic acid	- (-/0)	- (-/0)	0.258 (0.000/10)	- (-/0)	0.031 (0.000/10)
Glycolic acid	- (-/0)	0.007 (0.002/100)	- (-/0)	- (-/0)	0.011 (0.003/90)
Gamma-Glutamylcysteine	- (-/0)	0.026 (0.000/20)	- (-/0)	- (-/0)	0.006 (0.000/10)
Dimethylamine	- (-/0)	- (-/0)	- (-/0)	0.011 (0.002/75)	0.004 (0.000/10)
Guaiacol	- (-/0)	0.062 (0.024/40)	- (-/0)	- (-/0)	0.064 (0.026/50)
Ethyl isopropyl ketone	- (-/0)	0.733 (0.204/60)	1.546 (0.890/70)	- (-/0)	- (-/0)
Delta-Hexanolactone	- (-/0)	0.236 (0.207/20)	1.011 (0.000/10)	- (-/0)	- (-/0)
(S)-3-Hydroxyisobutyric acid	- (-/0)	- (-/0)	0.034 (0.005/20)	0.055 (0.012/50)	- (-/0)
Citraconic acid	- (-/0)	- (-/0)	- (-/0)	0.107 (0.000/25)	0.033 (0.011/50)
Dimethylglycine	- (-/0)	- (-/0)	- (-/0)	0.002 (0.001/100)	0.002 (0.001/60)
D-Fructose	- (-/0)	3.931 (0.512/20)	- (-/0)	- (-/0)	2.705 (1.244/60)
L-Fucose	- (-/0)	0.029 (0.025/40)	0.034 (0.020/20)	- (-/0)	- (-/0)
Dihydrothymine	- (-/0)	0.146 (0.000/10)	- (-/0)	- (-/0)	0.030 (0.003/20)
Trimethylamine	- (-/0)	- (-/0)	- (-/0)	0.002 (0.001/100)	0.003 (0.002/80)
o-Tyrosine	- (-/0)	0.114 (0.051/30)	- (-/0)	- (-/0)	0.082 (0.059/60)
L-Arabitol	- (-/0)	0.342 (0.000/10)	- (-/0)	- (-/0)	0.154 (0.000/10)
Benzoic acid	- (-/0)	- (-/0)	0.028 (0.000/10)	- (-/0)	0.007 (0.000/10)

Aminoadipic acid	0.224 (0.000/25)	- (-/0)	- (-/0)	- (-/0)	- (-/0)
Hydroxyisocaproic acid	- (-/0)	0.036 (0.008/30)	- (-/0)	- (-/0)	- (-/0)
L-Octanoylcarnitine	- (-/0)	0.004 (0.000/20)	- (-/0)	- (-/0)	- (-/0)
Guanosine	- (-/0)	0.001 (0.000/10)	- (-/0)	- (-/0)	- (-/0)
Deoxyadenosine monophosphate	- (-/0)	0.018 (0.002/20)	- (-/0)	- (-/0)	- (-/0)
Hexanoylcarnitine	- (-/0)	0.062 (0.000/10)	- (-/0)	- (-/0)	- (-/0)
Homocitrulline	- (-/0)	0.147 (0.000/10)	- (-/0)	- (-/0)	- (-/0)
1-Hexadecanol	- (-/0)	- (-/0)	0.161 (0.000/10)	- (-/0)	- (-/0)
Azelaic acid	- (-/0)	- (-/0)	0.196 (0.033/20)	- (-/0)	- (-/0)
Gamma-Aminobutyric acid	- (-/0)	- (-/0)	0.202 (0.030/20)	- (-/0)	- (-/0)
5-Aminopentanoic acid	- (-/0)	- (-/0)	0.519 (0.000/10)	- (-/0)	- (-/0)
Ethylmalonic acid	- (-/0)	- (-/0)	0.248 (0.000/10)	- (-/0)	- (-/0)
Ketoleucine	- (-/0)	- (-/0)	1.178 (0.000/10)	- (-/0)	- (-/0)
2-Hydroxyadipic acid	- (-/0)	- (-/0)	0.099 (0.000/10)	- (-/0)	- (-/0)
3-Hexanone	- (-/0)	- (-/0)	- (-/0)	1.309 (0.000/25)	- (-/0)
Glyceric acid	- (-/0)	- (-/0)	- (-/0)	1.460 (0.116/50)	- (-/0)
L-Iditol	- (-/0)	- (-/0)	- (-/0)	0.006 (0.000/25)	- (-/0)
Adenine	- (-/0)	- (-/0)	- (-/0)	1.590 (0.000/25)	- (-/0)
D-Xylitol	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.014 (0.002/40)
Levulinic acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.020 (0.005/60)
Acetamide	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.131 (0.088/20)
Glucaric acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.009 (0.003/30)
Malonic acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.017 (0.000/10)
Guanine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.008 (0.003/40)
Creatinine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.084 (0.000/10)
Dihydroxyacetone	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.045 (0.000/10)
Diethanolamine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.083 (0.000/10)
L-Serine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.021 (0.000/10)

Propylene glycol	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.187 (0.070/50)
Cyclohexanone	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.041 (0.000/10)
Diaminopimelic acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.032 (0.000/10)
Homocysteine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.128 (0.000/10)
L-Homoserine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.035 (0.000/10)
Heptanoic acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.030 (0.000/10)
Gamma-Caprolactone	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.006 (0.000/10)
Adipic acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.049 (0.000/10)
Benzaldehyde	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.014 (0.000/10)
2,3-Butanediol	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.039 (0.000/10)
Isocitric acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.002 (0.000/10)
Glutaric acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.401 (0.000/10)
Spermidine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.116 (0.000/10)
Cytosine	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.223 (0.000/10)
Glycerol 3-phosphate	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.019 (0.000/10)
Pyroglutamic acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.103 (0.000/10)
Tetrahydrofuran	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.005 (0.000/10)
Phthalic acid	- (-/0)	- (-/0)	- (-/0)	- (-/0)	0.065 (0.000/10)
D-Ribose	0.078 (0.041/100)	0.039 (0.011/90)	0.080 (0.033/100)	0.058 (0.029/75)	0.036 (0.014/80)
Orotic acid	2.734 (0.989/100)	0.713 (0.025/20)	1.681 (0.391/70)	3.007 (0.582/100)	1.386 (0.565/90)
Hypotaurine	0.100 (0.006/50)	0.042 (0.018/80)	0.083 (0.034/100)	0.017 (0.000/25)	0.032 (0.008/70)
Dimethylmalonic acid	1.837 (0.645/100)	0.445 (0.117/70)	1.171 (0.639/100)	1.013 (0.462/100)	0.350 (0.139/50)

8 Appendix 3: Supplemental tables for Chapter 4

Table A3- 1: Metabolites from chicken feather pulp processed using ultrafiltration

Average ppm	Metabolite	Number
8.263, 8.274, 8.723, 8.953	Niacinamide*	151
8.603	Adenosine diphosphate* and Adenosine monophosphate*	52, 53
8.465	Formate*	90
2.705, 6.087, 8.284. 8.392	S-Adenosylhomocysteine[a,c]	176
8.365	FAPy-adenine[a,b]* and Inosine*	89, 117
4.287, 4.292, 6.108, 8.227	Inosine*	117
8.216	Oxypurinol*	158
8.038	Imidazole[a,b]*	115
8.020	Guanosine and Theophylline*	107, 188
8.001	Histamine*	111
7.972	3-Methylxanthine*	33
7.915	FAPy-adenine[a,b]*	89
7.906	Carnosine[a]* and Xanthine*	68, 207
7.894	Histamine* and Terephthalic acid	111, 187
3.172, 7.881	Anserine[a,c]*	58
7.861	7-Methylxanthine* and Deoxyuridine[a]	44, 80
7.851	Deoxyuridine[a]	80
7.846	1,7-Dimethylxanthine*	6
7.215, 7.289, 7.742	Tryptophan*	200
4.370, 7.610	5-Hydroxymethyluracil	40
7.572	4-Pyridoxic acid and Tryptophan* and Uracil[a]*	37, 200, 204
3.098, 3.109, 3.992, 7.436	Phenylalanine*	163
7.373, 7.394	Phenylalanine* and Riboflavin*	163,174
7.383	Phenylalanine* and Thymine	163, 192
7.345	Phenylalanine* and Tryptophan*	163, 200
7.329	Imidazole[a,b]* and Phenylalanine*	115, 163
7.197	Tryptophan* and Tyrosine*	200, 201
7.135	Histamine* and 1-Methylhistidine*	111, 10
7.088	Homocarnosine[a,c]	113
2.693, 7.044	Carnosine[a]*	68
6.915	Anserine[a,c]* and Tyrosine*	58, 201
6.536	Argininosuccinic acid and Fumaric acid*	60, 91
6.167, 6.175	Adenosine diphosphate*	52
6.150	Cytidine monophosphate[a,b]* and Inosine triphosphate[a]	76, 118
6.139	Adenosine monophosphate* and Inosine triphosphate[a]	53, 118
6.128	Adenosine monophosphate*	53

5.618, 6.010	UDP-glucose[a]*	202
5.995, 6.000	Cytidine monophosphate[a,b]* and UDP-glucose[a]*	76, 202
5.985, 5.991	Cytidine triphosphate[a]	77
5.959, 5.968	Guanosine monophosphate[a,c]*	108
1.991, 4.217, 4.227, 4.232, 5.931, 5.94	5-Methylcytidine*	42
5.910, 5.922	1-Methylguanosine* and Deoxyuridine[a] and Guanosine	9, 80,107
5.811	Uracil[a]*	204
5.426	Glycogen and Maltose* and Sucrose	104, 129, 184
4.653, 4.667, 5.253	Glucose[c]* and Maltose*	98, 129
2.157, 2.170, 2.180, 2.451, 2.473, 3.476, 3.488, 3.506, 4.340, 4.351, 4.680	4-Hydroxyproline[a,b]	36
3.736, 3.757, 4.026, 4.537	Ascorbate*	61
4.450	1-Methylguanosine* and Inosine* and S-Adenosylhomocysteine[a,c]	9, 177,176
2.312, 2.318, 4.434	5-Hydroxymethyl-4-methyluracil	39
4.425	Guanosine	107
4.412, 4.419	Xylulose and Guanosine	209, 107
4.183, 4.189, 4.196, 4.391, 4.401	Xylulose	209
4.363	4-Hydroxyproline[a,b] and Tartrate	36, 185
4.328	N-Acetylmannosamine and Glycerophosphocholine	147, 102
2.373, 2.689, 4.300, 4.313	Malic acid*	126
3.728, 3.962, 4.282	Galactarate*	93
4.241, 4.251, 4.269, 4.276	Threonine[a,b]*	191
4.260	Galactonate* and Threonine[a,b]*	94, 191
4.207, 4.223	Sucrose	184
4.174	Allose and Xylulose and Phosphocholine*	57, 209, 164
3.225, 4.159, 4.163, 4.167	Phosphocholine*	164
4.149	Gluconate and Proline[a]*	97, 168
1.424, 4.109, 4.120, 4.131, 4.141	Lactate*	125
1.999, 2.008, 3.326, 3.335, 3.345, 3.364, 4.127, 4.136	Proline[a]*	168
4.082	myo-Inositol* and Tryptophan*	140, 200
4.067	Choline and Creatinine*	69, 74
3.210, 3.537, 3.544, 4.063	Choline	69
4.059	Choline and Xylulose and Tryptophan*	69, 209, 200
4.053	Xylulose and Tryptophan*	209, 200

4.045	Ascorbate* and Xylulose and Threonate	61, 209, 190
4.035	Ascorbate* and Xylulose and Gluconate	61, 209, 97
3.971, 3.997, 4.005, 4.014, 4.020	Serine	181
3.957	Serine and Tyrosine*	181, 201
3.950	1,7-Dimethylxanthine* and Tyrosine* and Serine	6, 201, 181
3.938	Creatine* and Tyrosine*	73, 201
3.662, 3.922, 3.928	Arabinitol[a,b]	59
3.913	1-Methylguanosine* and Betaine* and Glucose[c]*	9, 64,98
3.905	1-Methylguanosine* and 7-Methylxanthine*	9, 44
3.745, 3.752, 3.390, 3.834, 3.901	Glucose[c]*	98
3.895	1,5-Anhydrosorbitol and 1-Methylguanosine* and Glucose[c]*	5, 9,98
3.887	1-Methylguanosine* and Guanosine	9, 107
3.882	Glucose[c]* and Guanosine	98, 107
3.872	Glucitol[a,b] and Glucose[c]*	96, 98
3.862	Maltose*	129
3.844, 3.857	Glucose[c]* and Serine	98, 181
3.851	Glucose[c]* and Glycyl-glycine* and Serine	98, 105, 181
3.823, 4.156	Gluconate	97
3.811	2-Amino-3-phosphonopropionic acid*	13
3.804	2-Amino-3-phosphonopropionic acid* and Alanine*	13, 56
3.796	Ascorbate* and Glucose[c]* and Alanine*	61, 98, 56
3.657, 3.791	Glycerol*	101
3.785	Ascorbate* and Glucose[c]* and Guanidoacetic acid* and Alanine*	61, 98, 106, 56
3.770, 3.778	Ascorbate* and Glucose[c]* and Glutamate*	61, 98, 99
3.774	Alanine* and Methylacetoacetic acid	56, 98, 135
2.121, 2.132, 3.765	Glutamate*	99
3.760	Glucose[c]* and Glutamate*	98, 99
3.720	Anserine[a,c]* and Galactonate*	58, 94
3.713	Threonate	190
3.706	Allose and Galactonate* and Glucose[c]* and 1-Methylhistidine*	57, 94, 98, 10
3.699	Allose	57
3.694	Maltose* and Sucrose	129, 184
3.256, 3.687	Glycerophosphocholine	102
3.679	Arabinitol[a,b] and Isoleucine*	59, 121
3.667, 3.672	Arabinitol[a,b] and Glycerol*	59, 101
3.649	Glycerol* and Threonate	101, 190
3.553, 3.614, 3.628, 3.641	myo-Inositol*	140
2.101, 3.635	N-Acetylmannosamine	147
3.621	Xylulose and Sarcosine*	209, 177

3.598	1,5-Anhydrosorbitol and Phosphocholine* and Threonine[a,b]* and Valine*	5, 164, 191, 205
3.587	1,5-Anhydrosorbitol and Glycerol*	5, 101
3.586	Glycerol* and Glycine	101, 103
3.584	Glycerol* and Theophylline*	101, 188
3.571	Glycerol* and myo-Inositol*	101, 140
3.530	3-Methylxanthine* and Choline	33, 69
0.897, 3.520	Pantothenic acid	160
3.459	1,5-Anhydrosorbitol and Acetoacetic acid*	5, 47
3.443	1,5-Anhydrosorbitol	5
3.409, 3.418, 3.430	Taurine*	186
3.401	1,3,7-Trimethyluric acid	3
3.379	Methanol*	132
3.373	Theophylline*	118
3.355	1,3,7-Trimethyluric acid and Proline[a]*	3, 168
3.312	1,5-Anhydrosorbitol and 1-Methyluric acid* and Phenylalanine*	5, 11, 163
3.295	1-Methyluric acid* and Histamine* and Phenylalanine* and myo-Inositol* and Taurine*	11, 111, 163, 140 ,186
3.282	Histamine* and Phenylalanine* and myo-Inositol* and Taurine*	111, 163, 140, 186
3.271	Betaine* and Histamine* and Phenylalanine* and Taurine*	64 ,163, 111, 186
3.262	Trimethylamine N-oxide*	198
3.251	Agmatinium and Glycerophosphocholine	55, 102
3.244	Agmatinium and Acetylcholine*	55, 49
3.235	1,3,7-Trimethyluric acid and Agmatinium and Anserine[a,c]* and Glycerophosphocholine	3, 55, 58, 102
3.218	Acetylcholine* and Carnitine and Tyrosine*	49, 67,201
3.181, 3.110	Anserine[a,c]* and Beta-Alanine	58, 63
3.190	Anserine[a,c]* and Beta-Alanine and Tyrosine*	58, 63, 201
3.157	Dimethyl sulfone	82
3.151	Malonate*	128
3.120, 3.133	Phenylalanine* and N1-Acetylspermine*	163, 142
3.065, 3.077, 3.088	Tyrosine*	201
3.052	Agmatinium and Anserine[a,c]* and Phosphocreatine*	55, 58, 165
3.038	Creatine* and Creatinine*	73, 74
3.015, 3.026	Anserine[a,c]* and Histamine*	58, 111
2.936	N-Methylhydantoin*	152
2.928	Trimethylamine	197
1.660, 1.910, 1.916, 1.937, 1.941, 1.946, 2.525, 2.538, 2.551, 2.795, 2.821	Argininosuccinic acid	60
2.736	Sarcosine*	177

2.729	Dimethylamine* and Norspermidine[a,c]*	83, 154
2.635, 2.645, 2.699, 2.710, 2.719	Norspermidine[a,c]*	154
2.683	2,2-Dimethylsuccinic acid* and Malic acid*	12, 126
2.676	Carnosine[a]* and Phosphonoacetate*	68, 166
2.668	Carnosine[a]* and Malic acid*	68, 126
2.661	Anserine[a,c]* and Malic acid*	58, 126
2.658	Carnosine[a]* and Norspermidine[a,c]*	68, 154
2.653	Anserine[a,c]* and Carnosine[a]*	58, 68
2.622	Ketoleucine	124
2.571	Argininosuccinic acid and Beta-Alanine	60, 63
2.499	(S)-3-Hydroxybutyric acid[a]	2
2.489	(S)-3-Hydroxybutyric acid[a] and 4-Hydroxyproline[a,b]	2, 36
2.457	4-Hydroxyproline[a,b] and Pyruvic acid*	36, 173
1.688, 2.087, 2.407, 2.419	Saccharopine[a,b]	175
2.396	Pyruvate* and Saccharopine[a,b]	172, 175
2.385	Pyruvate*	172
2.378	Malic acid* and Proline[a]*	126, 168
2.368	3-Hydroxyisovalerate[a,c]	23
2.048, 2.093, 2.340, 2.352, 2.362	Glutamate* and Proline[a]*	99, 168
2.329	2-Piperidinone[a,b] and 4-Pyridoxic acid and Glutamate* and Proline[a]*	22, 37, 99, 168
2.306	Acetoacetic acid*	47
0.994, 1.045, 2.298	Valine*	205
2.289	Acetoacetic acid* and Valine*	47, 205
2.260, 2.269, 2.278	2-Piperidinone[a,b] and Valine*	22, 205
2.239	Riboflavin*	174
2.144	Dimethylsulfide and Glutamate*	85 , 99
2.080	2-Amino-3-phosphonopropionic acid* and Glutamate* and Proline[a]* and Saccharopine[a,b]	13, 99, 168, 175
2.069	Glutamate* and Proline[a]* and N-Acetylmannosamine	99, 168,147
2.060	Beta-N-Acetylglucosamine* and N-Acetylornithine	65, 149
2.036	Glutamate* and Proline[a]* and N-Acetyl-L-aspartic acid	99, 168, 145
2.028	Proline[a]* and N1-Acetylspermine* and N-Alpha-acetyllysine	168, 142, 150
2.018	Proline[a]* and N-Acetyl-L-alanine and N-Alpha-acetyllysine	168, 150
1.927	Acetic acid*	46
1.874	Thymine	192
1.830	2-Piperidinone[a,b] and N1-Acetylspermine*	22, 142
1.728, 1.753, 1.762, 1.804	2-Piperidinone[a,b]	22
1.782	2-Hydroxy-2-methylbutyric acid[a,c]* and 2-Piperidinone[a,b] and Ethylmalonate	16, 22, 88
1.702	2-Piperidinone[a,b] and Saccharopine[a,b]	22, 175

1.669, 1.677	2-Amino-3-phosphonopropionic acid* and Argininosuccinic acid	13, 60
1.497	Alanine*	56
1.014, 1.442, 1.454, 1.473	Isoleucine*	121
1.461	Dimethylmalonic acid* and Isoleucine*	84, 121
1.327	Lactate* and Isoleucine*	125, 121
1.278, 1.288	Isoleucine* and Methylmalonic acid[a]	121, 84
1.268	2,2-Dimethylsuccinic acid* and 3-Hydroxyisovalerate[a,c] and Isoleucine*	12, 23,121
1.248, 1.258	(S)-3-Hydroxybutyric acid[a] and Isoleucine*	2, 121
1.189	Isopropanol	122
1.065, 1.076	Isobutyrate[a,b]	119
0.964, 0.974	2-Hydroxy-2-methylbutyric acid[a,c]*	16
0.954	2-Hydroxy-2-methylbutyric acid[a,c]* and Isoleucine*	16, 121
0.933, 0.944	Ketoleucine and Isoleucine*	124, 121
0.892, 0.903, 0.914	Ethylmalonate	88
0.892, 0.903, 0.914	Ethylmalonate	88

All metabolites found in all three ages unless otherwise specified
[a] found in chicks
[b] found in 23-week-old chickens
[c] found in 45-week-old chickens
* Metabolite found using both extraction methods

Table A3- 2: Metabolites from chicken feather pulp processed using Bligh-Dyer methanol chloroform extraction

Average ppm	Metabolite	Number
7.610, 8.723, 8.953	Niacinamide[c]*	151
8.603	Adenosine diphosphate[c]* and Adenosine monophosphate* and Purine[b,c]	52, 53, 169
8.465	1-Methyladenosine[a,c] and Formate*	7, 90
6.087, 8.392	1-Methyladenosine[a,c]	7
7.921, 8.379	FAPy-adenine*	89
4.292, 3.844, 4.282, 4.287, 6.108, 8.364	Inosine[a,c]*	117
8.284	3-Methyladenine and Niacinamide[c]*	27, 151
8.274	1-Methyladenosine[a,c] and Niacinamide[c]*	7, 151
8.263	Adenosine diphosphate[c]* and Adenosine monophosphate* and Adenosine triphosphate	52, 53, 54
8.227	Hypoxanthine and Inosine[a,c]*	114, 117
8.216	Guanosine monophosphate[a,c]* and Hypoxanthine and Oxypurinol*	108, 114, 158
8.038	Carnosine[c]*	68
8.020	1-Methylguanosine* and Histamine[a]*	9, 111
8.001	3-Methylxanthine* and Anserine[c]* and Theophylline*	33, 58, 188
7.946	3-Nitrotyrosine and N-Acetyl-L-phenylalanine[a,c]	34, 146
7.936	N-Acetyl-L-phenylalanine[a,c]	146
7.927	3-Methylhistidine	30
3.706, 7.697, 7.9145	1-Methylhistidine*	10
7.906	dCTP[c] and Xanthine*	79, 207
7.894	dCTP[c] and Methylimidazoleacetic acid	79, 136
7.881	1-Methylguanine	8
7.861	7-Methylxanthine*	44
7.850	Histidine	112
3.335, 7.845	1,7-Dimethylxanthine*	6
3.478, 4.067, 7.215, 7.562, 7.741	Tryptophan[a,c]*	200
4.005, 7.329, 7.435	Phenylalanine*	163
7.373, 7.383, 7.394	2-Phenylpropionate and N-Acetyl-L-phenylalanine[a,c] and Phenylalanine*	21, 146, 163
7.345	Phenylalanine* and Tryptophan[a,c]*	163, 200
7.289	3-Hydroxymandelate and Imidazole* and Tryptophan[a,c]*	24, 115, 200
7.197	Tryptophan[a,c]* and Tyrosine*	200, 201
7.135	1-Methylhistidine* and 3-Methylphenylacetic acid	10, 32
3.326, 7.088	3-Methylhistamine	29
7.044	3-Methylhistidine and Methylimidazoleacetic acid	30, 136

6.915	Tyrosine*	201
6.526	Fumaric acid[a,c]*	91
6.167, 6.175	Adenosine diphosphate[c]*	52
1.937, 1.941, 1.946, 1.953, 6.159	Methyl propenyl ketone	134
6.150	Adenosine diphosphate[c]* and Adenosine monophosphate* and Adenosine triphosphate and Cytidine monophosphate[a,c]*	52, 53, 54, 76
6.139	Adenosine diphosphate[c]* and Adenosine monophosphate* and Adenosine triphosphate and dCMP[c] and Methyl propenyl ketone	52, 53, 54, 78, 134
6.128	dCMP[c] and dCTP	78, 79
6.010	Cytidine monophosphate[a,c]* and UDP-N-Acetylglucosamine[a,c]	76, 203
2.101, 4.196, 4.351, 6.000	UDP-N-Acetylglucosamine[a,c]	203
5.995	UDP-glucose[a,c]* and UDP-N-Acetylglucosamine[a,c]	202, 203
4.378, 5.985, 5.991	UDP-glucose[a,c]*	202
5.968, 5.978	Guanosine monophosphate[a,c]*	108
5.931, 5.940	5-Methylcytidine[a,c]*	42
5.910, 5.922	1-Methylguanosine*	9
5.811	Uracil*	204
5.426	Maltose* and Maltotriose[c]	129, 130
5.253	Glucose[c]* and Maltose* and Maltotriose[c]	98, 129, 130
3.598, 4.68	Maltose*	129
4.667	Glucose[c]* and Maltose*	98, 129
3.250, 3.262, 4.653	Glucose[c]*	98
4.026, 4.035, 4.044, 4.527	Ascorbate*	61
4.450	1-Methylguanosine* and Inosine[a,c]*	9, 117
4.391	Adenosine diphosphate[c]* and Adenosine monophosphate* and Adenosine triphosphate and UDP-glucose[a,c]*	52, 53, 54, 202
4.328	Cytidine monophosphate[a,c]* and UDP-N-Acetylglucosamine[a,c]	76
2.378, 4.300, 4.313	Malic acid*	126
4.276	Galactarate* and Inosine[a,c]* and Threonine*	93, 117, 191
4.269	Galactarate* and Threonine*	93, 191
4.260	5-Methylcytidine[a,c]* and Galactonate* and Threonine*	42, 94, 191
4.251	5-Methylcytidine[a,c]* and Threonine*	42, 191
4.241	1-Methylguanosine* and 5-Methylcytidine[a,c]* and Threonine*	9, 42, 191
4.232	1-Methylguanosine* and Threonine*	9, 191
4.223	dCTP[c]	79

4.217	dCTP[c] and UDP-glucose[a,c]*	79, 202
1.327, 4.109, 4.120, 4.131, 4.141	Lactate*	125
3.312, 3.556, 3.628, 3.641, 4.082	myo-Inositol*	140
4.063	Creatinine*	74
4.058	7-Methyladenine and Tryptophan[a,c]*	43, 200
4.052	2-Methylhippuric acid	20
4.020	Paraxanthine	161
4.014	Sedoheptulose[a,c]	178
3.997	3-Methyladenine	27
3.120, 3.992	Histidine and Phenylalanine*	112, 163
3.984	Galactose[a,c]	95
3.971	Galactarate*	93
3.949	Phosphocreatine*	165
3.938	Creatine*	73
3.913	Betaine*	64
3.905	5-Methoxytryptamine and Melatonin[a,c]	41, 131
3.901	trans-Ferulic acid	195
3.834, 3.857	Glycyl-glycine*	105
3.850	Trehalose[c]	196
3.823	2-Amino-3-phosphonopropionic acid* and Galactose[a,c]	13, 95
3.816	N-Nitrosodimethylamine	153
3.774, 3.796, 3.804	Alanine*	56
2.457, 2.489, 3.791	Glutamine	100
3.785	Alanine* and Glutamine and Guanidoacetic acid*	56, 100, 106
2.121, 3.778	Glutamate* and Glutamine	99, 100
2.036, 2.093, 2.361, 3.765, 3.770	Glutamate*	99
3.760	Ascorbate* and Glutamate*	61, 99
3.751	Methylimidazoleacetic acid	136
2.705, 2.975, 3.728	Anserine[c]*	58
3.720	3-Methylhistamine and 3-Methylhistidine	29, 30
3.679	Isoleucine[a,c]* and Sedoheptulose[a,c]	121, 178
3.243, 3.345, 3.649, 3.672	Xylose	208
3.667	Galactose[a,c] and Sedoheptulose[a,c] and Xylose	95, 178, 208
3.662	Maltotriose[c]	130
3.656	Indoleacetic acid and Xylose	116, 208
1.044, 2.278, 3.621	Valine*	205
3.614	myo-Inositol* and Phosphocholine[a,c]* and Threonine*	140, 164, 191
3.582	Glycerol[c]* and Ortho-Hydroxyphenylacetic acid[b,c]	101, 156
3.571	Glycerol[c]* and Glycine	101, 103

3.564	Glycerol[c]* and Theophylline*	101, 188
3.543	Glycerol[c]* and myo-Inositol*	101, 140
3.534	3-Methylxanthine* and Pantethine[a,c]	33, 159
0.892, 3.379, 3.527	Pantethine[a,c]	159
3.520	Galactose[a,c] and Pantethine[a,c]	95, 159
3.510	Glucose[c]* and Methylimidazoleacetic acid	98, 136
3.496	3-Hydroxyphenylacetic acid and Glucose[c]* and Melatonin[a,c]	26, 98, 131
3.485	Melatonin[a,c]	131
3.471	Maltotriose[c] and p-Hydroxyphenylacetic acid	130, 167
3.443	Maltose* and trans-Aconitate	129, 194
3.430	1,3-Dimethyluric acid and Taurine[a,c]*	4, 186
3.409, 3.418	Taurine[a,c]*	186
3.401	Itaconic acid[a,c]	123
3.373	Vanylglycol	206
3.364	Tryptaminium	199
3.355	Methanol* and Theophylline* and Tryptaminium	132, 188, 199
3.295	myo-Inositol* and Taurine[a,c]*	140, 186
3.282	1-Methyluric acid* and myo-Inositol* and Taurine[a,c]*	11, 140, 186
3.271	Betaine* and Taurine[a,c]*	64, 186
3.255	Trimethylamine N-oxide*	198
3.225	Acetylcholine*	49
3.218	Acetylcarnitine	48
3.210	Acetylcarnitine and Phosphocholine[a,c]*	48, 164
3.200	Octanoylcarnitine[c] and Tyrosine*	155, 201
3.190	Isobutyryl-L-carnitine and Tryptaminium and Tyrosine*	120, 199, 201
3.181	Anserine[c]* and Hexanoylcarnitine[c] and Methylmalonate and Tryptaminium	58, 110, 137, 199
3.172	5-Hydroxylysine[c] and N-Nitrosodimethylamine and Tryptaminium	38, 153, 199
3.165	1-Methylhistidine* and 5-Hydroxylysine[c] and Ethanolamine[a,c]	10, 38, 86
3.157	1-Methylhistidine* and Ethanolamine[a,c]	10, 86
3.150	Ethanolamine[a,c] and Phenylalanine*	86, 163
3.133	cis-Aconitate and Malonate*	70, 128
3.098	1-Methylhistidine* and 3-Nitrotyrosine	10, 34
3.065, 3.077, 3.088	1-Methylhistidine* and 3-Methylhistamine and 3-Nitrotyrosine and Tyrosine*	10, 29, 34, 201
3.052	Creatinine* and Tyrosine*	74, 201
3.038	Creatine* and Histamine[a]*	73, 111
3.026	Histamine[a]* and Phosphocreatine*	111, 165
3.015	Gabapentin[a,c] and Histamine[a]*	92, 111
2.928	N-Methylhydantoin*	152
2.864	N,N-Dimethylformamide[a,c]	141

2.841	Succinylacetone[a]	183
2.699, 2.795, 2.821	Aspartate	62
2.744	Dimethylamine*	83
2.736	Norspermidine[a,c]* and Sarcosine[a,c]*	154, 177
1.660, 1.669, 1.688, 2.653, 2.710, 2.719	Norspermidine[a,c]*	154
2.689	Aspartate and Malic acid*	62, 126
2.683	Citric acid	72
2.676	Aspartate and Phosphonoacetate*	62, 166
2.668	Malic acid* and Norspermidine[a,c]*	126, 154
2.661	Aspartate and Norspermidine[a,c]*	62, 154
2.657	Acetylcarnitine and Anserine[c]* and Citric acid	48, 58, 72
2.645	Phosphonoacetate*	166
2.566	Citric acid and Thiamine	72, 189
2.541	(+)-(S)-Carvone[c] and Acetylcarnitine and Thiamine	1, 48, 189
2.528	Acetylcarnitine and Citric acid	48, 72
2.515	Acetylcarnitine and Dimethylamine*	48, 83
2.501	Acetylcarnitine and Pyridoxal and Riboflavin[a,c]*	48, 170, 174
2.443, 2.479	3-Hydroxymethylglutaric acid and Glutamine	25, 100
2.468	Glutamine and Pyridoxine and Pyruvic acid*	100, 171, 173
2.431	Monomethyl glutaric acid and Gabapentin[a,c] and Glutamine and Succinylacetone[a]	139, 92, 100, 183
2.419	3-Hydroxymethylglutaric acid and Monomethyl glutaric acid and Succinylacetone[a]	25, 139, 183
2.407	Oxalacetic acid and Succinate	157, 182
2.396	2-Methylhippuric acid and 3-Hydroxymethylglutaric acid and Malic acid*	20, 25, 126
2.351, 2.385	Glutamate* and Malic acid*	99, 126
2.373	Glutamate* and Pyruvate*	99, 172
2.340	3-Methylindole and Glutamate*	31, 99
2.329	3-Methylphenylacetic acid and Glutamate*	32, 99
2.318	Glutamate* and Methyl propenyl ketone	99, 134
2.312	Methylacetoacetic acid	135
2.306	Cresol[a,c]	75
2.298	Acetoacetic acid*	47
2.289	Succinylacetone[a] and Valine*	183, 205
2.269	Cresol[a,c] and p-Cresol sulfate and Valine*	75, 162, 205
2.259	2-Aminoadipate[c] and 4-Hydroxy-3-methylbenzoic acid and Valine*	14, 35, 205
2.239	2-Aminoadipate c and 3-Methylglutarate	14, 28
2.170, 2.180, 2.195	Monomethyl glutaric acid	139
2.157	Acetylcarnitine and Glutamate* and Glutamine and Monomethyl glutaric acid	48, 99, 100, 139
2.143	Acetylcholine* and Glutamate* and Glutamine	49, 99,100

2.110, 2.132	2-Amino-3-phosphonopropionic acid* and Glutamate* and Glutamine	13, 99, 100
2.080	2-Amino-3-phosphonopropionic acid* and Glutamate*	13, 99
2.069	Glutamate* and N-Acetylneuraminic acid[a]	99, 148
2.059	Acetylglycine and Glutamate*	50, 99
2.048	Beta-N-Acetylglucosamine[a,c]* and Glutamate*	65, 99
2.028	Acetamide and Canthaxanthin and N1-Acetylspermine[a,c]* and Proline[c]*	45, 66, 142, 168
2.018	Proline[c]* and Selenomethionine[a,c]	168, 179
2.008	N6-Acetyl-L-lysine[a,c] and Proline[c]	143, 168
1.999	Canthaxanthin and Proline[c]	66, 168
1.991	3-Methylglutarate and Isoleucine[a,c]* and Proline[c]	28, 121, 168
1.981	5-Methylcytidine[a,c]* and Proline[c]	42, 168
1.961, 1.971	Proline[c]	168
1.927	Melatonin[a,c] and N-Acetyl-L-phenylalanine[a,c]	51, 131, 146
1.916	Hexadecanedioic acid[c]	109
1.910	5-Hydroxylysine[c] and Acetic acid* and Senecioic acid	38, 46, 180
1.782	Senecioic acid	180
0.974, 0.981, 1.761	2-Hydroxy-2-methylbutyric acid[a,c]*	16
1.752	(+)-(S)-Carvone[c] and 2-Amino-3-phosphonopropionic acid* and 2-Hydroxy-2-methylbutyric acid[a,c]*	1, 13, 16
1.727	2-Hydroxy-2-methylbutyric acid[a,c]* and (+)-(S)-Carvone c and 2-Amino-3-phosphonopropionic acid*	16, 1, 13
1.642, 1.702	Geraniol[a]	210
1.677	2-Amino-3-phosphonopropionic acid* and Norspermidine[a,c]*	13, 154
1.487	Alanine* and 2-Aminoisobutyric acid[a,c] and Gabapentin[a,c]	56, 15, 92
1.468	2-Hydroxy-2-methylbutyric acid[a,c]* and Isoleucine[a,c]*	16, 121
1.014, 1.258, 1.288, 1.453, 1.460	Isoleucine[a,c]*	121
1.442	Dimethylmalonic acid* and Isoleucine[a,c]*	84, 121
1.424	2-Phenylpropionate	21
1.362	Citramalic acid	71
1.278	2,2-Dimethylsuccinic acid* and Isoleucine[a,c]* and Methylmalonic acid	12, 121, 138
1.268	Isoleucine[a,c]* and Methylmalonic acid	121, 138
1.247	Isoleucine[a,c]* and Methylmalonate	121, 137
1.236	Methylmalonate	137
1.210	Dihydrothymine[a,c]	81
1.200	Canthaxanthin and Dihydrothymine[a,c]	66, 81
1.065, 1.076	2-Methylglutarate[a,c] and Ethyl isopropyl ketone[c]	19, 87
0.994	Valine* and 2-Hydroxy-2-methylbutyric acid[a,c]*	205, 16

0.954	2-Hydroxyvalerate[a,c] and Isoleucine[a,c]*	18, 121
0.933, 0.943	2-Hydroxyvalerate[a,c] and 3-Methylglutarate and Isoleucine[a,c]*	18, 28, 121
0.914	2-Hydroxyisocaproate[a,c]	17
0.897	Methyl isobutyl ketone[a,c]	133

All metabolites found in all three ages unless otherwise specified
[a] found in chicks
[b] found in 23-week-old chickens
[c] found in 45-week-old chickens
* Metabolite found using both extraction methods