Serum Proteomic Analysis of the Effects of Yeast Cell Wall Products on Broiler Chickens

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Declaration of Authorship

This thesis has not previously been submitted, in whole or in part, to this, or any other University, for any other degree. This thesis is the sole work of the author.

Niall Conlon BSc.

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Abbreviations

AP	Actigen®-Pak
AFBI	Agri-Food Biosciences Institute
ALT	Alanine Aminotransferase
A1AGP	Alpha-1-acid glycoprotein
AGP	Antibiotic Growth Promoters
ATP	Adenosine Triphosphate
AST	Aspartate Aminotransferase
AIV	Avian Influenza virus
CHAPS	(3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate)
CPLL	Combinatorial peptide ligand libraries
COST	Cooperation in Science and Technology
DIGE	Differential In Gel Electrophoresis
ELISA	Enzyme Linked Immunosorbent Assay
EO	Essential Oils
EU	European Union
emPAI	Exponentially Modified Protein Abundance Index
FAP	Farm Animal Proteomics
FASP	Filter Aided Sample Preparation
FDA	Food and Drug Administration
GO	Gene Ontology
GSH-Px	Glutathione Peroxidase
αGST	Glutathione-S-Transferase α
HSP	Heat Shock Protein
HAP	High Abundance Proteins
ID	Immunodepletion
IgA	Immunoglobulin A

IgG	Immunoglobulin G
IgY	Immunoglobulin Y
ICP-MS	Inductively Coupled Plasma Mass spectrometry
IL	Interleukin
LFQ	Label-Free Quantitative
LCR	LC-MS/MS Compatible Reagent
LC	Liquid Chromatography
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LAP	Low Abundance Proteins
MOS	Mannanoligosaccharides
MBL	Mannose Binding Lectin
MS	Mass Spectrometry
MALDI	Matrix Assisted Laser Desorption/Ionization
MQ	MaxQuant
NS	Natustat®
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PT	PowerTract®
PCA	Principal component analysis
PAI	Protein Abundance Index
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
ROS	Reactive Oxygen Species
RBP	Retinol Binding Protein
SeCys	Selenocysteine
SeMet	Selenomethionine
SDS	Sodium Dodecyl Sulfate
TRP	Transferrin receptor protein

TNF	Tumour Necrosis Factor
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
WHO	World Health Organisation
YCW	Yeast Cell Wall

Abstract

With an ever-growing market and continual financial pressures associated with the prohibition of antibiotic growth promoters, the poultry industry has had to rapidly develop non-antibiotic alternatives to increase production yields. A possible alternative is yeast and its derivatives, such as the yeast cell wall (YCW), which have been proposed to confer a number of beneficial effects on the host animal. Here, the effect of YCW supplementation on the broiler chicken was investigated using a quantitative proteomic strategy, whereby serum was obtained from three groups of broilers fed with distinct YCW feed supplements or a control basal diet. Development of a novel reagent enabled application of Proteominer™ technology for sample preparation and subsequent comparative quantitative proteomic analysis revealed proteins which showed a significant change in abundance (n = 167 individual proteins; p <0.05); as well as proteins which were uniquely identified (n = 52) in, or absent (n = 37) from, YCW-fed treatment groups versus controls. An average of 7.1% of proteins showed changes in abundance with YCW supplementation. A number of effects of these YCW supplements including immunostimulation (via elevated complement protein detection), potential alterations in the oxidative status of the animal (e.g., glutathione peroxidase & catalase), stimulation of metabolic processes (e.g., differential abundance of glyceraldehyde-3-phosphate dehydrogenase), as well as evidence of a possible hepatoprotective effect (attenuated levels of serum α-glutathione s-transferase) by one YCW feed supplement, were observed. It is proposed that specific protein detection may be indicative of YCW efficacy to stimulate broiler immune status, i.e. may be biomarkers of YCW efficacy. In summary, this work has developed a novel technology for the preparation of high dynamic range proteomic samples for LC-MS/MS analysis, is part of the growing area of livestock proteomics and, importantly, provides evidential support for beneficial effects that YCW supplementation has on the broiler chicken.

Chapter 1

Introduction

1.1 Overview of the Poultry Industry.

Currently, the global poultry industry produces approximately 100 million tonnes of meat and 73 million tonnes of eggs per annum (Motte & Tempio, 2017). The consumption of poultry products has increased threefold since 1970 with a 5% increase in annual production compared to 2.8% for total meat produce. This growth has allowed poultry meat to now represent 35% of global meat consumption. This rapid growth is expected to continue, with production of poultry meat expected to reach 181 million tonnes by 2050 (Alexandratos & Bruinsma, 2012).

In 2016, upwards of 23 billion poultry animals were farmed, and these fall into one of three production systems; Broilers, Layers and Backyard (Gerber *et al.*, 2013). Broilers are fully market-orientated chickens that have been selectively bred for meat production. They require high capital input but return high levels of flock productivity (Gerber *et al.*, 2013). Improvements in the growth and efficiency of this production system have resulted in the consumer price index for poultry meat growing at half the rate of all other products between 1960 and 2004, which is likely to have been a factor in the recent growth (Zuidhof *et al.*, 2014). Since first adapting genetic techniques to improve animal productivity in 1943, commercial breeding has seen massive improvements in broiler body weight, feed conversion rates and mortality rates (Hunton, 2006).

1.2 The use of Antibiotic Growth Promoters in Food Animals.

Antibiotics were first adopted as growth promoters in the 1950s after the discovery that their use could enhance growth efficiencies (Moore *et al.*, 1946). With the use of antibiotic growth promoters (AGP), weight gain may be observed, however, the main effect can be seen in feed efficiency (Coates *et al.*, 1955). A reduction in opportunistic pathogen infection, as well as sub-clinical infection, can also be seen with the use of AGPs (Dibner & Richards, 2005). The mode of action of AGP is via the modulation of gut microflora. This was shown in experiments by Coates *et al.* (1955) and (1963) which demonstrated that this enhancement of growth efficiency was not seen in germ-free animals. Adoption of AGPs became the norm throughout the poultry industry from the 1950s onwards (Dibner & Richards, 2005).

In 1969, the Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine recommended the discontinuation of a number of specific antibiotics due to antibiotic resistance concerns (Swann *et al.*, 1969). Information regarding the emergence of antibiotic resistance continued to surface throughout the 20th Century which led the World Health Organisation to publish two reports suggesting a link between antimicrobial growth promoter use in animal husbandry and antibiotic-resistant infection in humans, as well as recommending that Governments reduce the need for antimicrobials in animals (World Health Organization, 1997; World Health Organization, 2000).

Sweden was the first nation to adopt a ban of antimicrobials in 1986 (Wierup, 2001). This was followed by a number of bans of specific AGPs, such as Avoparcin in Denmark in 1995 (Aarestrup *et al.*, 2001) and the European Union (EU) with Council Directive 97/6/EC of 30 of January 1997 concerning additives in feeding-stuffs after a discovery of glycopeptide-resistant *Enterococci* (Bates *et al.*, 1993). Further bans were

introduced in Denmark as well as the EU in 1999, with bans on vancomycin and a number of antimicrobials used also to treat humans. In 2000, antimicrobials were restricted to therapeutic use in Denmark (Dibner & Richards, 2005).

Bans on the use of antibiotics in animal husbandry were introduced in the EU in 2006. The EU ratified Regulation (EC) No. 1831/2003 on additives for use in animal nutrition, which prohibited the use of coccidiostats and histomonostats. This came following statements by the Scientific Steering committee of the EU in 1999 and 2001 that the use of antimicrobials as growth promoting agents should be phased out as soon as possible in an effort to curb antibiotic resistance. The regulation allowed for "sufficient time such that alternative products to replace those antibiotics be developed" (European Commission, 2003). A two-year phasing out period led to an outright ban of antibiotic use in animal nutrition in January 2006.

In 2013 the United States Food and Drug Administration (FDA) issued a regulation that the "production use" of antibiotics should be eliminated in food-producing animals in an attempt to reduce the development of antibiotic-resistant bacteria (Food and Drug Administration, 2013). This ban came into effect in January 2017.

1.3 Performance Enhancing Alternatives to Antibiotic Growth Promoters

Regulation of the gut microbiome using prebiotics represents one alternative to the use of AGPs. These feed additives, or alternatives, have been seen to modulate the gut microbiota which can provide resistance to pathogenic bacteria as well as stimulate the immune system in a non-inflammatory manner thus improving the health of the animal and reducing the risk of food-borne disease (Gaggìa *et al.*, 2010).

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species in the colon (Gibson & Roberfroid, 1995). Dietary substrates must satisfy three criteria in order to be considered prebiotics: (i) the substrate must not be hydrolysed or absorbed in the stomach or small intestine, (ii) it must be selective for beneficial commensal bacteria in the colon and (iii) fermentation of the substrate should induce beneficial luminal/systemic effects within the host (Manning & Gibson, 2004).

1.3.1 Yeast and Yeast Derivatives in Poultry Industry

The use of yeast as a growth promoter was first reported in 1924 (Eckles *et al.*, 1924). Since then, yeast and its derivatives have been adopted as prebiotic growth promoters into many food-animal diets including ruminants, pigs and poultry (Gao *et al.*, 2003).

In the last number of decades, Yeast Cell Wall (YCW) products have been adopted in animal nutrition (Hooge, 2004). Approximately 30 - 60 % of YCW is composed of polysaccharides, of which the majority are β-glucans and mannanoligosaccharides (MOS). The remaining 40 - 70% of the YCW products are cell wall proteins, which are often complexed with MOS to yield mannoprotein complexes, lipids and chitin (Morales-Lopez *et al.*, 2009). A representation of the yeast cell wall can be seen in Figure 1.1.

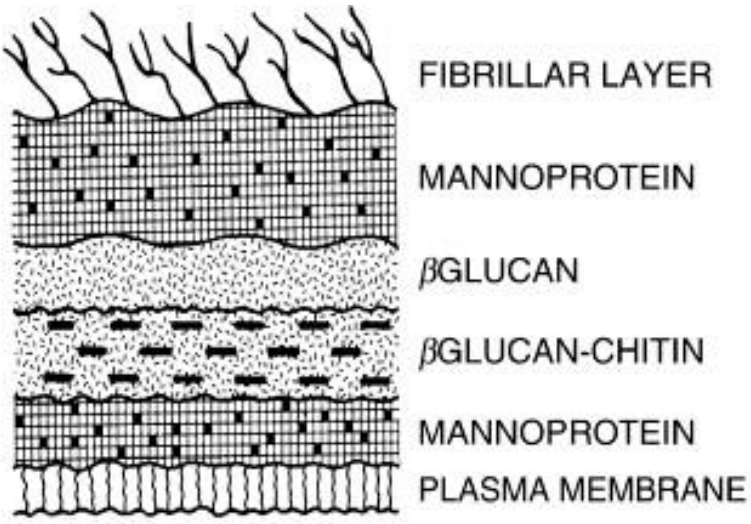

Figure 1.1: Schematic cross-section of the YCW showing major components (Kogan & Kocher, 2007).

1.3.2 β-glucans

β-glucans are naturally occurring polymers of glucose that are found in the cell wall of plants, bacteria and yeast. Typically found as either β-1, 3 glucan or β-1, 6 glucan these polysaccharides can have effects on the immune system of the host, and belong to a class of molecule known as biological response modifiers (Williams *et al.*, 1996). Immunological effects of these molecules have been seen in many species, including the broiler chicken (Abel & Czop, 1992; Chae *et al.*, 2006; Zokaeifar *et al.*, 2012).

In the early stages of the life of chickens, the immune system is underdeveloped and inept. β-glucans have been linked to enhancement of protective immunity during these early stages (Cox *et al.*, 2010). It has been suggested that many of these protective immunological effects are stimulated by the binding of β-glucans to monocyte and macrophage receptors which trigger an immunological cascade of events (Kogan & Kocher, 2007).

Supplementation of poultry diet with these polysaccharides has been found to have effects on both the innate and adaptive responses (Guo *et al.*, 2003). Proliferation and phagocytic activity of both macrophages and splenocytes have been seen to be improved in broiler chickens with β-glucan supplementation (Chen *et al.*, 2003). An activation of the humoral response has also been seen with rises in IgA and IgG levels recorded (Zhang *et al.*, 2008). T-cell levels were seen to be increased, with larger CD4+, CD8+, and CD4+:CD8+ T-cell populations found in β-glucan supplemented chickens (Chen *et al.*, 2003; Chae *et al.*, 2006). Several cytokines were found to be upregulated with β-glucan supplementation such as interleukin (IL)-1 (Gao *et al.*, 2003), IL-2 and Interferon (Zhang *et al.*, 2008). Increases in the size of primary and secondary lymphoid organs have also been seen (Gao *et al.*, 2003; Zhang, *et al*., 2008). Fungal components have been previously noted as potent activators of the complement system (Dlabač & Kawasaki, 1994; Bohn & BeMiller, 1995; Levitz, 2010; Song *et al.*, 2014; Mensink *et al.*, 2015).

1.3.3 Mannose Oligosaccharide

Mannose Oligosaccharide (MOS) is an indigestible sugar derived from the cell wall of a number of yeast species that has been linked to a wide variety of health benefits (Charachar *et al.*, 2017). These benefits include reducing the levels of pathogenic bacteria in the gut (Charachar *et al.*, 2017), enhancing growth of beneficial bacteria (Abel & Czop, 1992) and modulating the immune response (Shashidhara & Devegowda, 2003).

MOS have been found to modulate the gut microbiome by acting as high affinity ligands offering competitive binding sites for mannose-specific type-1 fimbriae of pathogenic bacteria (Spring *et al.*, 2000) and stimulating the production of mucins by goblet cells found in the digestive tract (Charachar *et al.*, 2017). Mucins are the

glycoprotein constituent of mucus (Pelaseyed *et al.*, 2014) which are involved in the binding and clearance of bacteria from the intestine. This reduction in pathogenic bacteria can create a more favourable environment for beneficial bacteria such as *Lactobacillus* and *Bifidobacterium spp.* in the broiler intestine (Baurhoo *et al.*, 2009).

The addition of MOS to the diet of broiler chickens can increase immune potential with improvements seen in cellular, humoral and mucosal immunities (Gomez-Verduzco *et al.*, 2009; Ozpinar *et al.*, 2010). Much like the effects of β-glucans, much of the immunostimulatory effect of MOS is mediated by the activation of macrophages. These macrophages exist in the intestine of poultry as part of the gut-associated lymphoid tissue and recognise pathogens through pathogen associated molecular pattern (PAMP) receptors (Shashidhara & Devegowda, 2003). Mannans from the YCW can bind these PAMP receptors which can lead to an improved cellular immune response (Chachar *et al.*, 2017). Ozpinar *et al.* (2010) showed significantly higher levels of IgG levels in broiler chickens supplemented with MOS compared to those supplemented with vitamin-based supplements.

The physiological effects of MOS can translate into improved performance of broilers. A number of studies have suggested that birds fed MOS as a supplement to a basal diet had significantly improved weight gain when compared to those fed the basal diet alone (Benites *et al*., 2008; Žikić et al., 2002; Shendare *et al*., 2008).

1.3.4 Potential of Essential Oils in Poultry Nutrition

Essential oils (EO) are a complex mix of plant secondary metabolites which are extracted from plant material such as roots, buds, leaves, flowers, bark, herbs, seeds, wood, and fruits (Greathead, 2003; Brenes & Roura, 2010). These products are often used as an additive in animal feed supplementation (Windisch *et al.,* 2008; Gong *et al.,* 2014; Zou *et al.,* 2016) and have been reported to convey a number of beneficial effects to the host animal. Natustat®, one of the YCW feed products used in this study, contains EO.

These phytogenic products have been previously reported to enhance the total antioxidative capacity of the host (Zeng *et al.,* 2015), stimulate the host immune system (Zeng *et al.,* 2015), stimulate digestive processes (Acamovic & Brooker, 2005) and regulate gut microflora, reducing levels of pathogenic bacteria (Jang *et al.,* 2007; Windisch *et al.,* 2008).

1.3.5 Role of Selenium in Poultry Nutrition

Selenium is an essential element in poultry nutrition and due to falling levels of selenium in soil due to commercial cropping, supplementation is essential in poultry nutrition (Peric *et al.,* 2009).

There are two main sources of selenium in poultry diet: inorganic selenium in the form of selenite or selenate and organic selenium in the form of selenomethionine (SeMet) (Surai & Fisinin, 2014). Organic selenium has been reported to be more bioavailable than its inorganic counterpart (Lönnerdal *et al.,* 2017). This trace element is essential for the production of selenoproteins which can participate in a number of physiological processes in production animals (Dalgaard *et al.,* 2018).

Selenium has been linked to the activation, proliferation and differentiation of cells that drive both the adaptive and innate immune responses (Huang *et al.,* 2012). Selenium deficiency was shown to impair thymus development in broiler chickens which reduced CD3+ and CD3+CD8+ T cell frequencies (Peng *et al*., 2011; Chang *et al*., 1994). Glutathione peroxidase is involved in signalling, peroxide scavenging and maintaining cellular redox homeostasis. Three of the eight glutathione peroxidases found in mammals contain selenocysteine residues at their catalytic site (Lubos *et al.*, 2015). Synthesis of selenoproteins, such as these glutathione peroxidases, is regulated by the availability of selenium and, in times of deficiency, synthesis of certain selenoproteins is reduced in favour of others (Howard *et al.,* 2013; Peric *et al.,* 2009). Macrophages stimulate cellular activation by controlling actin disassembly and re-assembly through the expression of methionine-R-sulfoxide reductase B1. This selenoprotein controls the state of actin through oxidation and reduction of methionine (Lee *et al.,* 2013). Selenium deficiency in poultry can lead to liver necrosis and muscular dystrophy (Koller & Exon, 1986).

1.4 Proteomics

Proteomics is the identification and analysis of the total protein in a biological system (Tsai *et al.,* 2015). Proteins provide a wealth of information about the status of an organism as they are direct functional molecules in living organisms (Zhang *et al.,* 2013). The examination of protein abundance, post-translational modification, structure as well as protein-protein interaction (Wright *et al.,* 2012) has allowed proteomics to become a powerful tool in the analysis of health status and disease.

Proteomic workflows involve two stages: separation of the proteomic sample through gel-based technologies or chromatographic techniques, and protein/peptide

identification using mass spectrometry(MS). Data analysis is then carried out using bioanalytical software and proteomic databases (Ramasamy *et al.,* 2014).

Proteomic analyses can be broken down into broad strategies, top-down and bottom-up (Ramasamy *et al.,* 2014). Top-down proteomics involves the analysis of specific intact proteins without proteolytic digestion (Campos & de Almeida, 2016). This technique has advantages such as post-translational modification and protein isoform identification (Zhang *et al.,* 2013). Bottom-up proteomics involves the analysis of complex proteomic samples using a combination of enzymatic digestion and high resolution chromatographic or electrophoretic techniques to deconvolute and analyse protein samples (Bogdanov & Smith, 2005; Gevaert *et al.,* 2007). Protein identification is then achieved by comparing mass spectra of fragmented peptides to the theoretical *in silico* digestion of the protein database (Zhang *et al.,* 2013). Bottom-up proteomic analysis has significant advantages in complex proteomic samples, such as overcoming the analytical challenges of analysing intact protein, which has led to it becoming the more universally adopted in modern proteomic analysis (Zhang *et al.,* 2013)

There are two predominantly used separation techniques in modern quantitative proteomics (Zhang *et al.,* 2013), shown in Figure 1.2: Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with an MS technique such as Matrix Assisted Laser Desorption/Ionization (MALDI) MS or Liquid Chromatography (LC) MS (Banks *et al.,* 2000), or Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS). 2D-PAGE was the first platform used in quantitative proteomics though limitations such as low resolution, labour intensive workflow and inadequate detection of low abundance proteins (LAP) in samples with large dynamic range, as well as the advancement of MS technology, have led LC-MS/MS-based analysis to become the preferred method (Zhang *et al.,* 2013). LC-MS/MS has now become the driving force in

the proteomic analysis of complex samples, delivering high throughput, in-depth proteomic analysis (Griffiths & Wang, 2009; Aebersold & Mann, 2003) and these developments have, in turn, fuelled the maturation of shotgun proteomics (Griffiths & Wang, 2009).

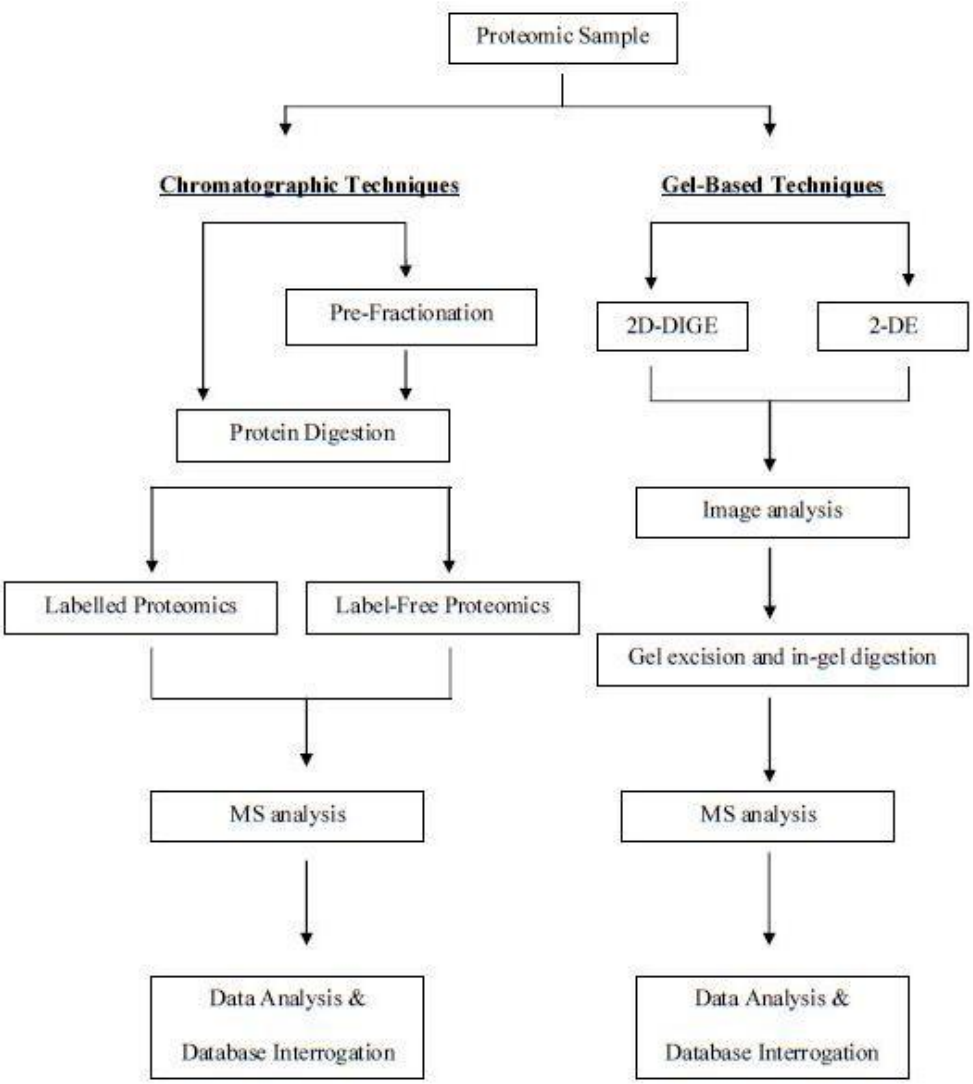

Figure 1.2. Workflow for quantitative proteomic analysis.

Label-free Quantitative (LFQ) proteomics has emerged as a high-throughput method for clinical proteomics (Griffiths & Wang, 2009). LFQ proteomics employs one of two methods to quantify protein abundance within a sample: Spectral Counting or Ion Intensity. In Spectral Counting, the protein abundance is correlated with the frequency of peptide spectral matches (Washburn *et al.,* 2001; Liu *et al.,* 2004; Gilchrist *et al.,* 2006). A protein abundance index (PAI) is constructed for each protein, which is the number of observed peptides divided by the number of theoretically identified peptides. This number is then converted to exponentially modified PAI (emPAI) which is used by bioanalytical software for protein quantification (Ishihama *et al.,* 2005). The Ion Intensity approach utilises the linear correlation between a peptide's peak area and its relative abundance to quantify changes in protein abundance. Peak areas can then be compared between conditions to give relative protein abundance (Bondarenko *et al.,* 2002; Chelius & Bondarenko, 2002).

Following LC-MS/MS, analysis algorithms must be used to annotate identified peptides and quantify protein changes. These algorithms can be developed or obtained as part of proteomic data analysis software packages (Drabik & Silberring, 2016). One such software package is MaxQuant. Maxquant is freely available software from the Max Planck Institue of Biochemistry (Germany). This software quantifies proteins using maximum peptide ratio information from extracted peptide ion signal intensities to accurately quantify fold changes in protein intensity over several orders of magnitude between comparator samples (Cox & Mann, 2008; Cox *et al.,* 2014).

1.5 Farm Animal Proteomics

Recently, proteomic investigation in farm animals has increased with a number of reviews published highlighting its potential (Bendixen *et al.,* 2011; Almeida & Bendixen, 2012; Di Girolamo *et al.,* 2014; Almeida *et al.,* 2015). This is largely due to the creation of the European Cooperation in Science and Technology (COST) action on Farm Animal Proteomics (FAP) which was formed to demonstrate the role proteomics can have in farm animal research (Rodrigues *et al.,* 2012). Despite this, farm animal proteomics still remains a minor component of proteomic study as a whole (Bili *et al.,* 2018). Livestock proteomic studies have largely focused on the understanding of traits linked to the animal welfare as well as the quality of food products such as meat and milk (Di Girolamo *et al.,* 2014; Almeida *et al.,* 2015).

1.5.1 Avian Proteomics

Despite the publication of the *Gallus gallus* genome in 2005 (International Chicken Genome Sequencing Consortium, 2005) and fowl species being a major food source to humans, avian proteomics has fallen behind that of other livestock (Almeida *et al.,* 2015).

Early avian proteomic research used MS to characterise tissues and structures, such as the components of the egg (Mann, 2007; D'Ambrosio *et al.,* 2008; Mann & Mann, 2011; Farinazzo *et al.,* 2009; Mann & Mann, 2008) and muscle meat (Bendixen, 2005). With developments in mass spectrometry and bioinformatic technologies in the last decade leading to advances in quantitative proteomics (Feng *et al.,* 2017), comparative proteomics has come to the forefront of proteomic research.

With these advances in proteomic technologies, a number of comparative avian proteomic investigations have been conducted in recent years. Proteomic analysis has

been used to investigate the effects of the stress of restraint and transport on chickens, results showed differential expression of proteins involved in cytoskeleton structure and carbohydrate metabolism (Hazard *et al.,* 2011). The effects of heat stress on broiler chickens was assessed by Zeng *et al.* (2013) revealing heat shock proteins HSP10 and HSP70 as potential biomarkers for heat stress in Muscovy and Pekin ducks. A proteomic study conducted by Zheng *et al.* (2014) on lean and obese Pekin ducks revealed a number of proteins related to glycolysis and ATP synthesis were increased in abundance in the obese group. Comparative proteomics has also been used to differentiate meat from different livestock species. Differences in the primary structure of proteins such as serum albumin, apolipoprotein and heat shock protein 27 were seen, which could be used as biomarkers for meat origin (Montowska & Pospiech, 2013).

1.6 Serum Proteomics

The blood of an animal flows through the entire body and contains a wealth of information regarding its pathological and physiological condition. Serum is a blood derivative which lacks cells and clotting factors (Issaq *et al.,* 2007; Biosa *et al.,* 2011). Serum proteins often originate from a variety of tissue and blood cells due to leakage or secretion which can provide valuable information on the status of the animal (Hu *et al.,* 2006).

Serum proteomics in livestock can be a useful tool for monitoring health status (Di Girolamo *et al.,* 2014). Investigations involving bovine, ovine, swine, avian and equine sera samples have been conducted on the effects of stress, infection as well as disease. Serum proteomic investigations by Marco-Ramell *et al.* (2011) and (2012) revealed changes in the oxidative status of cows in response to a harsher rearing habitat and elucidated actin as a potential biomarker for stress related to stocking density in pigs. Research conducted into *Mycobacterium* infection in cattle, using serum

proteomics revealed differential expression of α-1-acid glycoprotein and vitamin D-precursor in infected cows (Seth *et al.,* 2009). Serum proteomics has also been used to investigate a number of livestock diseases such as Spontaneous Equine Uveitis, which was investigated by Zipplies *et al*., (2009). Results showed reductions in the serum levels of Complement C1q and C4 as well as antithrombin in horses infected with this disease (Zipplies *et al*., 2009). Serum proteomic analysis of cows suffering from milk fever revealed upregulation of a number of proteins including serine peptidase inhibitor and endopin 2B (Xia *et al*., 2012).

A relatively small number of comparative proteomic studies have been conducted on avian serum in recent years (Table 1.1). Previous studies have principally focused on development, infection and disease. As far as could be found in the literature, no investigations using avian serum proteomics have been published on the effects of feed products on the broiler chicken.

Table 1.1. Previous proteomic studies conducted on avian serum/plasma.

Serum Proteomic Study	Year	Findings	Reference
Analysis of the effects of Lipopolysaccharide (LPS) on Broiler Chickens using Plasma proteomics.	2018	Pathways related with protein activation cascade and heterotopic cell-cell adhesion were affected by LPS-challenge.	Horvatić *et al.*, 2018
Analysis of the effects of LPS on Broiler Chickens using Plasma proteomics.	2016	Alterations in the abundance of proteins involved in immunomodulation, cytokine changes and defence mechanisms.	Packialakshmi *et al.*, 2016a
Analysis of the effects of Femoral Head Necrosis on Broiler Chickens using Plasma proteomics.	2016	Alterations in the abundance of proteins involved in Immunomodulation, nutrient transport and antimicrobial activity.	Packialakshmi *et al.*, 2016b
Analysis of the effects of Femoral Head Necrosis on Broiler Chickens using Plasma proteomics.	2015	Alterations in the abundance of proteins involved in transcription, angiogenesis and walking behaviour.	Packialakshmi *et al.*, 2015
Analysis of the effects of three strains of *Eimeria* on Broiler Chickens using serum proteomics.	2011	Elucidation of several candidate markers for early detection of *E. acervulina* infection.	Gilbert *et al*., 2011
Analysis of the effects of ovarian adenocarcinoma on Broiler Chickens using plasma proteomics.	2010	Ovomacroglobulin was increased in abundance across all time points in adenocarcinoma affected subject.	Hawkridge *et al.*, 2010
Analysis of the effects of Avian Pathogenic *E. coli* (APEC) on Broiler Chickens using serum proteomics.	2008	Outer Membrane Protein A was increased in abundance with APEC infection.	Tyler *et al.*, 2008
Investigation into changes in proteome of laying hens at different developmental stages.	2006	Alterations in the abundance of proteins involved in egg production	Huang *et al.*, 2006

1.6.1 Challenges in Serum Proteomics

One of the largest obstacles to serum proteomics is the large dynamic range of protein concentration. Current estimations are that protein concentration spans 10 orders of magnitude in serum samples, with biomarkers of disease and health status most often found in relatively low concentrations (Issaq *et al.,* 2007). Typical serum protein concentration in poultry has been described between 36 and 55 mg/ml (Bounous *et al.,* 2000) with the 22 most abundant proteins representing 99% of the total serum protein (Issaq *et al.,* 2007). Detection of lower abundance proteins (LAP) by MS technology can be obscured by the presence of high abundance proteins (HAP), such as serum albumin in the case of serum analysis (Anderson & Anderson, 2002; Ray *et al.,* 2011).

This suppression in detection of LAP occurs due to the mode of action of MS technologies. When ionised peptides enter the mass spectrometer, the most abundant peptides are isolated, individually fragmented and the mass/charge ratio of these daughter ions is obtained (MS/MS scan). The data obtained from these MS/MS scans is then used by bioanalysis software to predict peptide sequences. The next set of most abundant ions are then determined and separated for MS/MS and this process is repeated throughout the MS analysis. An ion exclusion list is collected to ensure the most abundant ions are not repeatedly collected for MS/MS. A highly complex proteomic sample with large dynamic range will have thousands of peptide ions detected in each MS scan resulting in MS/MS scans on a small percentage of the total ions present. This can result in excellent coverage of HAP but poor identification of LAP (Huber *et al.,* 2003; Brunet *et al.,* 2004; Reinhardt & Lippolis, 2006; Zolotarjova *et al.,* 2008; Lippolis & Nally, 2018).

Hence, in order to attain adequate coverage of the serum proteome, the use of serum pre-fractionation to selectively deplete interfering HAP or enrichment techniques

to augment LAP is vital (Millioni *et al.,* 2011; Doucette *et al.*, 2011). These steps must have good reproducibility and reasonable cost and time effectiveness to allow sufficient number of test sample preparations (Baiwir *et al.,* 2015)

1.6.2 Methods for reducing dynamic range in the serum proteome

The two main approaches in dynamic range reduction are selective reduction of specific HAP and enrichment of LAP using combinatorial ligand libraries (Zhang *et al.,* 2014).

The reduction of specific HAP approach exploits the disparity in serum protein concentration in which a small number of proteins are at extremely high concentration (Zheng *et al.,* 2014) and reduction of these specific HAP can improve the detection of lower abundance proteins in serum (Pieper *et al.,* 2003; Echan *et al.,* 2005). Reduction of specific HAP is typically achieved using commercial immunodepletion (ID) kits capable of binding and depleting the 7 (Hu-7, Agilent Technologies), 14 (Seppro IgY14, Sigma Aldrich) (MARS Hu-14, Agilent Technologies) or 20 (Proteoprep20, Sigma) most abundant serum proteins (Millioni *et al.,* 2011). However, due to the lack of suitable antibodies for most animal species (Di Girolamo *et al.*, 2014; Henning *et al.,* 2015), the ID approach is often not an option for use with animal serum. Production of kits for the major farm animals could be a valuable aid in livestock biomarker discovery (Almeida *et al.,* 2015).

Combinatorial peptide ligand libraries (CPLL) are made up of highly diverse synthetic hexapeptides capable of binding most, if not all of any given proteome and enriching LAP (Righetti *et al.,* 2012; Righetti *et al.,* 2015). These hexapeptide ligands are bound to poly(hydroxymethacrylate) substrate beads, in a 1:1 peptide:bead ratio (Righetti & Boschetti, 2007; Marco-Ramell & Bassols, 2010). These peptide ligands are synthetically produced through a 'split, couple, randomise' procedure. This procedure

randomly generates peptide ligands using 20 amino acids, theoretically resulting in 64 million different ligands bound to individual beads (Dowling *et al.,* 2015). Hexapeptide ligands are assumed at equimolar amounts and due to the limited number of ligand-bound beads, HAP saturate their equivalent ligands and surplus free protein is eluted (Zheng *et al.,* 2014). Lower abundant proteins, which have not saturated their corresponding ligands, consequentially become quantitatively bound (Gianazza *et al.,* 2016) and the dynamic range of the proteomic sample, reduced (Capriotti *et al.,* 2012). The enriched sample can then be eluted from their equivalent ligands yielding a proteomic sample with reduced dynamic range.

The first use of these ligand libraries in a proteomic study was published in 2005 (Thulasiraman *et al.,* 2005), and since then they have been used in the proteomic enrichment of many sample types such as: urine (Castagna *et al.,* 2005), red blood cells (Roux-Dalvai *et al.,* 2008), chicken egg white (D'Ambrosio *et al.,* 2008), plasma (Dowling *et al.,* 2015) and serum (Sennels *et al.,* 2007; Baiwir *et al.,* 2015; de Jesus *et al.,* 2017).

Proteominer™ (Bio-Rad Laboratories, CA, USA) is a commercial CPLL based protein enrichment technology capable of 'amplifying' lower abundance proteins in complex proteomic mixtures (Righetti *et al.,* 2006; Boschetti *et al.,* 2007; Righetti & Boschetti, 2007; Boschetti & Righetti, 2008) and allows species-independent enrichment of minor serum components (Righetti & Boschetti, 2007; Marco-Ramell & Bassols, 2010; Di Girolamo *et al.,* 2014). This process is represented in Figure 1.3.

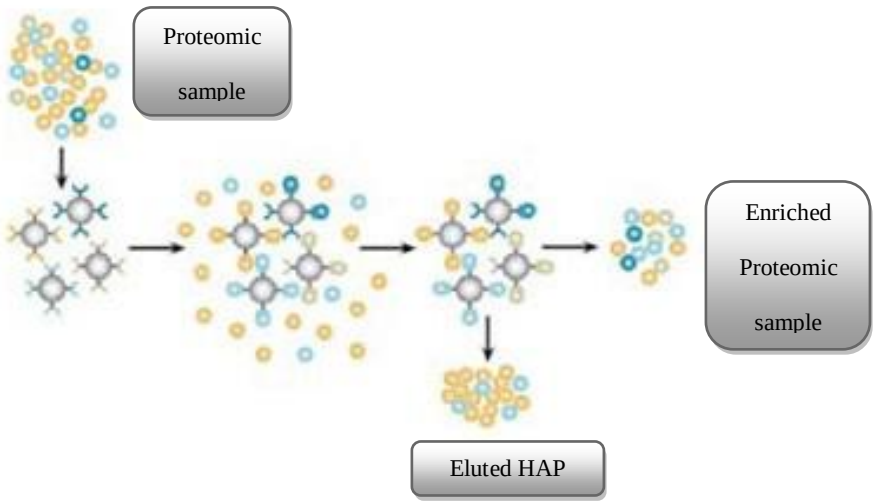

Figure 1.3: Representation of the reduction of the dynamic range of a proteomic sample using Proteominer™ technology. Adapted from Bio-Rad Laboratories, Inc representation of Proteominer™ technology.

Using CPLL as the method of dynamic range reduction, serum proteomics will be carried out on serum samples obtained from broilers fed a diet supplemented with one of three YCW-based products. This novel work could elucidate *in vivo* effects of the YCW and lead to advancements in the field of livestock feed supplementation.

The objectives of the work presented in this thesis are to:

i. Develop and optimise a reproducible method for the preparation and quantitative proteomic analysis of avian serum.
ii. Through serum proteomics, examine the effects of YCW feed on broiler chickens, through comparison with a control basal feed.
iii. Compare and contrast the effects of three individual, YCW-based, feed products.

Chapter 2

Materials & Methods

2.1 Materials

All products listed were obtained from Sigma-Aldrich Chemical Co. Ltd (U.K.) unless otherwise stated.

2.1.1 Solutions for pH Adjustment

2.1.1.1 5 M Hydrochloric Acid (HCl)

Hydrochloric acid (43.64 ml) was added to deionised water (40 ml) in a glass graduated cylinder. The final volume was adjusted to 100 ml with deionised water. The solution was stored at room temperature.

2.1.1.2 5 M Sodium Hydroxide (NaOH)

NaOH (20 g) was added to deionised water (80 ml). The solution was mixed and brought to a final volume of 100 ml with deionised water. The solution was stored at room temperature.

2.1.2 Serum Enrichment Reagents.

2.1.2.1 ProteoMiner™ Protein Enrichment Kit - Small Capacity (163-3006; BioRad)

Each kit contains 10 small capacity ProteoMiner™ spin columns, Wash Buffer (50 ml) (Phosphate Buffered Saline), Elution Reagent (8 M Urea, 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS)) (Lyophilised), Elution Reagent Rehydration Buffer (5 ml) (5% Acetic Acid). Capless collection tubes x 20. Capped collection tubes x 10. Kit stored at 4°C.

2.1.2.2 0.1 M Trizma® hydrochloride

Trizma® hydrochloride (1.57g) was dissolved in deionised water (80 ml). The solution was then adjusted to 100 ml using deionised water. The solution was stored at room temperature.

2.1.2.3 0.1 M Trizma® base

Trizma® Base (1.21g) was dissolved in deionised water (80 ml). The solution was then adjusted to 100 ml using deionised water. The solution was stored at room temperature.

2.1.2.4 0.1 M Trizma® pH 8.0

0.1 M Trizma® base (50 ml) (Section 2.1.2.3) was adjusted to pH 8.0 using 0.1 M Trizma® hydrochloride (Section 2.1.2.2). The solution was stored at room temperature.

2.1.2.5 LC-MS/MS Compatible Reagent (LCR)

6M Urea (36 g), 2M Thiourea (14.4 g) were dissolved in 0.1 M Trizma® pH 8.0 (Section 2.1.2.4) (40 ml). The solution was then made up to 50 ml using 0.1 M Trizma® pH 8.0 (Section 2.1.2.4) The solution was stored at room temperature.

2.1.2.6 0.15 M Trizma® hydrochloride

Trizma® hydrochloride (2.35 g) was dissolved in deionised water (80 ml). The solution was then adjusted to 100 ml using deionised water. The solution was stored at room temperature.

2.1.2.7 0.15 M Trizma® base

Trizma® Base (1.82 g) was dissolved in deionised water (80 ml). The solution was then adjusted to 100 ml using deionised water. The solution was stored at room temperature.

2.1.2.8 0.15 M Trizma® pH 9.0

0.15 M Trizma® base (50 ml) (Section 2.1.2.7) was adjusted to pH 9.0 using 0.15 M Trizma® hydrochloride (Section 2.1.2.6). The solution was stored at room temperature.

2.1.2.9 Label-Free solubilisation Buffer

8 M Urea (24 g) was dissolved in 0.15 M Trizma® buffer pH 9.0 (section 2.1.2.8) (40 ml). The solution was then made up to 50 ml using 0.15 M Trizma® buffer pH 9.0. The solution was stored at room temperature.

2.1.2.10 Sample Buffer

Acetonitrile (10 ml) and Trifluoroacetic Acid (TFA) (1 ml) were added to deionised water (30 ml). The solution was then made up to 50 ml using deionised water. The solution was stored at room temperature.

2.1.3 Protein Digestion Reagents

2.1.3.1 50 mM Ammonium Bicarbonate (NH_4HCO_3)

NH_4HCO_3 (197 mg) was dissolved in deionised water (50 ml). The solution was prepared immediately before use.

2.1.3.2 0.05 M Dithiothreitol (DTT)

DTT (77 mg) was dissolved in 50 mM Ammonium Bicarbonate (1 ml) (Section 2.1.3.1). The solution was then diluted one in ten by adding 0.1 ml of the DTT solution to 0.9 ml 50 mM Ammonium Bicarbonate buffer. The solution was prepared immediately before use.

2.1.3.3 0.11 M Iodoacetamide (IAA)

IAA (102 mg) was dissolved in 50 mM Ammonium Bicarbonate (1 ml) (Section 2.1.3.1). The solution was then diluted one in ten by adding 0.1 ml of the IAA solution

to 0.9 ml 50 mM Ammonium Bicarbonate buffer. The solution was prepared immediately before use and protected from light.

2.1.3.4 Lys-C (0.25 µg/µl)

Sequencing Grade Lys-C (20 µg) (90051 Thermo Scientific) was dissolved in 80 µl of deionised water. The solution was kept on ice until use. Remaining solution was aliquoted and snap-frozen using liquid nitrogen. Snap-frozen aliquots were stored at -20°C.

2.1.3.5 Trypsin (0.25µg/µl)

Sequencing Grade Trypsin (20µg) (V5111 Promega) was dissolved in 50 mM acetic acid (80 µl) (supplied with Trypsin). The solution was kept on ice until use. Remaining solution was aliquoted and snap-frozen using liquid nitrogen. Snap-frozen aliquots were stored at -20°C.

2.1.3.6 0.25% (w/v) ProteaseMax™

ProteaseMax™ (1 mg) (Promega) was dissolved in 50 mM Ammonium Bicarbonate (2.5 ml). The solution was kept on ice until use. Remaining solution was aliquoted and snap frozen using liquid nitrogen. Snap frozen aliquots were stored at -20°C.

2.1.4 Zip-tipping Reagents

2.1.4.1 Zip-tip Resuspension Buffer

TFA (5 µl) was added to deionised water (995 µl). The solution was prepared immediately before use.

2.1.4.2 Zip-tip Wetting Buffer

TFA (1 µl) was added to LC-MS grade Acetonitrile (800 µl) and deionised water (199 µl). The solution was prepared immediately before use.

2.1.4.3 Zip-tip Equilibration Buffer

TFA (1 µl) was added to deionised water (999 µl). The solution was prepared immediately before use.

2.1.4.4 Zip-tip Wash Buffer

TFA (1 µl) was added to deionised water (999 µl). The solution was prepared immediately before use.

2.1.4.5 Zip-tip Elution Buffer

TFA (1 µl) was added to LC-MS grade Acetonitrile (600 µl) and deionised water (399 µl). The solution was prepared immediately before use.

2.1.5 Q-Exactive: LC-MS/MS Solvents

2.1.5.1 Solvent A: 0.1% (v/v) TFA in Acetonitrile (3%(v/v))

LC-MS grade Acetonitrile (30 ml) and TFA (1 ml) were added to deionised water (969 ml). The solution was prepared immediately before use.

2.1.5.2 Solvent B: 0.1% (v/v) TFA in Acetonitrile (80% (v/v))

Deionised water 199 ml and TFA (1 ml) were added to LC-MS grade Acetonitrile (800 ml). The solution was prepared immediately before use.

2.1.6 Protein Characterisation Reagents

2.1.6.1 Phosphate Buffered Saline (PBS)

Ten PBS tablets (Oxoid) were dissolved in deionised water (1L). The solution was sterilised by autoclaving at 121°C for 15 min and stored at room temperature.

2.1.6.2 Bradford Solution

Bradford reagent (BioRad) was diluted 1/5 using Phosphate Buffered Saline (PBS) Section (2.1.6.1). This solution was prepared immediately before use.

2.2 Methods

2.2.1 Experimental Design, Sample Collection and Preservation

A total of 492 day-of-hatch male broiler chickens were used in the poultry feeding trial. Clean concrete-floor pens were used to house the birds in a medium scale trial facility on-site at Agri-Food Biosciences Institute (AFBI) (Belfast, UK). Animals were randomly split into four groups of 3 pens, with 12 pens in total (41 birds/pen; 123 birds/group) using a randomized complete block design. The pens were divided into four groups: group 1, fed a basal diet; group 2-4, fed a basal diet which included supplements 1, 2 and 3 respectively, at the manufacturers recommended inclusion levels (supplement 1 = Natustat® (Alltech Inc. supplement 2 = Actigen®-pak (Alltech Inc.), supplement 3 = PowerTract® (Alltech Inc.)). These supplements were mannan-rich fractions extracted from the yeast cell wall of *Saccharomyces cerevisiae*. Basal diets were prepared by a commercial feed mill and consisted primarily of wheat and soybean meal, as outlined in Table 2.1. Starter diets were fed from day 0 to day 10, grower diets from day 11-25 and finisher diets, day 26 to day 35. Feed and water were provided *ad libitum* throughout the study. Each pen was dressed with fresh litter for bedding from day zero. The temperature was initially set at 30^{o}C per day up to day 10 and then decreased linearly by 1 ^{o}C every second day. During the experiment, the birds received a lighting regimen of 16 h light and 8 h darkness until day 35. All conditions were kept uniform for all four groups. On days 7, 21 and 35, blood samples from necropsied birds were collected into 100 ml sterile sample cups (75.562.105; Sarstedt) and then transferred to BD Vacutainer® blood collection tubes using 10 ml wide-bore serological pipettes (86.1688.010; Sarstedt). The whole blood was allowed to clot at room temperature for 30 – 60 min. The clot was removed by centrifugation at 4500 rpm for 10 min at 4 °C. The resulting serum supernatant was apportioned into 0.5 ml aliquots

and snap frozen using liquid nitrogen. Aliquots were stored at -80 °C and thawed on ice before use. These steps are shown in Figure 2.1. All procedures were subject to the approval of the local Animal Welfare Ethics Review Board and subsequent approval by a Home Office Inspector. All procedures were carried out under the strict guidelines of the Animal (Scientific Procedures) Act 1986 (Corrigan & Corcionivoschi, 2017).

Table 2.1. Basal diet composition of starter, grower and finisher rations, obtained from Corrigan & Corcionivoschi (2017).

Ingredients	**Starter**	**Grower**	**Finisher**
Wheat	54.62	57.55	61.30
Soya	12.00	12.00	12.00
Soybean meal	25.00	21.00	17.00
Limestone	0.72	0.70	0.50
Di-calcium phosphate	1.65	2.00	2.15
Soyabean oil	4.00	5.00	5.50
Salt	0.20	0.20	0.20
Sodium bicarbonate	0.20	0.17	0.16
DL-Methionine	0.49	0.44	0.38
L-Lysine	0.37	0.32	0.28
Threonine	0.25	0.13	0.03
Vitamin-mineral premix	0.50	0.50	0.50
Nutrient analysis % or as indicated			
Metabolizable Energy (Kcal/kg)	2999.00	3081.00	3133.80
Crude Protein	23.12	21.53	20.04
Lysine	1.45	1.31	1.17
Methionine + Cysteine	1.09	1.00	0.91
Calcium	0.97	0.91	0.85
Available Phosphorous	0.49	0.41	0.41
Vitamin-Mineral Premix			
Copper (mg)	16	16	16

Iodine (mg)	1.1	1.1	1.1
Iron (mg)	30	30	30
Manganese (mg)	110	110	110
Selenium (mg)	0.3	0.3	0.3
Zink (mg)	105	105	105
Synergen (g)	200	200	200
Vitamin A (IU)	13000	11000	10000
Vitamin D3 (IU)	5000	4750	4500
Vitamin E (IU)	80	60	50
Vitamin K (mg)	3	3	2.5
Thiamin (B1) (mg)	3	2.5	2
Riboflavin (B2) (mg)	9	7	6
Niacin (mg)	60	55	50
Pantothenic Acid (mg)	20	15	12
Pyridoxine (B6) mg	5	4	3
Biotin (mg)	0.25	0.225	0.2
Folic Acid (mg)	2	1.8	1.6
Vitamin B12 (µg)	200	175	150
Vitamin C (mg)	200	200	200
Choline (mg)	500	450	400

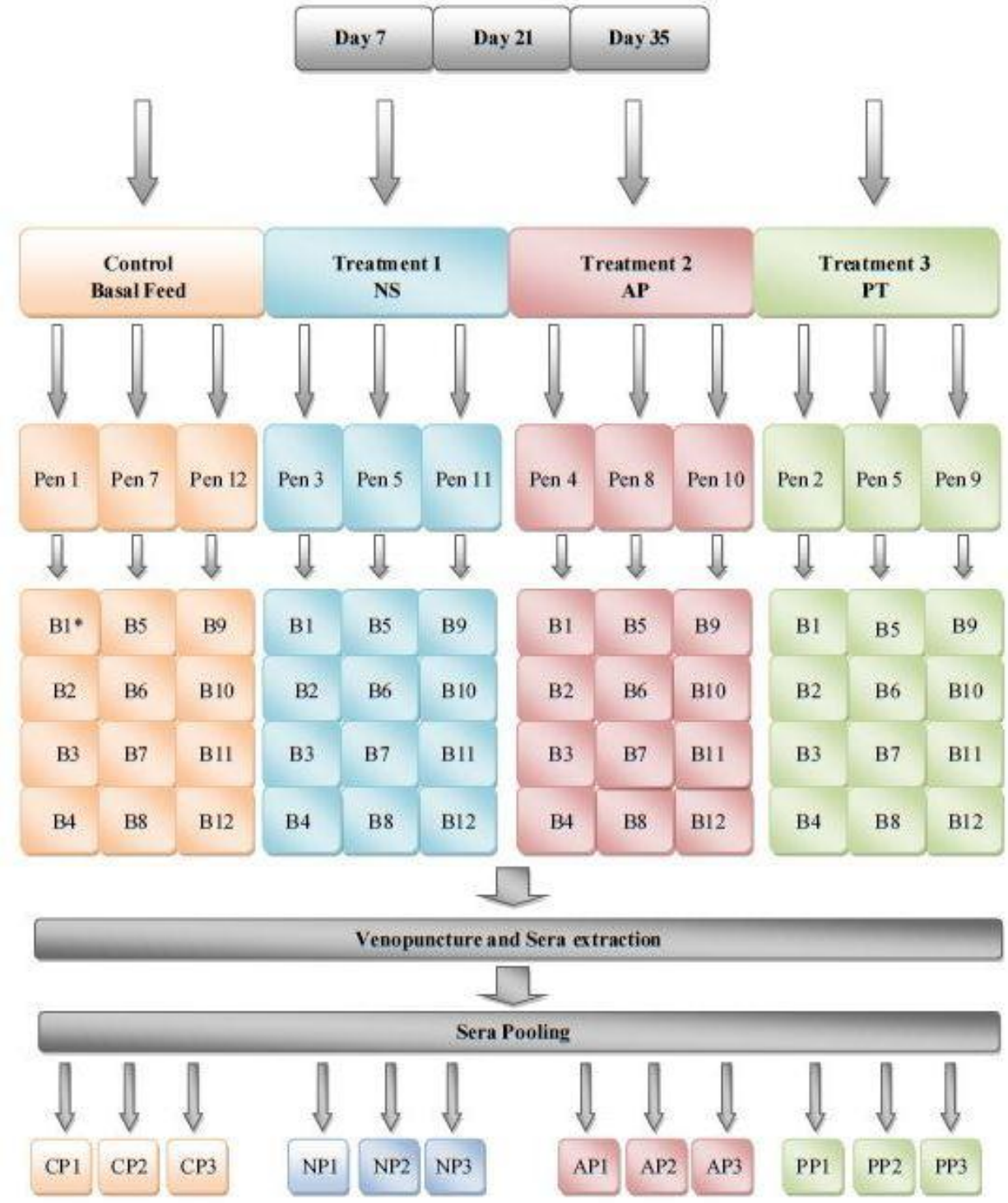

Figure 2.1. Overview of sera sample collection, preparation and analysis from sample collection to serum pooling as described in section 2.2.1. * CP denotes Control Pen, NP denotes Natustat Pen, AP denotes Actipak Pen and PP denotes PowerTract Pen.

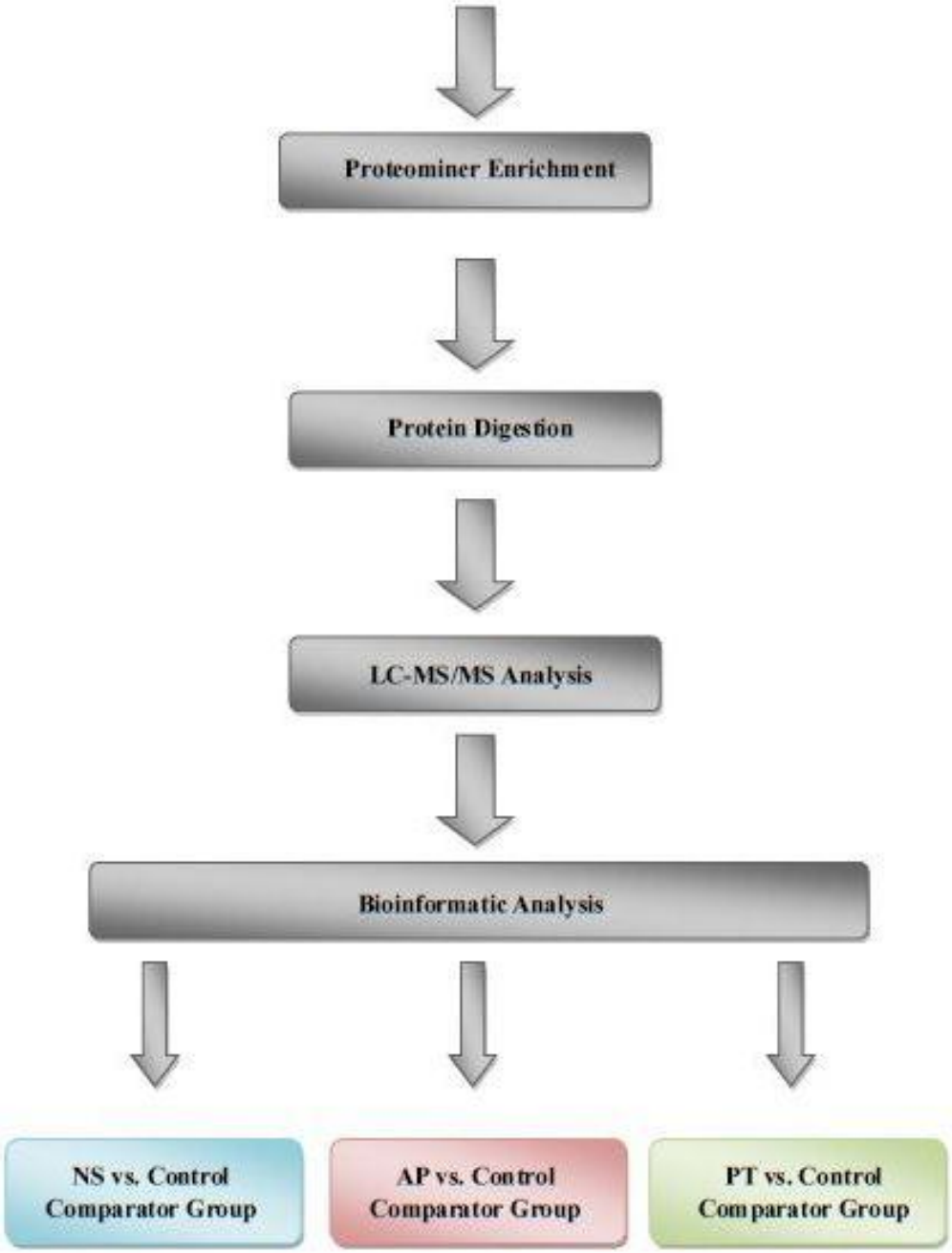

Figure 2.1 (continued). Overview of sera sample collection, preparation and analysis from Proteominer enrichment to Bioinformatic Analysis, as described in section 2.2.1.

*B1 denotes Bird 1.

2.2.2 Protein Characterisation Methods

2.2.2.1 Bradford Protein Assay

Bradford reagent (Bio-Rad) was diluted 1 in 5 in PBS (Section 2.1.7.1) prior to use. Protein samples to be assayed was diluted appropriately, and the sample (20 µl) was added to diluted Bradford reagent (980 µl) followed by vortexing. The final sample (1 ml) was then transferred to a plastic cuvette (1.5 ml) and incubated at room temperature for 5 min. The absorbance (595 nm) was read using a spectrophotometer (BioPhotometer; Eppendorf) relative to an appropriate blank. Protein concentrations were then determined based on a standard curve (0.1 - 1.5mg/ml)

2.2.3 ProteoMiner™ Serum Enrichment

All pooled serum samples were first cleared of precipitate by centrifugation (5415 D; Eppendorf) (10,000 g 10 min). Serum samples (200 µl) were then applied to the Proteominer™ Protein Enrichment kit (163-3006; BioRad) according to manufacturer's instructions. ProteoMiner™ enrichment columns were first drained of storage solution by removing caps and placing columns in a capless collection tube and centrifuging (Minispin; Eppendorf) (1000 g, 1 min). Collected material was discarded. Columns were then washed by replacing caps and applying 200 µl wash buffer. Columns were rotated end-over-end several times for 5 min and then drained through centrifugation (1000 g, 1 min). This wash step was repeated twice. Precipitate-free serum samples (200 µl) were then applied to the washed ProteoMiner™ columns. Columns were rotated for 2 h at room temperature on a rotational shaker. Columns were drained of sera by removing caps and placing columns in a capless collection tube and centrifuging (1000 g, 1 min). Columns were then washed by replacing caps and applying 200 µl wash buffer. Columns were again rotated end-over-end several times over 5 min and then

drained through centrifugation (1000 g, 1 min). This wash step was repeated twice. Deionised water (200 µl) was applied to columns which were rotated end-over-end for 1 min and drained through centrifugation (1000 g, 1 min). 20 µl of either the provided ProteoMiner elution buffer or LCR (Section 2.1.2.5) was applied to the column. Columns were then lightly vortexed for 5 s over 15 min. Caps were removed and columns placed in capped collection tubes and centrifuged (1000 g, 1 min). This elution step was repeated twice giving 60 µl of enriched serum sample. Enriched serum samples were stored -20°C until further processing.

2.2.4 Mass Spectrometry Methods

2.2.4.1 Protein Digestion from Q-Exactive Liquid Chromatography - Mass Spectrometry (LC-MS/MS) analysis

Enriched serum samples were removed from -20 °C and allowed to reach room temperature. Any precipitate was resuspended through light vortexing. Samples were tested for protein concentration by Bradford assay (Section 2.2.3.1). 10 µl of the enriched sample was then separated. Remaining samples were returned to -20°C storage. The pH of the sample was then adjusted to between 8.5 and 9.0 using 3 to 5 volumes of Label-Free Solubilisation Buffer (Section 2.1.2.9). The volume of diluted enriched serum sample corresponding to 5 µg protein was then removed for further processing. Protein samples (5 µg each) were then brought to 10 mM DTT using 0.05 M DTT (Section 2.1.3.2) and incubated at 56°C for 30 min. Samples were then brought to 25 mM IAA using 0.11 M IAA (Section 2.1.3.3). Lys-C (Section 2.1.3.4) (0.5 µl) was then added to samples at 1:100 ratio (Protease: Protein) and samples were incubated at 37°C for 4 h. 50 mM Ammonium Bicarbonate (3 volumes) was then added to samples. Trypsin (Section 2.1.3.5) (0.8 µl) was then added to samples at 1:25 ratio (Protease:

Protein). Protease Max (Section 2.1.3.6) (0.45 µl) was then added. Samples were incubated at 37°C overnight. Using Sample Buffer (Section 2.1.2.10) (0.33 volumes) samples were then diluted. Peptide samples were evaporated to dryness in a SpeedyVac™ (DNA 120; Thermo Scientific) and stored at -20°C.

2.2.4.2 ZipTip® Pipette Tip Protocol

Peptide samples were resuspended in Resuspension buffer (20 µl) (Section 2.1.4.1) and sonicated for 2 min to aid peptide resuspension. Samples were then centrifuged briefly to collect peptide digests. ZipTips were wetted by aspirating and dispensing Wetting buffer (10 µl) (Section 2.1.4.2) into the tip using a pipette. This was repeated five times. The ZipTip was equilibrated by aspirating and dispensing Equilibration buffer (10 µl) (Section 2.1.4.3) into the tip using a pipette. This was repeated five times. Peptide samples were then individually applied to the ZipTips by aspirating and dispensing resuspended samples (10 µl) into the tip using a pipette. This was repeated fifteen times. The ZipTip bound peptide sample was then washed by aspirating Wash buffer (10 µl) (Section 2.1.4.4) into the tip and dispensing into a waste container, using a pipette. This was repeated five times. ZipTip bound peptides were then eluted by aspirating Elution buffer (10 µl) (Section 2.1.4.5) into the tip and dispensing into a clean Eppendorf tube, using a pipette. This was repeated five times. Eluted samples were evaporated to dryness in a SpeedyVac™ (DNA 120; Thermo Scientific) and stored at -20°C. On the day of analysis, samples were resuspended in Buffer A (20 µl) (Section 2.1.5.1). Samples were sonicated for 2 min to aid peptide resuspension. The samples were then centrifuged (minispin; Eppendorf) (13,400 rpm for 10 min). The peptide sample (15 µl) was then transferred to a Q Exactive vial for injection.

2.2.4.3 Q-Exactive: LC-MS/MS Analysis of Protein samples

Peptide samples were analysed using a Thermo Scientific Q Exactive connected to a Dionex Ultimate 2000 (RSLnano) chromatography system. Each sample was loaded onto an EASY-Spray PepMap RSLC C18 column (75 μm x 500 mm), and separated by an increasing acetonitrile gradient over 120 min flow rate of 250 nl/min. The mass spectrometer was operated in positive mode with MS^n carried out on the 15 most abundant precursor ions at each time point. Singly charged ions were excluded from analysis.

2.2.5 Bioinformatic Analysis

2.2.5.1 Label-Free quantitative (LFQ) proteomic analysis using MaxQuant and Perseus

Peptide-Spectral mapping and protein peptide matching of raw files from Q-Exactive analysis of LFQ proteomic analysis were carried out using MaxQuant (version 1.5.7.0; http://maxquant.org) (Tyanova *et al.,* 2015). This utilised the Andromeda database search to match MS/MS data with the *Gallus gallus* 9031 reference proteome (UP000000539 - *Gallus gallus*) from UniProt (http://uniprot.org). Search parameters included: peptide tolerance (ppm) of 20 for first search and 4.5 for main search, carbidomethylation of cysteine as a fixed modification, oxidation of methionine and acetylation of N-termini as variable modifications, maximum 2 missed cleavage sites, and a minimum 1 peptide detected per protein. The maximum protein/ peptide false discovery rates were set at 1% based on a comparison to a reverse database (decoy database). The LFQ algorithm was used to generate normalised spectra intensities to infer relative protein abundance.

Subsequently, protein groups were exported and processed in Perseus (version 1.5.6.0; http://coxdocs.org/doku.php?id=:perseus:start) for data filtering (Tyanova *et al.,* 2016). Proteins (i) only identified by site (ii) only identified by modification site, or (iii) identified by the decoy database were removed. Extracted LFQ intensities measured for each run were grouped according to treatment and time point. Protein abundances were $\log_2$ transformed. At each time point (Day 7, Day 21 and Day 35) treatment groups were compared to the control group creating three comparator groups (AP vs. Control, NS vs. Control and PT vs. Control). A 2-sample t-test was performed to identify proteins with significant ($p < 0.05$) within each comparator group. Proteins uniquely detected in a treatment group and absent from control or proteins absent from treatment and present in control groups were identified and tabulated.

Pathway mapping was carried out using Reactome Pathway Database (https://reactome.org/). Proteins were specifically analysed for features and/or functions in UniProt (http://uniprot.org).

2.2.5.2 Software Graphing and Statistical Analysis

All statistical analyses and graphing was carried out using GraphPad Prism (version 5.0; GraphPad Software Inc.) or Microsoft Excel.

Chapter 3

Optimisation of Serum Pre-fractionation Strategy

3.1 Introduction

Proteins possess tremendous importance in the analysis of the status of living organisms as they are direct biofunctional molecules and changes in protein abundance can be indicators of an animal's wellbeing (Zhang *et al.*, 2013). Consequently, the identification and quantification of changes in protein concentration is an effective means of investigating the health and disease status of subjects.

With very little previous information about avian serum proteomics available in the literature, see Table 1.1, there is currently no established protocol for this type of proteomic analysis. Therefore, the development of a protocol for the pre-fractionation and preparation of serum for LC-MS/MS analysis was required before quantitative proteomics could be conducted on trial samples.

Serum pre-fractionation is a necessary step in proteomic analysis due to the disparity between the dynamic range of serum protein concentrations, which is over 10-12 orders of magnitude (Issaq *et al.*, 2007), and the dynamic range of detection of current MS-based technologies (4-5 orders of magnitude) (Makarov *et al.*, 2006). This disparity can lead to poor identification of low abundance proteins which reduces the depth of analysis. Reduction in the concentration of a number of high abundance proteins (HAP) is a key step in reducing dynamic range of serum protein concentration and should allow greater visibility of the low abundance proteome (Doucette *et al*., 2011). Selective immunodepletion of these HAP is often the method of choice in proteomic analysis of serum from heavily studied organisms such as human or mouse (Echan *et al.*, 2005; Haudenschild *et al.*, 2014). However, this technology has not yet been developed for livestock serum and so was not initially an option for the pre-fractionation of broiler serum samples (Di Girolamo *et al.*, 2014; Henning *et al.*, 2015).

Proteominer™ is a saturation based, enrichment technology that narrows the dynamic range of applied proteomes and facilitates identification and quantification of low abundance proteins (LAP) which would otherwise be undetectable. Randomly generated, equimolar, hexapeptide ligands allow species-independent LAP enrichment, which represents a valuable alternative to immunodepletion techniques (Millioni *et al.*, 2011). This technology has the added benefit of being non-depleting, meaning that HAP are not selectively depleted, this reduces the risk of co-depletion or non-specific depletion of proteins of interest (Righetti & Boschetti, 2007; Marco-Ramell & Bassols, 2010; Di Girolamo *et al.*, 2014). Due to these advantages, Proteominer™ technology was selected as the pre-fractionation technique to be used in the quantitative proteomic analysis of the effects of Alltech feed products on broilers.

The commercial elution buffer provided within the Proteominer™ kit contains 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), a detergent used to solubilise proteins and ensure elution from the enrichment column. Detergents can not only cause ion suppression during MS analysis (Yeung *et al.*, 2008) but also have deleterious effects on C18 columns (Deschamps, 1986) which are used for protein separation in LC-MS/MS analysis. Therefore, to ensure maximum protein identification as well as column integrity, it proved necessary to alter a reagent used in protein elution from Proteominer™ columns.

The overall objective of the work presented in this Chapter was to develop and optimise a protocol for preparation of broiler serum samples for quantitative LC-MS/MS analysis.

3.2 Results

3.2.1 Preliminary proteomic analysis of broiler serum

Twelve serum samples were used in preliminary proteomic analysis. These serum samples were from individual broiler chickens harvested on Day 35, three serum samples from each treatment group. Serum samples were enriched for LAP using Proteominer™ enrichment technology. Enriched serum samples were then analysed through LC-MS/MS. Figure 3.1 shows a frequency distribution graph of the number of proteins and sequence coverage of proteins detected across the serum samples (n = 12) prepared using the commercial Proteominer™ buffer. A total of 380 proteins were detected across samples applied to the LC-MS/MS in preliminary analysis. Of detected proteins, 35% were detected with less than 10% sequence coverage.

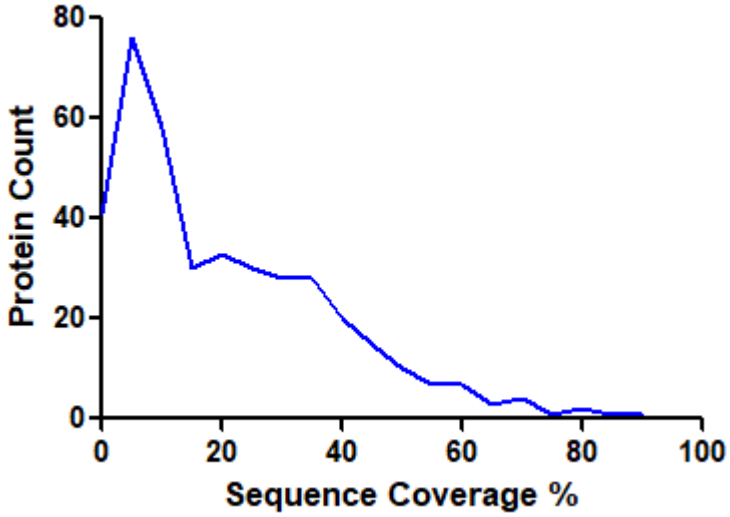

Figure 3.1 Frequency distribution graph of samples prepared using the commercial Proteominer™ elution buffer showing number of proteins identified and protein sequence coverage.

Figure 3.2 shows a total ion chromatogram of a serum sample prepared using the commercial Proteominer™ elution buffer. Detection levels are low throughout this LC-MS/MS analytical run until 125 min, when a large peak can be seen. The *m/z* for this peak is 615.4. This *m/z* corresponds to CHAPS, a component of the commercial Proteominer™ elution buffer. This large CHAPS peak (125 min-130 min)(Blue Arrow) caused suppression in peptide detection within the enriched serum sample. The base peak of this chromatogram is 1.39E10.

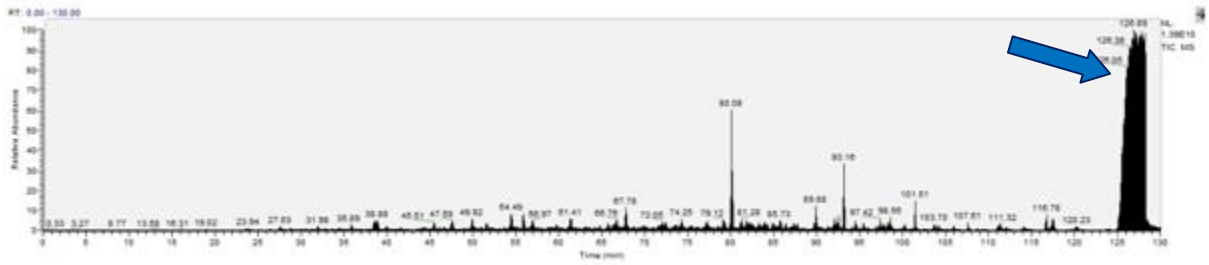

Figure 3.2 Total Ion Chromatograph of one representative sample prepared using the Proteominer™ elution buffer. Arrow indicates CHAPS elution.

3.2.2 Development and analysis of LC-MS/MS Compatible Reagent (LCR)

In order to avoid the signal suppression effect seen in proteomic samples prepared using the commercial Proteominer™ elution buffer, an alternative reagent was developed, lacking CHAPS but capable of eluting proteins from the enrichment column. This buffer, LCR (Section 2.1.2.5), was used in place of the commercial Proteominer™ elution buffer during protein enrichment. One serum sample was enriched for LAP using the Proteominer™ technology with LCR used in place of the commercial elution buffer. The enriched serum sample was then analysed by LC-MS/MS. Figure 3.3 shows a total ion chromatogram of the serum sample prepared using LCR This total ion chromatogram lacks the large peak between 125 min and 130 min that was previously seen in samples prepared using the commercial Proteominer™ elution buffer (Figure 3.2). Higher levels of peptide detection can be seen throughout this LC-MS/MS analysis when compared to Figure 3.2. The base peak for this total ion chromatogram is 1.30E10.

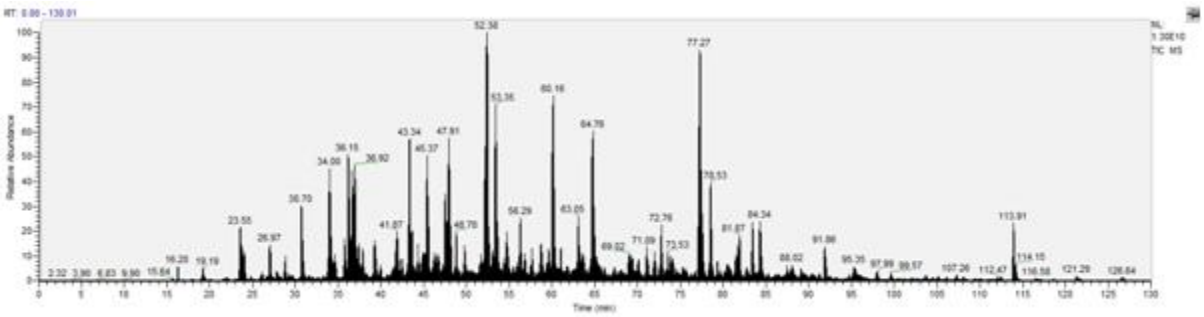

Figure 3.3 Total Ion Chromatograph of serum sample prepared using LCR.

The number of proteins detected and sequence coverage of detected proteins were compared between data obtained from analyses done on a serum sample prepared using the commercial Proteominer™ elution buffer and the same serum sample prepared using LCR. It can be seen in Figure 3.4 that a greater number of proteins were detected and higher sequence coverage was achieved using LCR whereby 218 proteins were detected in the serum sample prepared using the commercial Proteominer™ buffer, but 475 proteins were detected in the serum sample prepared using LCR.

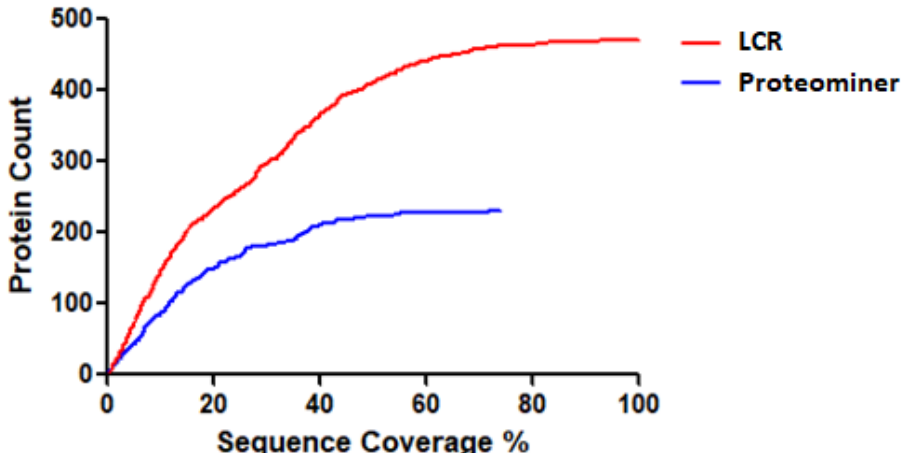

Figure 3.4 Cumulative frequency distribution graph comparing proteins detected in samples prepared using the commercial Proteominer™ elution buffer and LCR.

Proteins (*n* =218) were identified in the serum sample prepared using the commercial Proteominer™ buffer (Figure 3.5). However, 475 proteins were detected in the serum sample prepared using LCR. Of these proteins, 196 were identified by both analyses. Proteins (*n* = 22) were uniquely identified in the sample prepared using the Proteominer™ elution buffer and 279 proteins (over 10-fold more) were uniquely identified in the sample prepared using LCR.

Figure 3.5 Venn Diagram comparing proteins identified during LC-MS/MS analyses of serum samples prepared using the commercial Proteominer™ elution buffer and LCR.

3.2.3 Comparison of sample groups prepared using Proteominer Elution Buffer and LCR

In order to further evaluate the effects of the replacement elution buffer on larger sample groups, twelve serum samples prepared using the Proteominer elution buffer were compared to twelve serum samples prepared using LCR. Samples prepared using the commercial Proteominer™ buffer are from three individual birds obtained from each feed group on Day 35. Samples prepared using LCR are serum samples pooled by pen on Day 35. All serum samples were enriched for low abundance proteins using the Proteominer™ small-capacity enrichment kit and analysed through LC-MS/MS. It can be seen in Figure 3.6 that a greater number of proteins were detected and higher sequence coverage was achieved using the LCR reagent. There was a 55% increase in the total number of proteins identified and an 88% increase in the number of proteins identified with greater than 10% sequence coverage in samples prepared using LCR, when compared with samples prepared using the commercial Proteominer™ elution buffer.

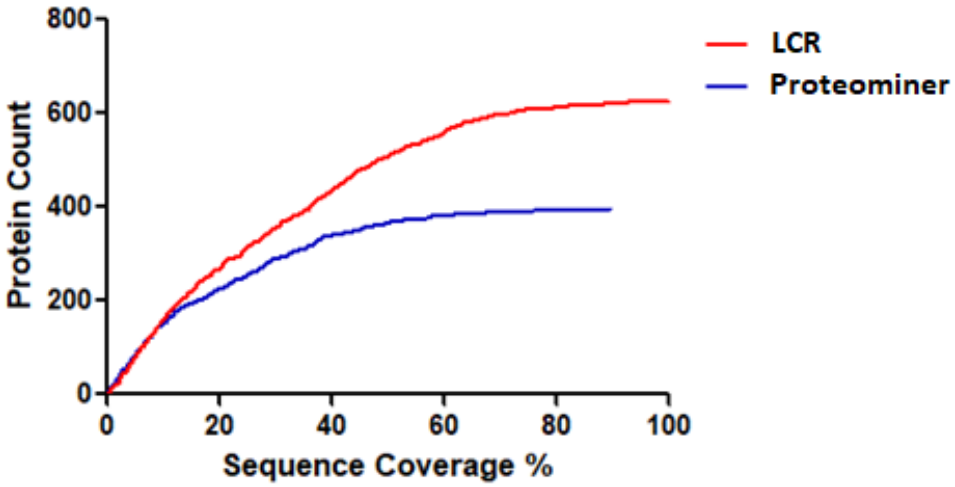

Figure 3.6 Cumulative frequency distribution graph comparing proteins detected in samples prepared using the commercial Proteominer™ elution buffer and LCR.

3.3 Discussion

Proteominer technology is pre-fractionation technique used to enrich proteomic samples for LAP and increase the numbers of identified proteins. This technology was developed for use with 2D-PAGE and contains CHAPS, a detergent in the elution buffer responsible for ensuring the elution of proteins from the enrichment column. However, detergents can cause signal suppression in MS analysis and have a deleterious effect on C18 chromatography columns (Deschamps, 1986; Yeung *et al.*, 2008). This signal suppression is evident in Figure 3.2, a representative total ion chromatogram of one sample prepared for LC-MS/MS using the commercial Proteominer™ elution buffer. A large peak corresponding to CHAPS can be seen between 125 min and 130min in this chromatogram. This large peak represents the high concentration of CHAPS present in the peptide sample which caused signal suppression resulting poor ion detection throughout the analytical run. The result of this poor ion detection is evident in Figure 3.1. The total number of proteins identified was relatively low and 35% of these proteins were identified with less than 10% sequence coverage.

In order to prevent signal suppression and abolish any chance of column deterioration during LC-MS/MS analysis, it was essential that CHAPS be excluded from sample preparation. In order to exclude CHAPS from peptide samples and also ensure elution of proteins from the Proteominer™ column, an alternative reagent was developed. LCR, lacks detergent and contains high concentrations of Urea (6M) and Thiourea (2M) which solubilise and denature proteins and cause dissociation from hexapeptide ligands leading to the elution of proteins from the enrichment column.

The removal of CHAPS from the elution buffer allowed greater ion detection throughout LC-MS/MS analytical runs. It can be seen from Figure 3.3 that the large

peak, corresponding to CHAPS, which is visible in Figure 3.2, has been removed with the change in elution buffer. Improved ion detection can be seen throughout this analysis.

Base peak intensity is the intensity of the most abundant ion in a total ion chromatogram obtained during MS. This peak is used to scale the chromatogram and is assigned a relative abundance value of 100 (Price, 1991). When comparing Figure 3.2 and Figure 3.3, it can be seen that the base peak intensity, visible in the top right of the chromatogram, is similar in both chromatograms, 1.39E10 and 1.30E10 respectively. Thus, the increase in ion intensity visible in Figure 3.3 is due to an increase in ion detection and not due to scaling differences in the chromatograms.

The effect of this increase in detection can be seen in Figure 3.4. This cumulative frequency distribution graph demonstrates a considerable improvement in protein identification. Figure 3.4 shows the effects of the change in elution buffer on one serum sample prepared using the Proteominer™ elution buffer and LCR. A dramatic increase in the total number of proteins identified can be seen. The number of proteins detected increased from 218 to 475 proteins. The number of proteins detected with greater than 10% sequence coverage also increased from 169 to 339 with the change in elution reagent. This higher level of protein identification allows greater information to be obtained on the serum proteomic content of the source animal.

Figure 3.5 shows a comparison between proteins detected using the commercial Proteominer™ elution buffer and LCR. A total of 218 proteins were identified in the peptide sample prepared for LC-MS/MS analysis using the commercial Proteominer™ buffer. Proteins (n = 475) were identified in samples prepared using LCR. Proteins (n =196) were identified in both LC-MS/MS analyses with 179 proteins uniquely

identified using the newly developed LCR. Proteins (n = 22) that were detected in the peptide sample prepared using the provide Proteominer™ buffer, were not detected using in samples prepared using LCR. Though this small number of undetected proteins could represent potential protein identification loss with the newly developed elution buffer, the gain in total protein identification number as well as the removal of risk of any deleterious effect on the C18 chromatography column compensates for this potential shortcoming.

In comparison of samples prepared using LCR and those prepared using the commercial Proteominer™ buffer, it can be seen in Figure 3.6 that the implementation of LCR led to improved protein detection. Though these samples differ slightly in their preparation, one set from individual birds and one set consisting of pooled sera, the capability of LCR to improve protein identification is evident from this analysis. With the implementation of LCR there was an improvement in protein detection, with 55% more proteins identified in samples prepared using LCR. There was also an improvement in percentage sequence coverage. Proteins (35%) detected in samples prepared using the commercial Proteominer™ elution buffer were identified with less than 10% sequence coverage. This number was reduced to 25% in samples prepared using LCR which translated to an 88% increase in the total number of proteins detected with greater than 10% sequence coverage. This improved sequence coverage allows greater confidence in proteins that have been identified.

In comparison to previous studies completed on serum samples prepared using the Proteominer™ enrichment technology, the implementation of LCR proved effective. Many of the previous studies which employed Proteominer™ technology for the enrichment of serum/plasma proteins used in-gel techniques such as 2D-PAGE or differential in gel electrophoresis (DIGE) followed by analysis through an MS

technology (Cumová *et al.*, 2012; González-Iglesias *et al.*, 2014; Righetti *et al.*, 2014; de Seny *et al.*, 2016; de Jesus *et al.*, 2017), therefore are not suitable for the direct comparison of protein detection capabilities. A number of previous studies which have used Proteominer™ with LC-MS/MS employed several methods so that the enriched protein sample is eligible for LC-MS/MS analysis. In a recent study, Pisanu *et al.* (2018) implemented a reagent change, whereby the Proteominer™ elution buffer was substituted with 100 mM Tris-HCl, 0.8% SDS, pH 6.8. The elution procedure was more laborious with 1 h wash steps used instead of the 15 min wash steps recommended by the manufacturer. This change in elution reagent allowed the detection of 553 proteins from a single serum sample. The number of proteins detected in this serum sample was 16% higher than the number of proteins detected in a single serum sample prepared using LCR (n = 475). This improvement in protein detection could be an indication of a more effective enrichment step. However, the use of SDS in the elution buffer necessitated the introduction of a filter aided sample preparation (FASP) step which could, in larger sample sets, introduce significant variability. SDS can, if not completely removed from proteomic samples, also have deleterious effects on C18 columns and introduce signal suppression effects (Rundlett & Armstrong, 1996; Botelho *et al.*, 2010) which could further adversely affect the reproducibility of results using this method. Another approach that was taken in previous Proteominer™ LC-MS/MS studies was the precipitation of proteins from the commercial elution buffer prior to LC-MS/MS analysis. Millioni *et al.* (2011) precipitated proteins from the elution buffer using acetone, followed by strong cation exchange fractionation of tryptic peptides. This method allowed the detection of 318 proteins in a single serum sample. Another study conducted by Capriotti *et al.* (2012) precipitated proteins from the elution buffer using chloroform and methanol. The number of proteins detected across 3 technical replicates

were 238 proteins, 240 proteins and 250 proteins, respectively. The average number of proteins detected in one serum sample using this method was 243. Although these precipitation methods did allow LC-MS/MS analysis of the enriched serum proteins, they did not allow the same level of protein identification that was achieved in the present study and the addition of a precipitation step could reduce reproducibility in larger sample groups. With relatively high protein identification capabilities and without the need for further downstream steps prior to protein digestion, elution of proteins from the Proteominer™ column using LCR is therefore a valuable alternative to the methods used previously with Proteominer technology to prepare serum samples for LC-MS/MS analysis.

In conclusion, in order to achieve maximum identification of serum proteins and avoid issues with LC-MS/MS signal suppression, it was essential to develop an alternative elution reagent for the Proteominer™ enrichment kit. This new elution buffer allowed greater peptide detection which resulted in a larger number of serum proteins detected with higher sequence coverage, and should allow greater access to the serum proteome, giving a more in-depth view into the effects of feed treatments. In comparison to previous studies including application of Proteominer™ enriched serum samples to the LC-MS/MS, the induction of the new reagent proved effective and without the need for precipitation or FASP step prior to LC-MS/MS, could improve the reproducibility of sample preparation. The reagent may also have applications for the improvement in serum proteome coverage, following Proteominer™ extraction, for other animal species.

Chapter 4

Serum Proteomic Analysis of the effect of Yeast Cell Wall products on Broiler Chickens.

4.1 Introduction

With the introduction of bans on the use of antibiotic growth promoters (AGP) in animal husbandry, interest in alternative growth promoters has grown immensely. Yeast and its derivatives, such as yeast cell wall (YCW), have emerged as valuable alternatives to AGP and have been shown to impart a number of beneficial effects on the animal which have application in the field of animal husbandry. Some noted effects of yeast-based products include modulation of the gut microbiome (Yang *et al.,* 2009) reduced infection by pathogenic bacteria and beneficial stimulation of the host immune system (Gao *et al.,* 2003; Chae *et al.,* 2006; Goodridge *et al.,* 2009; Dalonso *et al.,* 2015; Song *et al.,* 2014; Hoving *et al.,* 2018). The effects of yeast and its derivatives has been widely studied (Brown, 2006; Kogan & Kocher, 2007; Song *et al.,* 2014) but the literature available for serum proteomic analysis of these effects is limited. With recent advances in serum proteomics, this area now represents an exciting opportunity to investigate the biological effects of YCW feed supplementation in broiler chickens.

Immunological responses triggered by the YCW are largely due to the action of two molecules, Mannose Oligosaccharide (MOS) and β-glucan. These molecules are highly abundant in YCW and modulate the immune system of the host through the binding of receptors which are expressed on the surface of macrophages and monocytes as well as in the serum of the host eliciting downstream immunological stimulation (Kogan & Kocher, 2007; Song *et al.,* 2014).β-glucan is bound by Complement receptor type 3 (Ross *et al.,* 1987), lactosylceramide (Zimmerman *et al.,* 1998), and Dectin-1 (Brown, 2006) as well as a number of scavenger receptors present in monocytes (Rice *et al.,* 2002). MOS is bound by the Mannose receptor which is a c-type lectin receptor (Gazi & Martinez-Pomares, 2009) capable of recognising self and mannan ligands and is present on the surface of macrophage and endothelial cells (Ringo *et al.,* 2010; Ringo

et al., 2014). Mannan ligands may also bind other receptors such as DC-SIGN and Dectin-2 which can elicit immunological responses such as leukocyte activation (Gazi & Martinez-Pomares, 2009).

YCW immunostimulation has been reported in a number of species including human (Rice et al., 2002), fish (Song *et al.,* 2014) and mice (Majtán *et al.,* 1998) as well as livestock such as cows (Kimura et al., 2008), pigs (Xiao *et al.,* 2004; Kogan & Kocher, 2007) and broiler chickens (Gao *et al.,* 2003) and has been shown to induce a range of immunological effects both *in vivo* and *in vitro*. Previous *in vitro* studies have shown that the binding of these receptors by fungal glycan ligands can lead to immunological responses such as enhancing the functional status of macrophages and neutrophils (Williams *et al.,* 1996; Tzianabos, 2000; Zeković *et al.,* 2005), release of TNF-α from macrophages (Majtán *et al.,* 1998; Engstad *et al.,* 2002; Gantner *et al.,* 2003; Brown, 2006) cytokine and chemokine production in macrophages (Adachi *et al.,* 1994; Olson *et al.,* 1996; Young *et al.,* 2001; Gantner *et al.,* 2003; Brown, 2006) and enhancing TNF-γ response in T-cells (Xiao *et al.,* 2004). The ability of epithelial cells to secrete macrophage inflammatory protein was also increased with the administration of β-glucan (Hahn *et al.,* 2003). PGG-glucan, another carbohydrate-based prebiotic was seen to increase the migration of neutrophils towards C5a (Tsikitis *et al.,* 2004).

In vivo immunological effects have also been reported. Addition of glycan ligands to the feed of animals has been shown to elicit immunological responses such as increased resistance to bacterial and parasitic infection in mice (Yun *et al.,* 1997; Yun *et al.,* 1998; Yun *et al.,* 2003; Kournikakis *et al.,* 2003). With the addition of glucan phosphate, another carbohydrate prebiotic, to the diet, an increased resistance to *Staphylococcus aureus* and *Candida albicans* was observed (Rice *et al.,* 2005). The ability of immature chickens to fight *Salmonella enterica* serovar *Enteritidis* was

increased with the addition of β-glucan to the diet (Lowry *et al.*, 2005), which was accompanied by increased phagocytic activity in heterophils and increased oxidative burst. An increase in LPS-induced TNF-α and IL-6 production was detected in the blood of weaned pigs with dietary β-glucan supplementation (Bohn & BeMiller, 1995).

The complement system is a non-cell mediated innate immune response which is present in the serum of the host. This response is mediated either through the binding of antigen-specific antibodies, direct binding of microbial cell surfaces or lectin-specific binding of carbohydrate residues (Levitz, 2010) and is shown in Figure 4.1. Complement activation leads to cleavage of proteolytic precursors to form complement components (Abbas *et al.*, 2017) which can be measured as increased serum titre of those components.

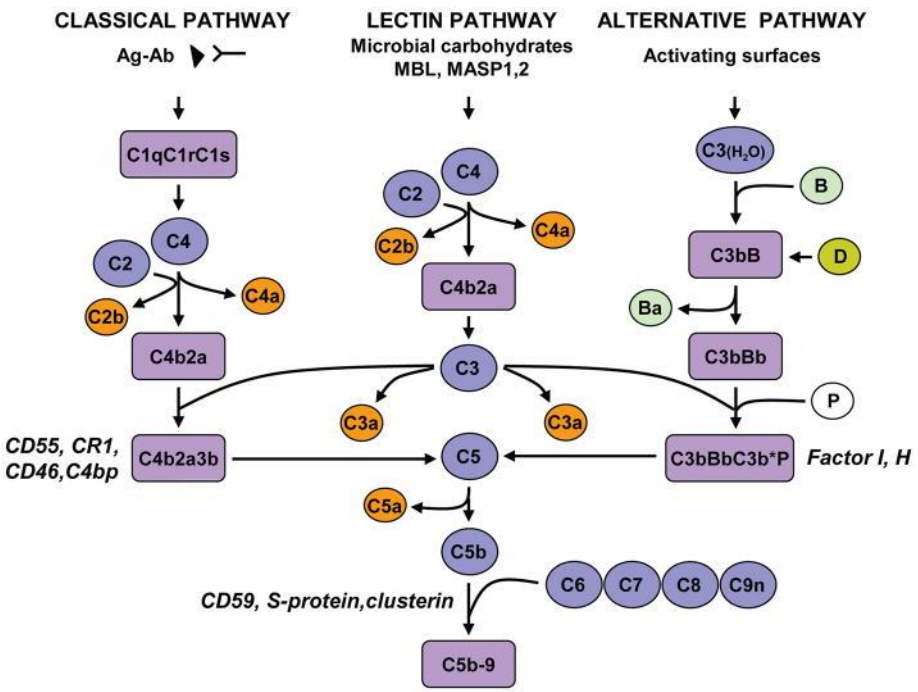

Figure 4.1. Graphical representation of complement activation pathways and assembly of terminal pathway, adapted from Tegla *et al.* (2011).

Fungal components are potent activators of the complement which leads to opsonisation and recruitment of inflammatory cells as a result of C3a and C5a generation. Fungal stimulation of the complement cascade can be activated through the classical, alternative or lectin pathways (Levitz, 2010). Although the effects of YCW feed supplementation on the complement has not been reported specifically in broiler chickens, as far as could be found in the literature, it has been previously seen that MOS and β-glucan can activate the complement cascade (Dlabač & Kawasaki, 1994; Bohn & BeMiller, 1995; Levitz, 2010; Song *et al.,* 2014) and the absorption of β-glucans into the circulatory system and their presence in the serum has been previously shown in mice (Rice *et al.,* 2005) which would allow for complement stimulation.

Selenium is an essential dietary mineral in poultry nutrition with vital roles in the detoxification of reactive oxygen species (ROS), immune function and productivity (Surai, 2002; Surai & Fisinin, 2014). Sources of selenium in the diet of poultry include inorganic selenium, such as selenite or selenate, or organic yeast-derived selenium which has the mineral incorporated into proteins and peptides in the form of selenomethionine (SeMet) or selenocysteine (SeCys) (Peric *et al.,* 2009). Organic selenium is taken up in the gastrointestinal tract of animals through the amino acid transport mechanism (Surai, 2002) and is reportedly more bioavailable than its inorganic forms (Rayman, 2004; Lönnerdal *et al.,* 2017). Inorganic selenium can also be seen to have pro-oxidative effects and can contribute to oxidative damage (Peric *et al.,* 2009).

In the last number of decades, consumer awareness of meat quality has dramatically improved. Water lost during meat preparation and cooking is referred to as drip-loss and has become synonymous with poor quality meat and for that reason should be mitigated (Northcutt *et al.,* 1994). Drip-loss has been linked to oxidative damage of cell

membranes which allows seepage of cellular contents (Mahan *et al.,* 1999). Antioxidants, such as the Se-dependant Glutathione Peroxidase (GSH-Px), have been previously linked with a reduction in drip-loss and improved poultry meat quality (Choct *et al.,* 2004). Selenium forms a vital part of GSH-Px (Arthur *et al.,* 1992) and dietary supplementation has been previously seen to increase the level of GSH-Px in the serum (Wang & Xu, 2008; Jiang *et al.,* 2009; Pappas *et al.,* 2005; Wang *et al.,* 2011; Cai *et al.,* 2012; Yoon *et al.,* 2007) and muscle (Pappas *et al.,* 2005; Wang *et al.,* 2011; Zhou & Wang, 2011; Cai *et al.,* 2012) of chickens. Increased levels of selenium in the serum and muscle of the broiler chicken are not only of protective benefit to the chicken but also of benefit to the consumer, as it has been found that the intake of selenium in most Europeans' diet is below recommended values and that selenium-enriched foods are a helpful supplement in maintaining selenium levels (Thiry *et al.,* 2013). The selenium source included in the control, Natustat® (NS) and Actigen®-Pak (AP) diets was inorganic selenite/selenate. PowerTract® (PT)-supplemented broilers were fed an organic selenium yeast source as part of the feed supplement.

Essential oils (EO) are another feed additive used in feed animal supplementation (Windisch *et al.,* 2008; Gong *et al.,* 2014; Zou *et al.,* 2016). These products have been shown to elicit effects such as enhancing total antioxidant capacity (Zeng *et al.,* 2015), immunostimulation (Zeng *et al.,* 2015) and antimicrobial activity (Rota *et al.,* 2007) all of which can beneficially contribute to the health and immune status of the animal. The YCW supplement Natustat® used in this study contains EO.

The blood of an animal is an excellent reservoir of biomarkers of health and disease. This biofluid flows through the entire body of the animal so can offer exceptional insights into internal biological activity. Serum, a blood derivative lacking cells as well as clotting factors, is an excellent indicator of health and immune status

(Issaq *et al.*, 2007; Biosa *et al.*, 2011) and may offer a potential insight into how these YCW products are affecting the broiler chicken. Using Liquid Chromatography-tandem mass spectrometry (LC-MS/MS) analysis and with the implementation of LC-MS/MS Compatible Reagent (LCR) in sample preparation, the effects of three YCW-based feed supplements, AP, NS and PT on the health status of the broiler chicken was investigated. These three feed supplements are yeast-derivative based with some supplementation. NS is supplemented with essential oils and PT contains an organic selenium source. For each treatment group, proteins that were significantly different in abundance ($p < 0.05$) in comparison to control group, as well as proteins that were uniquely identified or absent in serum samples from supplemented birds, when compared to control, were tabulated and analysed. No cut-off value was applied to fold changes.

The objectives of the work presented in this chapter were:

i. Through serum proteomics, examine the effects of YCW feed on broiler chickens, through comparison with a control basal feed.
ii. Compare and contrast the effects of three individual, YCW-based, feed products.

4.2 Results.

4.2.1 Overview of LC-MS/MS results

Tryptic digests, from 36 pooled serum samples, were analysed by LC-MS/MS across three time points, Day 7, Day 21 and Day 35. Within each time point, each treatment group was compared to control which created three comparator groups: AP vs. control, NS vs. control and PT vs. control, for each time point. Within each comparator group, proteins which were significantly changed in abundance ($p < 0.05$) as well as proteins which were uniquely present in treatment groups in comparison to the control group or uniquely absent in treatment groups versus control, were tabulated and analysed. Between 5% and 12% of the total identified proteins within each comparator group showed changes in abundance (Table 4.1). The largest change in protein abundance was seen on Day 7, with an average of 9.11% of proteins changed in abundance or unique to/absent from the serum of treated birds when compared to control birds. NS-treated birds showed the largest change in protein abundance throughout the three time points with an average of 7.78% of proteins changed in abundance. The largest individual change in abundance was seen in Day 7 NS vs. control samples with 11.06% of proteins changed.

Inter-day and inter-treatment analysis were also conducted on the total proteins identified throughout all serum samples analysed. This was achieved by applying LC-MS/MS data obtained from each sample to the data analysis software MaxQuant (MQ), first grouped by Day and then, in a separate analysis by treatment. Proteins ($n = 1007$) were identified in at least one serum sample from all time points (Figure 4.2). The majority of proteins ($n = 512$) (51% of total proteins) were shared between at least two time points. Proteins ($n = 169$) were uniquely identified in pooled serum samples obtained on Day 7, on Day 21 ($n = 117$) and on Day 35 ($n = 209$). Proteins ($n = 602$)

were identified in at least one serum sample from all treatment groups (Figure 4.3). The majority of proteins (n = 733) (74% of total proteins) were shared between at least two treatment groups. Proteins (n = 27) were uniquely identified in pooled serum samples from the control treatment group, in the AP treatment group (n = 22), in the NS treatment group (n = 51) and the PT treatment group (n = 150).

Table 4.1. Overview of proteins with significantly changed abundance within comparator groups.

Comparator Group	Total Identified Proteins	Significant Changes*	Unique / Absent Changes**	Total Changes	Total Changes (%)
D7 AP vs. control*		38	26	64	9.83
D7 NS vs. control	651	51	21	72	11.06
D7 PT vs. control		14	28	42	6.45
D21 AP vs. control		25	9	34	5.78
D21 NS vs. control	588	34	9	43	7.31
D21 PT vs. control		23	10	33	5.61
D35 AP vs. control		21	17	38	6.09
D35 NS vs. control	624	21	10	31	4.97
D35 PT vs. control		29	14	43	6.89

* Significant changes denotes proteins which have changed in abundance with $p < 0.05$ in a two-sample t-test

** Unique/Absent Changes denotes proteins that were uniquely present in a treatment group or absent from a treatment group when compared to control.

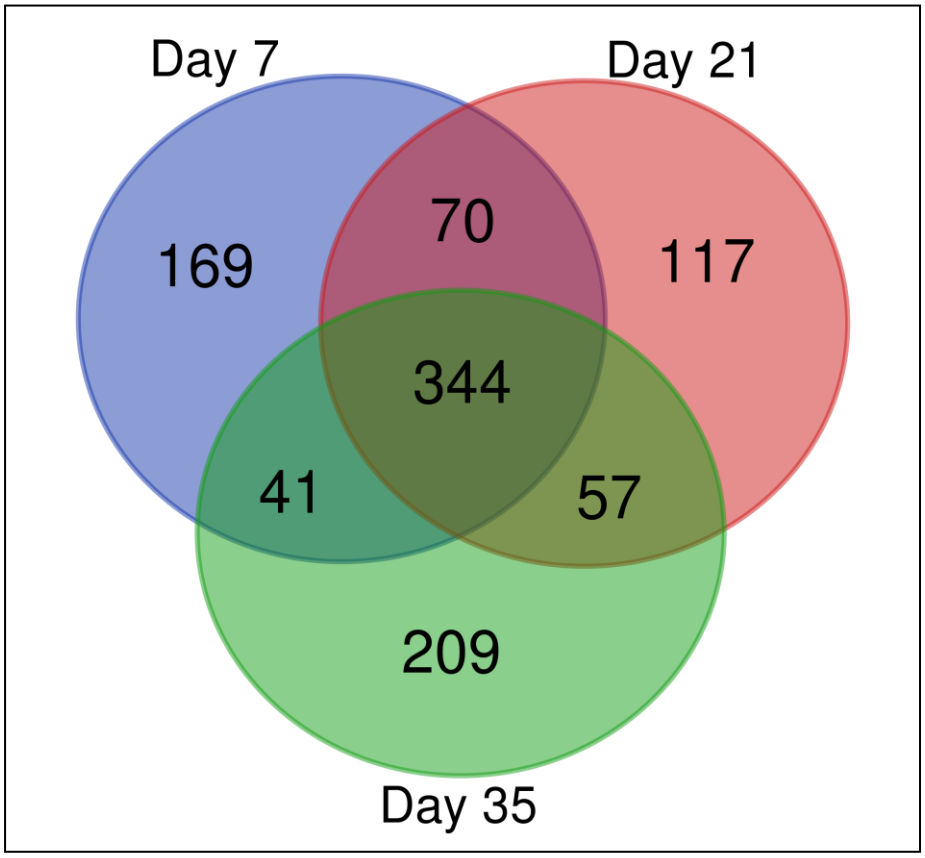

Figure 4.2. Shared and distinct proteins between experimental time points.

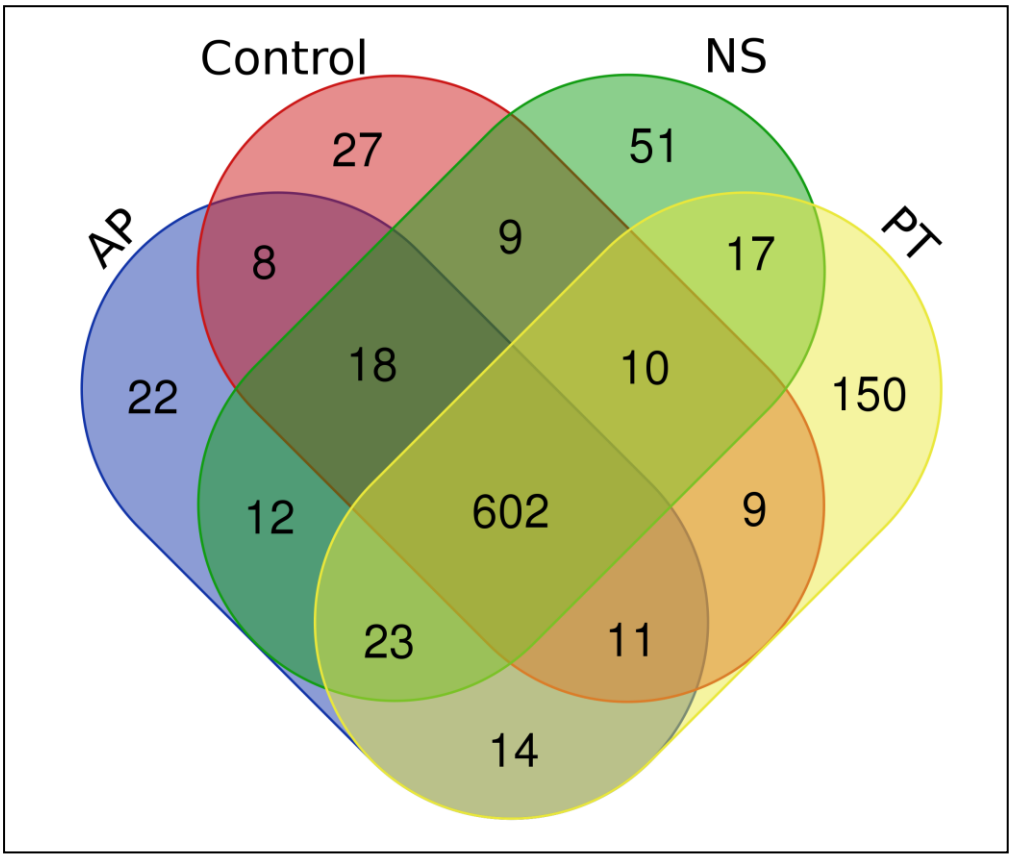

Figure 4.3. Shared and distinct proteins between control and treatment groups

4.3 Quantitative proteomic analysis of serum from broilers fed an Actigen®-Pak supplemented diet vs. serum from broilers fed a control diet

A total of eighty-two proteins were found to be significantly increased in abundance (p <0.05) or uniquely present in AP samples with comparison to control (Table 4.2). Proteins (n =55) were found to be significantly decreased (p <0.05) in abundance or absent in serum of AP samples (Table 4.3).

Principal component analysis (PCA) was conducted on proteomic data obtained from LC-MS/MS analysis of AP and control serum samples (Figure 4.4). These data show that AP samples and control samples cluster separately on Day 7, Day 21 and Day 35. Hierarchal clustering was also conducted on proteomic data which matched PCA results on Day 7, Day 21 and Day 35 (data not shown).

Nineteen proteins, characterised to be involved in the innate immune system by Reactome pathway mapping software, were significantly increased (p <0.05) in abundance in the AP serum samples: namely Proteasome subunit beta 1 (A0A1L1RYR5), Chromogranin A (F1NLZ2), Proteasome 26S subunit, ATPase 5 (F1NU79), Uncharacterized protein (Q5F491), Surfactant protein A (Q90XB2), Pantetheinase precursor (E1BUA6), Uncharacterized protein (F1NPN5), Alpha-1,4 glucan phosphorylase (E1BSN7), Uncharacterized protein (A0A1D5PW77), T-complex protein 1 subunit theta (F1NEF2), Proteasome subunit beta type (A0A1L1RUE7), Transthyretin (P27731), Elongation factor 1-alpha 1 (Q90835), Proteasome subunit alpha type (F1NEQ6), Metalloproteinase inhibitor 2 (R4GIL5), Cathepsin D (Q05744), Complement C4 Precursor (A0A1D5P5V5), Complement C6 (B8ZX71) and Complement C7 (E1C6U2). These are represented in a Reactome pathway map (Figure 4.5). Mannose-binding protein (Q98TA4) was increased in abundance and approaching significance (p = 0.2) on Day 35 in AP samples.

A number of proteins involved in carbohydrate metabolism were also significantly increased in abundance in AP samples, specifically Glyceraldehyde-3-phosphate dehydrogenase (P00356) and phosphoglycerate Kinase (F1NU17) were significantly increased ($p < 0.05$) in AP samples on Day 7.

Individual proteins of note also showed changes in abundance in AP samples. Osteocalcin (P02822), a marker for bone resorption, was uniquely identified on Day 21. Gastrokine-2 (A0A1D5PFM9) is uniquely present in the serum of AP treated birds on Day 35. Insulin-like growth factor II was significantly decreased ($p <0.05$) in abundance on Day 35. Transferrin receptor protein 1 (F1NTM6) was increased in abundance and approaching significance ($p = 0.06$) (Appendix 1-Table 7.1) on Day 35.

Proteins ($n = 3$) involved in vitamin A transport (Zabetian-Targhi *et al.*, 2015; Hu *et al.*, 2017) were increased in abundance in AP samples. Transthyretin (P27731) was significantly increased in abundance on Day 35, Retinol binding protein 7 (E1C0M1) was uniquely present on Day 7 and Retinol-binding protein 4 (P41263) was increased in abundance and approaching significance ($p = 0.08$) on Day 21(Appendix 1-Table 7.1).

A number of proteins involved in nucleotide metabolism were significantly altered in abundance ($p < 0.05$) in AP samples. These were 3'-phosphoadenosine 5'-phosphosulfate synthase 1 (E1C8P2) which was uniquely present and Adenylate kinase isoenzyme 1 (P05081) that was significantly increased ($p < 0.05$) on Day 7. Nucleoside diphosphate kinase (O57535) was uniquely present on Day 35 whereas Adenosine Deaminase (A0A1D5PDK4) was significantly decreased in abundance ($p <0.05$) on Day 35.

Finally, Alpha-1-Acid Glycoprotein was increased in abundance on Day 35 in samples from AP supplemented birds with a large fold change (Appendix 1-Table 7.2). However, this change was not significant ($p = 0.37$).

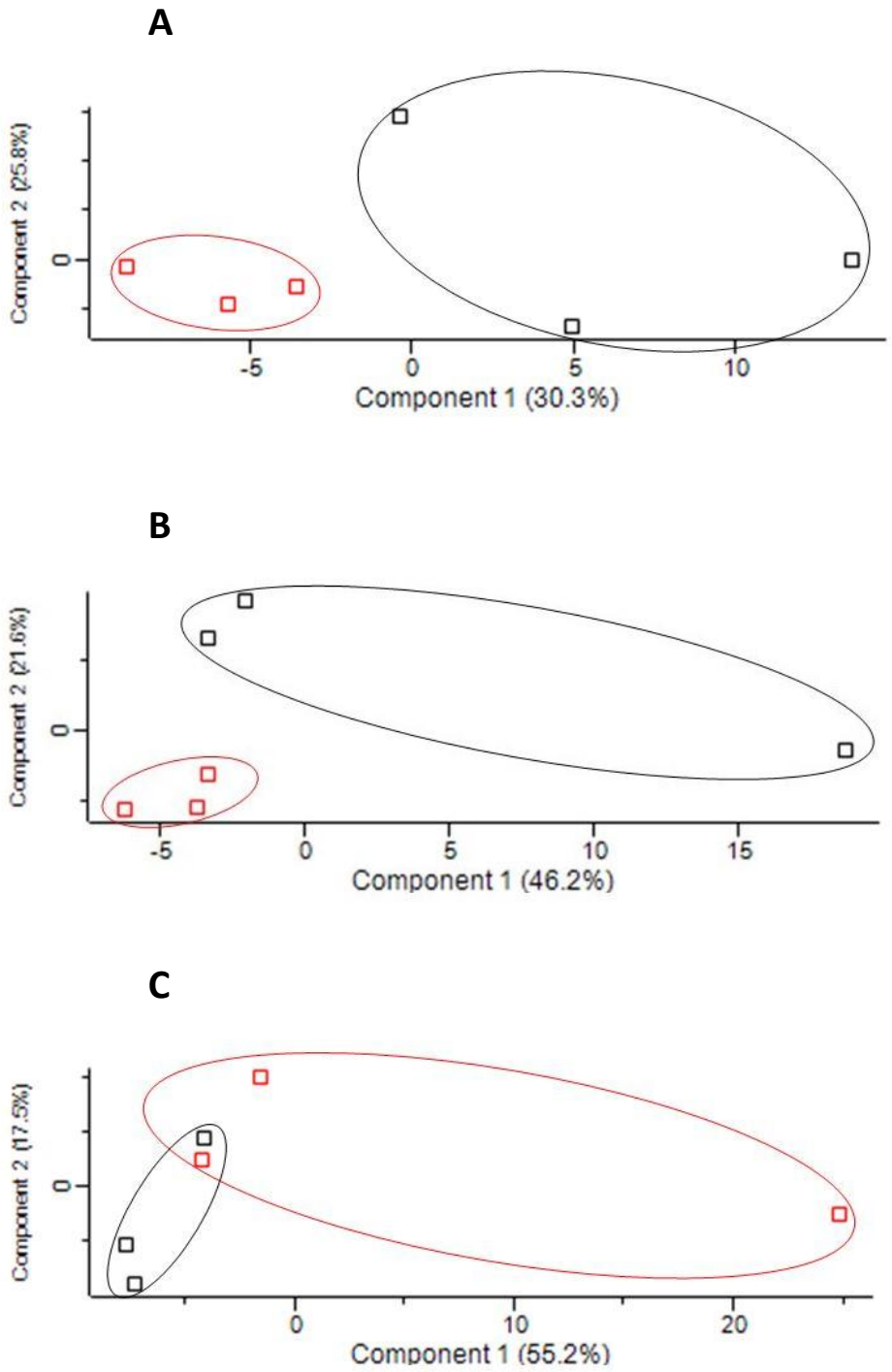

Figure 4.4. Principal Component Analysis (PCA) comparing AP serum samples (Red) vs. control serum samples (Black) for A. Day 7, B. Day 21, and C. Day 35.

Table 4.2. Proteins with significantly ($p < 0.05$) increased abundance or **unique** in AP treatment samples. Proteins are listed in order of change of abundance.

Protein Description	Fold change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Barrier to autointegration factor 1	Unique	2	42.2	Day 7	A0A1D5NXY4
Uncharacterized protein	Unique	2	1.0	Day 7	A0A1D5NZ61
Dynactin subunit 2	Unique	6	22.1	Day 7	A0A1D5PGQ9
Legumain	Unique	2	11.8	Day 7	A0A1L1RX51
Proteasome subunit beta 1	Unique	3	36.4	Day 7	A0A1L1RYR5
Retinol binding protein 7	Unique	3	32.1	Day 7	E1C0M1
Ankyrin repeat domain 2	Unique	2	8.9	Day 7	E1C1Q6
Mediator of cell motility 1	Unique	3	21.9	Day 7	E1C6C0
3'-phosphoadenosine 5'-phosphosulfate synthase 1	Unique	5	12.3	Day 7	E1C8P2
Uncharacterized protein	Unique	4	30.8	Day 7	F1N8Y3
Elongation factor 1-alpha	Unique	15	49.9	Day 7	F1N9H4
Succinyl-CoA:3-ketoacid-coenzyme A transferase	Unique	3	11.0	Day 7	F1N9Z7
Four and a half LIM domains 1	Unique	4	18.5	Day 7	F1NED9
Myeloid protein 1	Unique	6	30.1	Day 7	F1NEF7

Protein Description	Fold change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Small nuclear ribonucleoprotein 13	Unique	3	30.5	Day 7	F1NII6
Chromogranin A	Unique	2	10.7	Day 7	F1NLZ2
PDZ and LIM domain 5	Unique	4	8.2	Day 7	F1NTC8
Proteasome 26S subunit, ATPase 5	Unique	3	12.7	Day 7	F1NU79
Cofilin-2	Unique	3	25.9	Day 7	P21566
Uncharacterized protein	Unique	2	3.7	Day 7	Q5F491
Profilin	Unique	2	27.9	Day 7	Q5ZL50
Endophilin-A2	Unique	4	20.4	Day 7	Q8AXV0
Surfactant protein A	Unique	3	16.2	Day 7	Q90XB2
Seryl-tRNA synthetase	Unique	2	4.3	Day 7	R4GJ59
Disintegrin and metalloproteinase domain-containing protein 33 precursor	Unique	2	3.6	Day 21	A0A1D5NV10
Calpastatin	Unique	2	3.9	Day 21	A0A1D5PFJ2
Tubulointerstitial nephritis antigen like 1	Unique	2	10	Day 21	F1N8G6
Osteocalcin	Unique	3	45.4	Day 21	P02822

Protein Description	Fold change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Tenascin	Unique	8	8.7	Day 21	P10039
Tenascin	Unique	10	10.8	Day 35	P10039
Gastrokine-2	Unique	2	18.6	Day 35	A0A1D5PFM9
N-acetylglucosamine-1-phosphate transferase gamma subunit	Unique	2	16	Day 35	E1BS68
Pantetheinase precursor	Unique	10	27	Day 35	E1BUA6
Adhesion G protein-coupled receptor G6	Unique	2	2.5	Day 35	E1C8C2
Alpha-1-anti-ase	Unique	9	27.8	Day 35	F1NPN5
Nucleoside diphosphate kinase	Unique	3	28.1	Day 35	O57535
Apolipoprotein B (Fragment)	Unique	7	24.7	Day 35	P11682
Glycerol-3-phosphate dehydrogenase	1.55	9	37.5	Day 7	A0A1D5P1Y7
Adenylate kinase isoenzyme 1	1.44	11	63.4	Day 7	P05081
Glyceraldehyde-3-phosphate dehydrogenase	1.33	20	75.1	Day 7	P00356
Myosin regulatory light chain 2, skeletal muscle isoform	1.22	9	64.3	Day 7	P02609
Heat shock protein beta-1	1.15	18	79.4	Day 7	F1P593
Myosin light chain 1, skeletal muscle isoform	1.08	9	60.9	Day 7	P02604

Protein Description	Fold change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Phosphoglycerate kinase	1.07	17	60.9	Day 7	F1NU17
Proline and arginine rich end leucine rich repeat protein	1	10	32.9	Day 7	A0A1D5PAN0
Uncharacterized protein	0.97	7	25.5	Day 7	F1NIP5
Protein/nucleic acid deglycase DJ-1	0.91	5	59	Day 7	A0A1D5PN39
Actin, alpha skeletal muscle	0.89	13	24.4	Day 21	A0A1I7Q414
Uncharacterized protein	0.84	2	3.7	Day 21	F1NMN2
Carbonic anhydrase 2	0.83	7	44.6	Day 35	P07630
Alpha-1,4 glucan phosphorylase	0.81	5	7.8	Day 35	E1BSN7
Uncharacterized protein	0.8	54	24	Day 21	A0A1D5PW77
Receptor of-activated protein C kinase 1	0.75	9	56.9	Day 7	A0A1I7Q3Y2
Complement C7	0.73	28	54.3	Day 35	E1C6U2
Low molecular weight phosphotyrosine protein phosphatase	0.69	10	60.8	Day 7	A0A1D5P9Z1
Uncharacterized protein	0.69	7	24.1	Day 7	A0A1L1RQM3
T-complex protein 1 subunit theta	0.65	8	21	Day 7	F1NEF2
Proteasome subunit beta type	0.65	14	25.1	Day 21	A0A1L1RUE7

Protein Description	Fold change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Transthyretin	0.64	9	72.7	Day 35	P27731
Cystatin A	0.62	4	18.3	Day 21	F1NHG8
Tropomyosin alpha-1 chain	0.61	16	57.2	Day 7	A0A1D5NVL7
Serpin H1	0.58	12	44	Day 7	P13731
60 kDa heat shock protein	0.57	12	34.4	Day 7	Q5ZL72
Complement C6	0.56	29	40.3	Day 35	B8ZX71
Elongation factor 1-alpha 1	0.53	18	58.9	Day 7	Q90835
Ribosomal protein L23a	0.53	3	24.5	Day 7	E1BS06
Proteasome subunit alpha type	0.53	15	27.9	Day 21	F1NEQ6
Collagen type V alpha 1 chain	0.49	11	57	Day 21	F1NI79
Eukaryotic translation initiation factor 5A-1	0.47	6	63.9	Day 7	Q09121
C-type lectin domain family 3-member B	0.45	6	40.5	Day 21	Q9DDD4
Metalloproteinase inhibitor 2	0.42	11	67.7	Day 21	R4GIL5
F-actin-capping protein	0.38	6	6.7	Day 21	P14315

Protein Description	Fold change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Uncharacterized protein	0.37	5	41.1	Day 21	A0A1L1S0T3
Cathepsin D	0.37	5	87.6	Day 21	Q05744
Sortilin	0.35	13	67.7	Day 21	A0A1D5PNT8
Sortilin	0.28	14	25.6	Day 7	A0A1D5PNT8
Uncharacterized protein	0.33	5	52.9	Day 7	A0A1D5PH37
Complement C4 precursor	0.3	61	59.2	Day 35	A0A1D5P5V5
Heparan sulfate proteoglycan 2	0.24	8	42.6	Day 21	A0A1L1RJ69
Fibromodulin (FM)	0.14	24	62.7	Day 21	P51887
Far upstream element-binding protein 2	0.13	13	44.1	Day 21	Q8UVD9

[1]Fold change refers to the $\log_2$ fold change in protein abundance in response to AP treatment. [2]Coverage (%) refers to the % of protein sequence represented by identified peptides. [3]Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial.

Table 4.3. Proteins with significantly ($p < 0.05$) decreased abundance or **absent** in AP feed treatment samples. Proteins are listed in order of change of abundance.

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Growth differentiation factor 11	Absent	2	6.4	Day 7	A0A1D5P7V6
Uncharacterized protein	Absent	5	10.3	Day 7	F1NZV7
Uncharacterized protein	Absent	3	9	Day 21	A0A1D5PF52
Glutathione S-transferase	Absent	2	9.5	Day 21	Q08392
Catalase	Absent	2	8.9	Day 21	Q5ZL24
T-complex protein 1 subunit theta	Absent	3	6.8	Day 21	Q6EE31
NSF attachment protein alpha	Absent	2	8.2	Day 35	A0A1D5NUZ0
Uncharacterized protein	Absent	3	2.5	Day 35	A0A1D5NW21
Fibulin-1	Absent	6	36.5	Day 35	A0A1L1RU28
ERH, mRNA splicing and mitosis factor	Absent	17	39.4	Day 35	A0A1L1RZP8
Endoplasmic reticulum lectin 1	Absent	2	8.5	Day 35	F1NCV8
Nuclear transport factor 2	Absent	2	63	Day 35	F1NLL4
Nuclear transport factor 2	-0.6	4	40.7	Day 21	F1NLL4
TAR DNA-binding protein 43 (TDP-43)	Absent	4	14.5	Day 35	Q5ZLN5

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Heterogeneous nuclear ribonucleoprotein A2/B1	Absent	3	20.1	Day 35	Q5ZME1
Junction plakoglobin	-1.45	33	7.1	Day 7	E1C1V3
Uncharacterized protein	-1.23	12	0.7	Day 7	A0A1L1RLW1
Insulin like growth factor binding protein 5	-1.17	3	15.9	Day 35	F1ND88
Fibromodulin	-1.08	4	6.3	Day 35	P51887
Fibulin 5	-1.02	7	16.7	Day 35	A0A1L1RQ98
Serpin family D member 1	-1.00	21	37.9	Day 21	A0A1D5PLZ2
Uncharacterized protein	-0.93	2	37.5	Day 7	F1NSC7
Uncharacterized protein	-0.33	3	37.5	Day 21	F1NSC7
Olfactomedin-like protein 3	-0.92	20	50.5	Day 35	Q25C36
Ovoinhibitor	-0.82	9	60.6	Day 7	P10184
EGF containing fibulin like extracellular matrix protein 1	-0.78	19	53.7	Day 35	A0A1D5P380
Hemoglobin subunit alpha-D	-0.77	7	90.1	Day 7	P02001
Fibrinogen beta chain	-0.65	8	26.9	Day 7	Q02020

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Carboxypeptidase	-0.62	7	16	Day 21	A0A1L1RXB2
Fibrinogen gamma chain	-0.61	6	34.1	Day 7	E1BV78
Insulin-like growth factor II	-0.59	2	11	Day 35	P33717
Uncharacterized protein	-0.59	21	23.5	Day 7	A0A1D5PSJ4
Histone H2B 8	-0.58	11	19	Day 7	Q9PSW9
Uncharacterized protein	-0.53	3	36.4	Day 21	F1NC22
Chemerin	-0.52	7	43.3	Day 35	A0A0K0PUH6
Uncharacterized protein	-0.52	2	38.5	Day 21	F1NSC8
Matrilin-3	-0.5	53	27.9	Day 7	O42401
Matrilin-3	-0.38	7	16.8	Day 35	O42401
Uncharacterized protein	-0.48	25	52	Day 7	R9PXM5
Hyaluronan binding protein 2	-0.48	15	31.4	Day 21	F1NEB3
Uncharacterized protein	-0.47	8	58.7	Day 21	A0A1D5PV72
Ig lambda chain C region	-0.45	9	50.7	Day 21	P20763
Protein-lysine 6-oxidase	-0.43	7	28	Day 35	A0A1D5P1U0
Adenosine deaminase	-0.4	17	66	Day 35	A0A1D5PDK4

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
HGF activator	-0.37	11	15.6	Day 7	E1BZN8
Collagen alpha-1(VI) chain	-0.34	10	12.7	Day 35	A0A1D5PWN6
Apolipoprotein A-I	-0.25	2	90.3	Day 7	P08250
Transforming growth factor beta induced	-0.25	8	70.3	Day 7	A0A1D5NX81
CD74 molecule	-0.21	4	20.8	Day 35	F1NYL5
ADAM metallopeptidase with thrombospondin type 1 motif 13	-0.12	29	27.1	Day 35	A0A1D5PEF7

[1]Fold change refers to the $\log_2$ fold change in protein abundance in response to AP treatment. [2]Coverage (%) refers to the % of protein sequence represented by identified peptides. [3]Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial.

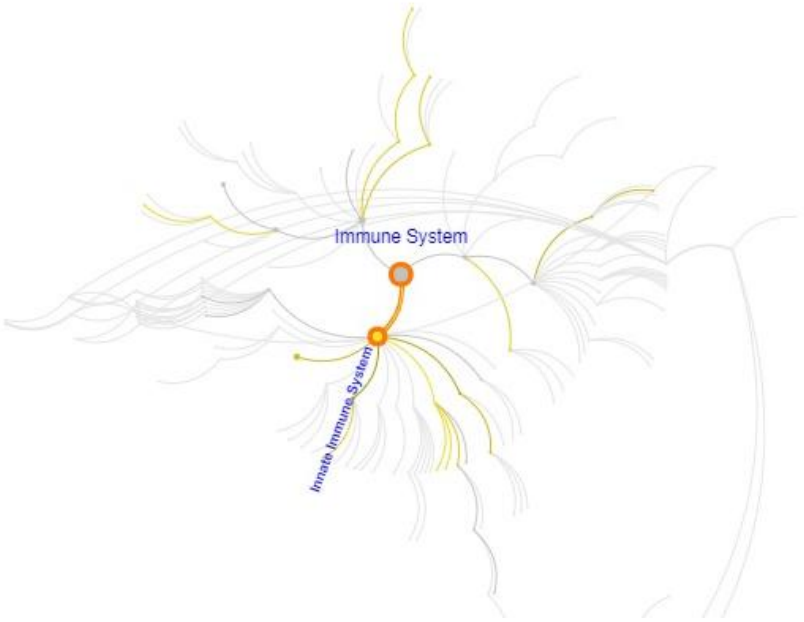

Figure 4.5. Pathway map of 19 proteins involved in the immune system that were significantly increased in abundance in the serum of AP supplemented broiler chickens, obtained using Reactome software. Highlighted lines and dots represent proteomic pathways within the immune system that contain proteins which have been increased in abundance with AP supplementation.

4.4 Quantitative proteomic analysis of serum from broilers fed a Natustat® supplemented diet vs. serum from broilers fed on a control diet

A total of 84 proteins were found to be significantly increased ($p < 0.05$) in abundance or uniquely present in the serum of NS supplemented birds across the three time points (Table 4.4). Proteins ($n = 62$) were significantly decreased in abundance ($p < 0.05$) or absent from NS samples in comparison to control samples across the three time points (Table 4.5).

Principal component analysis and hierarchal clustering were conducted on proteomic data obtained through LC-MS/MS analysis of NS and control serum samples. Figure 4.6 shows PCA of NS serum samples versus control serum samples. These data show that NS samples and control samples cluster separately on Day 7, Day 21 and Day 35. Hierarchal clustering was also conducted on proteomic data which matched PCA results on Day 7 and Day 21. However, one control sample on Day 35 clustered more closely with NS samples than the control group.

Using Reactome pathway mapping software, 21 proteins that were significantly increased ($p < 0.05$) in abundance or uniquely present in NS samples were identified as involved in the innate immune system. These proteins are: Proteasome endopeptidase complex (A0A1L1RSU8), Proteasome subunit beta 1 (A0A1L1RYR5), Proteasome 26S subunit, ATPase 5 (F1NU79), Uncharacterized protein (F1NPN5), Alpha-1-acid glycoprotein (Q8JIG5), Proteasome subunit alpha type (F1NC02), Proteasome subunit alpha type (A0A1L1S0K9), Proteasome subunit beta type (A0A1L1RUE7), Proteasome subunit alpha type (A0A1D5PHL0), Proteasome subunit alpha type (F1NEQ6), Vascular cell adhesion molecule 1 (F1P201), Elongation factor 1-alpha 1 (Q90835), Proteasome subunit alpha type (Q5ZJX9), Peroxiredoxin-6 (F1NBV0), Complement Factor H (E1C7P4), Interleukin 6 signal transducer (A0A1D5PMY8),

Glutaminyl-peptide cyclotransferase (A0A1D5PRR0), Complement C4 precursor (A0A1D5P5V5), Complement C5 (E1BRS7), Complement C6 (B8ZX71) and Complement C7 (E1C6U2). These proteins are represented in a Reactome pathway map (Figure 4.7). Mannose Binding Protein (Q98TA4) was also increased in abundance and approaching significance (p = 0.16) on Day 35 in NS samples (Appendix 1-Table 7.3b).

A number of proteins involved in ROS detoxification were significantly increased (p <0.05) in abundance on Day 7: specifically, Peroxiredoxin 6 (F1NBV0), Glutathione Peroxidase (F1NPJ8) and Glutaredoxin 3 (A0A1D5NW30).

A number of proteins involved in Vitamin A transport and metabolism were increased in abundance in NS samples. Retinol binding Protein 4 (P41263) level was significantly increased and Retinol binding Protein 7 (E1C0M1) was uniquely present in NS samples on Day 7.

The largest significant (p <0.05) Log_2 fold-change detected across all serum samples was seen in the Day 35 NS vs. control comparator group. Alpha-1-acid glycoprotein (A1AGP) (Q8JIG5) had a Log_2 fold change of 2.12 on Day 35. In order to see if a similar result could be obtained from another test, an A1AGP Chicken ELISA kit (ab157690) (abcam®) was used to test pooled serum samples. This assay was conducted on control and NS samples from Day 35, twice. Assay 1 (Appendix, Figure 7.1) showed an increased in the mean A1AGP concentration in NS samples when compared to the control, increasing from 1.64 mg/ml to 2.27 mg/ml. This increase in abundance in NS samples was in accordance with the change observed in LC-MS/MS analysis, however this change was not significant (p = 0.169). The average CV% was 17.7 in assay 1 with CV% for two replicates reaching above 30%. Assay 2 (Appendix, Figure 7.2) again showed an increase in the mean concentration of A1AGP in NS

samples, from 2.2 mg/ml to 2.3 mg/ml though this change was not significant ($p = 0.6$). The average CV% for assay 2 was 2.3%. However, the change in concentration between NS and control samples was smaller than the change detected in assay 1.

A number of proteins of note showed changes in abundance in NS samples. Osteocalcin (P02822) was uniquely present in NS samples on Day 21. Gastrokine-2 (A0A1D5PFM9) was uniquely present in NS samples on Day 35. Transferrin Receptor protein was significantly increased ($p < 0.05$) with a relatively large Log_2 fold change (1.7) on Day 35. Insulin like growth factor binding protein 5 (F1ND88) was significantly decreased ($p < 0.05$) on Day 35.

Proteins ($n = 5$) with involvement in carbohydrate metabolism were altered in abundance ($p < 0.05$) with NS supplementation. These include N-phosphoglycerate kinase (F1NU17), glyceraldehyde-3-phosphate dehydrogenase (P00356) and mimecan (Q9W6H0) which were significantly increased ($p < 0.05$) in abundance in NS samples. N-acetyl-alpha-glucosaminidase (A0A1D5NU78) was uniquely present on Day 7. Beta-hexosaminidase (F1NTQ2) was increased in abundance and approaching significance ($p = 0.09$) on Day 7 (Appendix 1-Table 7.2). α-Enolase (A0A1D5PSH6) was significantly decreased ($p < 0.05$) on Day 21. α-Amylase was increased in abundance and approaching significance ($p = 0.08$) on Day 7 (Appendix 1-Table 7.3b). β-Enolase (P07322) showed decrease in abundance and approaching significance ($p = 0.09$) on Day 21(Appendix 1 Table -7.3b) and significantly decreased ($p < 0.05$) in abundance on Day 35 (Table 4.5).

Proteins involved in nucleotide metabolism were significantly altered in abundance ($p < 0.05$) or unique to/absent from, NS samples. Nucleoside diphosphate kinase (O57535) and cytidine/uridine monophosphate kinase 2 (R4GJC4) were uniquely

present in NS samples on Day 21 and Day 7, respectively. Adenylate kinase isoenzyme (P05081) was significantly increased ($p < 0.05$) on Day 21 and adenosine deaminase (Q5ZKP6) was significantly decreased ($p < 0.05$) on Day 21 and Day 35.

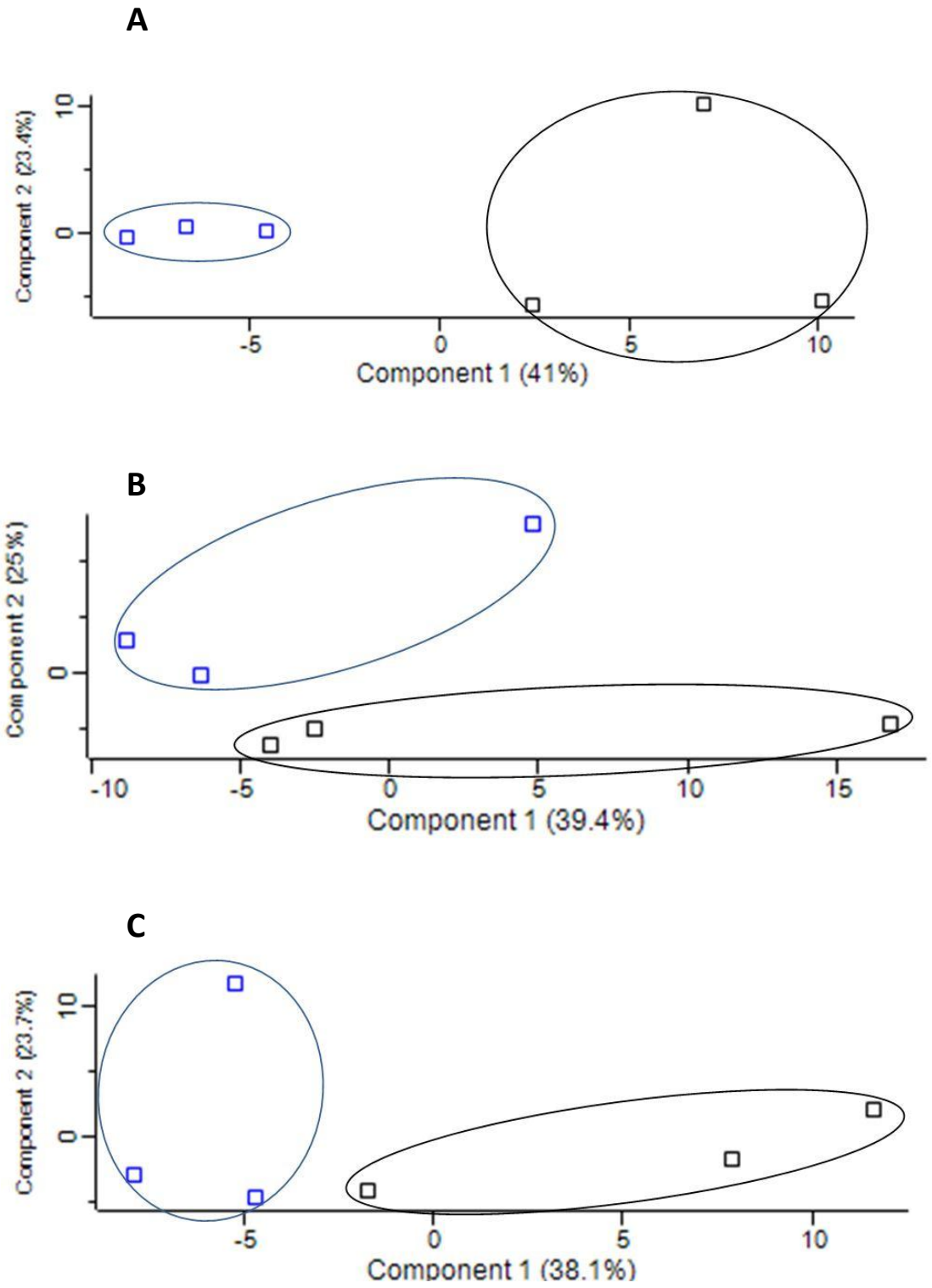

Figure 4.6. Principal Component Analysis (PCA) comparing NS (Blue) vs. control (Black) pooled serum samples for A. Day 7, B. Day 21, and C. Day 35.

Table 4.4. Proteins with significantly (p<0.05) increased abundance or **unique** in NS feed treatment samples. Proteins are listed in order of change of abundance.

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
N-acetyl-alpha-glucosaminidase	Unique	2	3.7	Day 7	A0A1D5NU78
Uncharacterized protein	Unique	2	1	Day 7	A0A1D5NZ61
Dynactin subunit 2	Unique	6	22.1	Day 7	A0A1D5PGQ9
Uncharacterized protein	Unique	5	41.4	Day 7	A0A1D5PQ15
Proteasome endopeptidase complex	Unique	3	43.7	Day 7	A0A1L1RSU8
Proteasome subunit beta 1	Unique	3	36.4	Day 7	A0A1L1RYR5
Retinol binding protein 7	Unique	3	32.1	Day 7	E1C0M1
Uncharacterized protein	Unique	4	30.8	Day 7	F1N8Y3
Elongation factor 1-alpha	Unique	15	49.9	Day 7	F1N9H4
Succinyl-CoA:3-ketoacid-coenzyme A transferase	Unique	3	11	Day 7	F1N9Z7
Endoplasmic reticulum lectin 1	Unique	3	12	Day 7	F1NCV8
Small nuclear ribonucleoprotein 13	Unique	3	30.5	Day 7	F1NII6
PDZ and LIM domain 5	Unique	4	8.2	Day 7	F1NTC8
Proteasome 26S subunit, ATPase 5	Unique	3	12.7	Day 7	F1NU79

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Natriuretic peptides A (Prepronatriodilatin)	Unique	3	17.9	Day 7	P18908
Profilin	Unique	2	27.9	Day 7	Q5ZL50
Twisted gastrulation protein homolog 1	Unique	2	15.6	Day 7	Q98T89
Cytidine/uridine monophosphate kinase 2	Unique	6	26.5	Day 7	R4GJC4
Nucleoside diphosphate kinase	Unique	2	20.3	Day 21	O57535
Osteocalcin	Unique	3	45.4	Day 21	P02822
Tenascin	Unique	8	8.7	Day 21	P10039
Gastrokine 2	Unique	2	18.6	Day 35	A0A1D5PFM9
N-acetylglucosamine-1-phosphate transferase gamma subunit	Unique	2	16	Day 35	E1BS68
N-acetylglucosamine-1-phosphate transferase gamma subunit	0.34	5	27.2	Day 7	E1BS68
Adhesion G protein-coupled receptor G6	Unique	2	2.5	Day 35	E1C8C2
Alpha-1-anti-ase	Unique	9	27.8	Day 35	F1NPN5
Aggrecan core protein	Unique	3	2.3	Day 35	F1NZX1
Alpha-1-acid glycoprotein	2.12	7	13.9	Day 35	Q8JIG5
Transferrin receptor protein 1	1.7	10	10.5	Day 35	Q90997

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Heat shock protein beta-1	1.3	18	77.9	Day 7	F1P593
Myosin light chain 1, skeletal muscle isoform	1.23	9	54.9	Day 7	P02604
Myosin regulatory light chain 2, skeletal muscle isoform	1.22	9	57.7	Day 7	P02609
Adenylate kinase isoenzyme 1	1.15	11	58.1	Day 7	P05081
Glyceraldehyde-3-phosphate dehydrogenase	1.13	20	72.1	Day 7	P00356
Proline and arginine rich end leucine rich repeat protein	1.13	10	27.9	Day 7	A0A1D5PAN0
Glycerol-3-phosphate dehydrogenase	1.11	9	24	Day 7	A0A1D5P1Y7
Proteasome subunit alpha type	1.08	7	36.2	Day 7	F1NC02
Uncharacterized protein	1.08	7	21.1	Day 7	F1NIP5
Phosphoglycerate kinase	0.94	17	51.9	Day 7	F1NU17
Complement C5	0.93	82	54.8	Day 35	E1BRS7
Dynactin subunit 3	0.89	6	32.9	Day 7	A0A1D6UPU1
Glutaredoxin 3	0.88	8	29.8	Day 7	A0A1D5NW30

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Uncharacterized protein	0.86	7	15.2	Day 7	A0A1L1RQM3
Low molecular weight phosphotyrosine protein phosphatase	0.84	10	58.5	Day 7	A0A1D5P9Z1
Proteasome subunit alpha type	0.84	9	46.3	Day 7	A0A1L1S0K9
Protein/nucleic acid deglycase DJ-1	0.84	5	51.4	Day 7	A0A1D5PN39
Complement C6	0.83	29	33.4	Day 35	B8ZX71
Proteasome subunit beta type	0.79	13	56.6	Day 7	A0A1L1RUE7
Proteasome subunit alpha type	0.79	5	36.1	Day 7	A0A1D5PHL0
Periostin	0.77	16	24.5	Day 21	F1P4N9
Receptor of-activated protein C kinase 1	0.76	9	40.9	Day 7	A0A1I7Q3Y2
Proteasome subunit alpha type	0.76	6	23.9	Day 7	F1NEQ6
Serpin H1	0.76	12	34.9	Day 7	P13731
Vascular cell adhesion molecule 1	0.75	6	13	Day 21	F1P201

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Ribosomal protein S14	0.69	4	34.9	Day 7	Q5ZHW8
T-complex 1	0.68	9	17.6	Day 7	Q5ZMG9
Uncharacterized protein	0.67	8	38.3	Day 7	A0A1D5PAH2
Complement C7	0.66	28	52.3	Day 35	E1C6U2
T-complex protein 1 subunit zeta	0.64	20	51.8	Day 7	Q5ZJ54
Elongation factor 1-alpha 1	0.63	18	54	Day 7	Q90835
Retinol-binding protein 4	0.61	11	68.9	Day 7	P41263
Proteasome subunit alpha type	0.6	6	41.5	Day 7	Q5ZJX9
Nuclear transport factor 2	0.55	3	33.9	Day 7	F1NLL4
Chaperonin containing TCP1 subunit 5	0.55	17	34.4	Day 7	Q5F411
40S ribosomal protein S12	0.54	7	61.4	Day 7	P84175
Uncharacterized protein	0.54	6	39.8	Day 35	A0A1D5PK48
Peroxiredoxin-6	0.53	12	66.4	Day 7	F1NBV0
Uncharacterized protein	0.51	6	40.2	Day 35	A0A1D5PZU8
Eukaryotic translation initiation factor 5A-1	0.5	6	56.6	Day 7	Q09121

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Glia maturation factor beta	0.5	5	31.6	Day 7	A0A1D6UPR3
Complement C4 precursor	0.48	61	45.6	Day 35	A0A1D5P5V5
Collagen type V alpha 1 chain	0.46	6	4.8	Day 21	F1NI79
Insulin like growth factor binding protein acid labile subunit	0.44	8	15.4	Day 21	F1NI07
TRK-fused gene	0.41	4	12.9	Day 7	A0A1L1RK44
Complement factor B-like protease	0.39	6	36.8	Day 7	P81475
Nidogen 2	0.36	20	16.3	Day 35	F1NDL4
Uncharacterized protein	0.35	5	17.6	Day 7	Q5ZMC1
Complement factor H	0.32	93	71.5	Day 7	E1C7P4
Glutathione peroxidase	0.3	11	45.9	Day 7	F1NPJ8
Interleukin 6 signal transducer	0.29	3	5	Day 7	A0A1D5PMY8
Sortilin	0.28	14	21.3	Day 7	A0A1D5PNT8

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Glutaminyl-peptide cyclotransferase	0.26	13	58.5	Day 7	A0A1D5PRR0
Fibromodulin	0.23	2	3.7	Day 21	P51887
Mimecan	0.15	9	32.7	Day 35	Q9W6H0

[1]Fold change refers to the $\log_2$ fold change in protein abundance in response to NS treatment. [2]Coverage (%) refers to the % of protein sequence represented by identified peptides. [3]Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial.

Table 4.5. Proteins with significantly ($p<0.05$) decreased abundance or **absent** in NS feed treatment samples. Proteins are listed in order of change of abundance.

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Malate dehydrogenase	Absent	3	23.3	Day 7	A0A1D5PZS3
Junction plakoglobin	Absent	6	10.4	Day 7	E1C1V3
Uncharacterized protein	Absent	2	19.1	Day 7	R4GIC2
DEAD-box helicase 17	Absent	3	6.1	Day 21	A0A1D5PD32
Uncharacterized protein	Absent	3	9	Day 21	A0A1D5PF52
V-type proton ATPase subunit B, brain isoform	Absent	3	11.3	Day 21	A0A1D5PP57
Splicing factor proline and glutamine rich	Absent	3	18.4	Day 21	A0A1D5PPW4
Glutathione S-transferase	Absent	2	9.5	Day 21	Q08392
T-complex protein 1 subunit theta	Absent	3	6.8	Day 21	Q6EE31
NSF attachment protein alpha	Absent	2	8.2	Day 35	A0A1D5NUZ0
Uncharacterized protein	Absent	3	2.5	Day 35	A0A1D5NW21
Glia maturation factor beta	Absent	2	28	Day 35	A0A1D6UPR3
Uncharacterized protein	Absent	5	11.7	Day 35	E1BSP1
Heterogeneous nuclear ribonucleoprotein A2/B1	Absent	5	20.1	Day 35	Q5ZME1

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Serpin family F member 2	-2.17	13	24.2	Day 7	F1NAR5
Beta-enolase	-1.65	12	40.9	Day 35	P07322
Receptor of-activated protein C kinase 1	-1.33	6	21.9	Day 21	A0A1I7Q3Y2
Serpin family G member 1	-1.24	12	15.8	Day 7	F1NA58
Uncharacterized protein	-1.18	2	0.7	Day 7	A0A1L1RLW1
Tubulin beta-7 chain	-1.18	12	40.1	Day 21	P09244
Uncharacterized protein	-1.11	11	8.6	Day 21	A0A1L1RJ91
Phosphoglycerate kinase	-1.03	11	27.8	Day 21	A0A1D5NZW9
Fibulin 5	-0.85	7	17.2	Day 35	A0A1L1RQ98
Elongation factor 1-alpha 1	-0.84	16	49.7	Day 21	Q90835
Pyruvate kinase PKM	-0.83	26	45.6	Day 35	P00548
Adenosine deaminase	-0.8	17	60.4	Day 35	Q5ZKP6
DAZ associated protein 1	-0.79	4	15.4	Day 21	Q5ZM92
Alpha-enolase	-0.74	14	34.3	Day 21	A0A1D5PSH6
Chaperonin containing TCP1 subunit 5	-0.73	9	22.25	Day 21	Q5F411

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Macrophage receptor with collagenous structure	-0.71	10	21.4	Day 21	A0A1D5PJZ3
Adenosine deaminase	-0.7	20	69.8	Day 21	A0A1D5PDK4
Tubulin alpha chain	-0.68	11	30.3	Day 21	A0A1D5PC38
Uncharacterized protein	-0.68	10	30.3	Day 21	E1C477
Heterogeneous nuclear ribonucleoprotein K	-0.68	3	8.4	Day 21	A0A1L1S010
T-complex protein 1 subunit zeta	-0.67	14	31.2	Day 21	Q5ZJ54
Phosphoglycerate mutase 1	-0.62	9	25.4	Day 35	Q5ZLN1
Tubulin beta-6 chain	-0.62	14	38.2	Day 21	P09207
LSM8 homolog, U6 small nuclear RNA associated	-0.6	4	55.9	Day 21	E1BZ75
Chaperonin containing TCP1 subunit 2	-0.57	11	26.5	Day 21	Q5F424
Alpha-actinin-1	-0.56	4	4.8	Day 21	A0A1D5P9P3
Uncharacterized protein	-0.54	7	18.5	Day 21	F1NIP5
Complement C1q C chain	-0.52	5	24	Day 21	A0A1D5PGB2
Kininogen 1	-0.52	15	28.1	Day 7	A0A1L1RNR4
Complement C1q B chain	-0.51	6	33.6	Day 21	F1NH19
Insulin like growth factor binding protein 5	-0.5	3	19.3	Day 35	F1ND88

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Vitronectin	-0.48	15	39.5	Day 7	E1C7A7
Fibulin-1	-0.46	23	39.3	Day 35	O73775
Ubiquilin 4	-0.46	5	12.4	Day 21	A0A1D5P624
Methylthioribose-1-phosphate isomerase 1	-0.46	9	46.6	Day 21	A0A1D5PN97
EGF containing fibulin like extracellular matrix protein 1	-0.45	19	52.7	Day 35	A0A1D5P380
Far upstream element binding protein 1	-0.45	12	21.5	Day 21	A0A1D5P2H3
Apolipoprotein H	-0.44	14	42.7	Day 35	A0A1L1RTQ4
Epiphycan	-0.43	5	13.7	Day 7	Q90944
Ig lambda chain C region	-0.42	9	53.3	Day 35	P20763
Aggrecan core protein	-0.38	16	7.7	Day 7	F1NZX1
Fibulin-1	-0.38	18	31.8	Day 21	A0A1L1RU28
Uncharacterized protein	-0.34	43	25.4	Day 35	F1NEQ4
Far upstream element-binding protein 2	-0.3	13	18.7	Day 21	Q8UVD9
F-actin-capping protein subunit beta isoforms 1 and 2	-0.29	11	36.2	Day 21	P14315

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Uncharacterized protein	-0.28	2	38.5	Day 21	F1NSC8
Vimentin	-0.27	30	64.6	Day 21	A0A1L1RXL9
Fibrinogen gamma chain	-0.18	9	33.7	Day 7	E1BV78

[1]Fold change refers to the $\log_2$ fold change in protein abundance in response to NS treatment. [2]Coverage (%) refers to the % of protein sequence represented by identified peptides. [3]Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial.

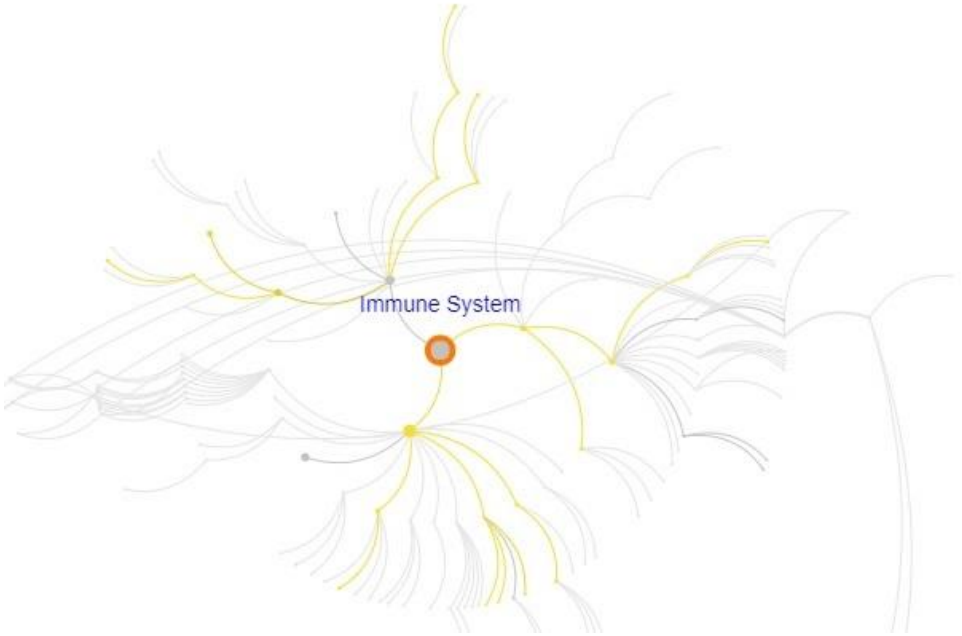

Figure 4.7. Pathway map of 19 proteins involved in the immune system that were significantly increased in abundance or uniquely present in the serum of NS supplemented broiler chickens, obtained using Reactome software.

4.5 Quantitative proteomic analysis of serum from broilers fed a PowerTract® supplemented diet vs. serum from broilers fed on a control diet.

Proteins (n = 64) were found to be significantly increased ($p < 0.05$) in abundance or uniquely present in PT samples when compared to control, across all time points (Table 4.6). Proteins (n = 54) were significantly decreased ($p < 0.05$) or absent from PT samples across all time points (Table 4.7).

Principal component analysis and hierarchal clustering were conducted on proteomic data obtained through LC-MS/MS analysis of PT and control serum samples. Figure 4.8 shows PCA of PT serum samples versus control serum samples. These data reveal that PT samples and control samples cluster separately on Day 7, Day 21 and Day 35. Hierarchal clustering was also conducted on proteomic data which did not match PCA grouping on Day 35, although samples largely grouped together on Day 7 and Day 21 (data not shown).

Using the Reactome pathway mapping software, 17 proteins that were found to be significantly increased ($p < 0.05$) in abundance or uniquely present in PT samples, were identified as involved in the innate immune response. These proteins are: Uncharacterised protein (F1NPN5), Proteasome subunit beta 1 (A0A1L1RYR5), Complement factor H (E1C7P4), Proteasome 26S subunit, ATPase 5 (F1NU79), Dual specificity phosphatase 3 (A0A1L1S0I4), Chromogranin A (F1NLZ2), Transthyretin (P27731), Alpha-1,4 glucan phosphorylase (E1BSN7), Proteasome subunit alpha type (F1NEQ6), Beta-2-microglobulin (P21611), Beta-hexosaminidase (F1NTQ2), Complement C2 (A0A1D5P4P1), Complement C4 precursor (A0A1D5P5V5), Complement C5 (E1BRS7), Complement C6 (B8ZX71), Complement C7 (E1C6U2) and Mannose-binding protein (Q98TA4). These proteins are represented in a Reactome pathway map in Figure 4.9.

Proteins (n = 4) involved in carbohydrate metabolism showed changes in abundance in PT samples. Glyceraldehyde-3-phosphate dehydrogenase (P00356) was significantly increased ($p < 0.05$) on Day 7. Glutamate dehydrogenase 1 and Beta-1,4-galactosyltransferase 4 (E1C9B0) were absent from PT samples on Day 7.

Proteins (n = 4) involved in nucleotide metabolism showed significant ($p < 0.05$) alterations in abundance in PT samples. Adenylate kinase isoenzyme significantly increased ($p < 0.05$) on Day 7. Guanine Deaminase (F1NJD6) significantly decreased ($p <0.05$) on Day 21. Adenosine deaminase (A0A1D5PDK4) significantly decreased ($p < 0.05$) on Day 35. Deoxythymidylate kinase (A0A1D5PKC2) was absent from PT samples on Day 7.

Proteins (n = 2) involved in Vitamin A transport increased in abundance in PT samples. Retinol binding protein 4 (P41263) was increased in abundance and approaching significance ($p = 0.06$) on Day 21. Transthyretin (P27731) was significantly increased ($p < 0.05$) in abundance on Day 35.

Many key proteins involved in the detoxification of ROS were significantly decreased ($p < 0.05$) in abundance or absent from PT samples. Two Catalase proteins were absent from PT samples on Day 7 and Day 21, respectively, Catalase (A0A1D5PPU9) and Catalase (Q5ZL24). Peroxiredoxin-1 (P0CB50) was significantly decreased in abundance on Day 35. Glutathione Peroxidase (F1NPJ8) was increased in abundance and approaching significance ($p = 0.08$ and 0.09 respectively) in PT samples on Day 21 (Appendix 1- Table 7.5) and Day 35 (Appendix 1- Table 7.5b). Glutathione-S-Transferase (GST) (Q08392) was absent from PT samples in all time points, this protein was identified as GST alpha-class.

A number of proteins showed changes in abundance in PT samples. Transferrin Receptor Protein was significantly increased ($p < 0.05$) on Day 35. Gastrin releasing peptide (A0A1D5PXC4) was significantly decreased ($p < 0.05$) in abundance on Day 35.

PT-supplemented birds were the only group fed an organic source of selenium in their diet instead of the inorganic source selenium selenite. In order to examine any potential effects of this change in selenium source, control and PT samples were examined for a SeMet/SeCys substitution for methionine/cysteine using MQ. Control and PT samples were also searched for known selenoproteins. There was one protein identified as having SeMet/SeCys substitution in control samples (Table 4.8) this protein was SEC31 homolog B, COPII coat complex component (E1BXC8). The protein was detected in only one control sample with a sequence coverage of 3.6% and 1 peptide detected. Proteins ($n = 4$) were identified as having a SeMet/SeCys substitution in PT samples (Table 4.9), Coatomer subunit alpha (A0A1D5P185), Golgin A4 (A0A1D5PNT3), Nuclear factor related to kappaB binding protein (E1BZI6) and Translocase of outer mitochondrial membrane 34 (F1P4X4). The sequence coverage of proteins identified was low, namely 0.8-5.4%. Of the four proteins identified with a SeMet/SeCys substitution, three were identified in one PT pooled serum sample obtained on Day 7.

Using a list of previously identified selenoproteins obtained from Liu *et al.* (2017) (Appendix 1-Table 7.7), one selenoprotein was identified in PT and control samples, Glutathione Peroxidase (F1NPJ8). This protein was increased in abundance with *p*-value approaching significance on Day 21 ($p = 0.08$) and Day 35 ($p = 0.09$) (Table 4.10). Selenoprotein F precursor (A0A1D5PFR6) was uniquely detected in PT samples, but was not detected in AP, NS and control samples.

Alpha-1-Acid Glycoprotein was increased in abundance on Day 35 in samples from PT supplemented birds with a large fold change (Appendix 1-Table 7.6). However, this change was not significant ($p = 0.36$).

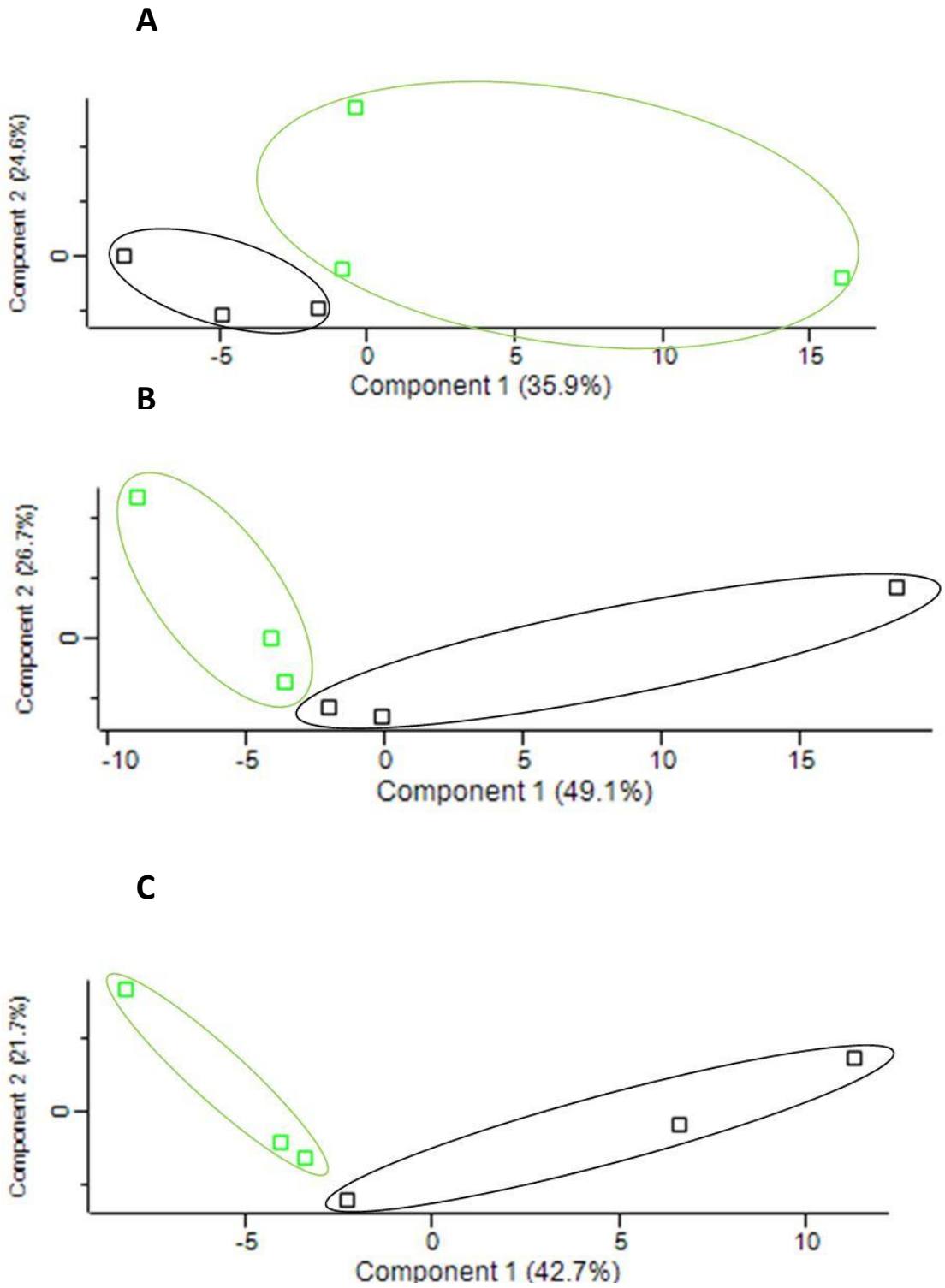

Figure 4.8. Principal Component Analysis (PCA) graphs comparing PT (Green) vs. control (Black) pooled serum samples for A: Day 7, B: Day 21, and C: Day 35.

Table 4.6. Proteins with significantly (p<0.05) increased abundance or **unique** in PT feed treatment samples. Proteins are listed in order of change of abundance.

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Barrier to autointegration factor 1	Unique	2	42.2	Day 7	A0A1D5NXY4
HSPA (Hsp70) binding protein 1	Unique	5	12.1	Day 7	A0A1D5P628
Uncharacterized protein	Unique	9	41.4	Day 7	A0A1D5PQ15
Proteasome subunit beta 1	Unique	4	36.4	Day 7	A0A1L1RYR5
Ankyrin repeat domain 2	Unique	5	8.9	Day 7	E1C1Q6
Plexin domain containing 2	Unique	3	5.9	Day 7	E1C486
Mediator of cell motility 1	Unique	2	21.9	Day 7	E1C6C0
3'-phosphoadenosine 5'-phosphosulfate synthase 1	Unique	3	12.3	Day 7	E1C8P2
Elongation factor 1-alpha	Unique	3	49.9	Day 7	F1N9H4
Four and a half LIM domains 1	Unique	3	18.5	Day 7	F1NED9
Small nuclear ribonucleoprotein 13	Unique	4	30.5	Day 7	F1NII6
Chromogranin A	Unique	3	10.7	Day 7	F1NLZ2
PDZ and LIM domain 5	Unique	3	8.2	Day 7	F1NTC8

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Proteasome 26S subunit, ATPase 5	Unique	2	12.7	Day 7	F1NU79
Uncharacterized protein	Unique	2	14.1	Day 7	F1NWB2
Natriuretic peptides A	Unique	2	17.9	Day 7	P18908
Cofilin-2	Unique	4	25.9	Day 7	P21566
Stratifin	Unique	15	28.5	Day 7	R4GF89
Seryl-tRNA synthetase	Unique	3	4.3	Day 7	R4GJ59
Disintegrin and metalloproteinase domain-containing protein 33 precursor	Unique	2	3.6	Day 21	A0A1D5NV10
Gastrokine 2	Unique	3	18.6	Day 35	A0A1D5PFM9
Dual specificity phosphatase 3	Unique	2	26.3	Day 35	A0A1L1S0I4
Alpha-1-anti-ase	Unique	2	27.8	Day 35	F1NPN5
Aggrecan core protein	Unique	2	2.3	Day 35	F1NZX1
Insulin-like growth factor I	Unique	3	19.0	Day 35	P18254
Zyxin	Unique	5	4.8	Day 35	Q04584
Adenylate kinase isoenzyme 1	1.12	11	60.3	Day 7	P05081
Transferrin receptor protein 1	1.07	10	9.7	Day 35	F1NTM6

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Myosin regulatory light chain 2, skeletal muscle isoform	1.07	9	57.8	Day 7	P02609
Uncharacterized protein	1.04	2	19.2	Day 35	A0A1D5P1L5
Complement C5	1.04	82	55.5	Day 35	E1BRS7
Heat shock protein beta-1	1.03	18	77.9	Day 7	F1P593
Transthyretin	1.00	9	71.4	Day 35	P27731
Glyceraldehyde-3-phosphate dehydrogenase	0.95	20	72.0	Day 7	P00356
Uncharacterized protein	0.94	4	37.9	Day 21	A0A1L1RQF3
Myosin light chain 1, skeletal muscle isoform	0.90	9	53.9	Day 7	P02604
Alpha-1,4 glucan phosphorylase	0.89	5	6.5	Day 35	E1BSN7
Uncharacterized protein	0.79	6	39.8	Day 35	A0A1D5PK48
Actin, alpha skeletal muscle	0.78	13	34.8	Day 21	A0A1I7Q414
Low molecular weight phosphotyrosine protein phosphatase	0.75	10	58.5	Day 7	A0A1D5P9Z1
Uncharacterized protein	0.70	7	21.5	Day 7	F1NIP5
Complement C6	0.68	29	34.6	Day 35	B8ZX71

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Complement C7	0.68	28	52.0	Day 35	E1C6U2
Uncharacterized protein	0.67	4	37.1	Day 35	A0A1L1RQF3
Uncharacterized protein	0.63	18	22.3	Day 35	A0A1L1S0T3
Uncharacterized protein	0.60	2	23.0	Day 21	A0A1D5P058
Uncharacterized protein	0.56	5	42.3	Day 35	F1NSC7
Complement C4 precursor	0.55	61	48.1	Day 35	A0A1D5P5V5
Mannose-binding protein	0.50	8	27.5	Day 35	Q98TA4
Proteasome subunit alpha type	0.45	6	23.5	Day 7	F1NEQ6
Beta-2-microglobulin	0.45	5	60.5	Day 21	P21611
Uncharacterized protein	0.44	4	38.5	Day 35	F1NSC8
Beta-hexosaminidase	0.42	20	49.2	Day 7	F1NTQ2
Sortilin	0.41	14	20.9	Day 7	A0A1D5PNT8
RAD23 homolog B, nucleotide excision repair protein	0.41	28	9.0	Day 7	F1N9B7

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Complement C2	0.41	2	15.7	Day 35	A0A1D5P4P1
Vascular cell adhesion molecule 1	0.39	9	12.3	Day 21	F1P201
Uncharacterized protein	0.38	6	21.3	Day 21	A0A1L1S0T3
Complement factor H	0.28	15	71.9	Day 21	E1C7P4
Plasminogen	0.23	94	69.7	Day 21	R4GMH5
Apolipoprotein A-I	0.20	57	92.8	Day 21	P08250
Uncharacterized protein	0.19	55	17.3	Day 7	Q5ZMC1
Lumican	0.17	5	39.2	Day 35	P51890
Prolyl 4-hydroxylase subunit alpha-1	0.13	11	21.5	Day 7	P16924

[1]Fold change refers to the $\log_2$ fold change in protein abundance in response to PT treatment. [2]Coverage (%) refers to the % of protein sequence represented by identified peptides. [3]Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial.

Table 4.7. Proteins with significant (p <0.05) decreased abundance or **absent** in PT feed treatment samples. Proteins are listed in order of change of abundance.

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Glutamate dehydrogenase 1, mitochondrial	Absent	4	9.9	Day 7	A0A1D5NT61
Growth differentiation factor 11	Absent	2	6.4	Day 7	A0A1D5P7V6
Catalase	Absent	3	5.3	Day 7	A0A1D5PPU9
Malate dehydrogenase	Absent	3	23.3	Day 7	A0A1D5PZS3
Integral membrane protein 2B	Absent	2	46.2	Day 7	A0A1L1RIU5
Uncharacterized protein	Absent	2	0.9	Day 7	A0A1L1RLW1
Beta-1,4-galactosyltransferase 4	Absent	2	8.5	Day 7	E1C9B0
Carbamoyl-phosphate synthase 1	Absent	4	3.3	Day 7	F1N9N8
Glutathione S-transferase	Absent	2	9.5	Day 7	Q08392
NSF attachment protein alpha	Absent	2	8.2	Day 21	A0A1D5NUZ0
Uncharacterized protein	Absent	3	9	Day 21	A0A1D5PF52
Deoxythymidylate kinase	Absent	3	22.6	Day 21	A0A1D5PKC2
Lysophospholipase II	Absent	2	19	Day 21	E1BRI5
Small nuclear ribonucleoprotein 13	Absent	3	36.7	Day 21	F1NII6

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Proteasome 26S subunit, ATPase 5	Absent	3	12.7	Day 21	F1NU79
Glutathione S-transferase	Absent	2	9.5	Day 21	Q08392
Catalase	Absent	2	8.9	Day 21	Q5ZL24
Carbohydrate sulfotransferase 3	Absent	4	13.3	Day 21	Q92179
NSF attachment protein alpha	Absent	2	8.2	Day 35	A0A1D5NUZ0
Uncharacterized protein	Absent	3	2.5	Day 35	A0A1D5NW21
Reversion inducing cysteine rich protein with kazal motifs	Absent	6	8.9	Day 35	A0A1D5PTW4
Uncharacterized protein	Absent	3	29.6	Day 35	A0A1D5PZ95
Glutathione S-transferase	Absent	2	9.5	Day 35	Q08392
TAR DNA-binding protein 43	Absent	3	14.5	Day 35	Q5ZLN5
Heterogeneous nuclear ribonucleoprotein A2/B1	Absent	5	20.1	Day 35	Q5ZME1
Cytidine/uridine monophosphate kinase 2	Absent	5	25.7	Day 35	R4GJC4
Gastrin-releasing peptide	-1.30	3	19.7	Day 35	A0A1D5PXC4

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Fibromodulin	-1.20	4	7.7	Day 35	P51887
Tubulin beta-7 chain	-1.20	12	39.7	Day 21	P09244
Beta-tropomyosin	-1.18	27	62.2	Day 35	Q05705
Integrin-linked kinase	-1.11	4	10.3	Day 35	Q9DF58
cAMP-dependent protein kinase type I-alpha regulatory subunit	-1.02	11	27.4	Day 35	Q5ZM91
Fibulin 5	-0.90	7	16.8	Day 35	A0A1L1RQ98
cAMP-dependent protein kinase type I-alpha regulatory subunit	-0.89	6	20.3	Day 21	Q5ZM91
Tropomyosin alpha-1 chain	-0.84	12	47.6	Day 21	A0A1D5P342
MHC class II beta chain 2	-0.82	3	14.8	Day 21	A5HUL4
Elongation factor 1-alpha 1	-0.77	16	50.0	Day 21	Q90835

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Beta-tropomyosin	-0.71	22	61.3	Day 21	Q05705
Hyaluronan binding protein 2	-0.70	15	32.5	Day 21	F1NEB3
Phosphatidylcholine-sterol acyltransferase	-0.68	13	37.4	Day 21	P53760
Heat shock protein beta-1	-0.67	17	79.4	Day 35	F1P593
Collagen alpha-2(I) chain	-0.65	11	10.1	Day 35	P02467
Complement C7	-0.61	21	43.8	Day 7	E1C6U2
Alpha-actinin-1	-0.48	4	4.37	Day 21	A0A1D5P9P3
DAZ associated protein 1	-0.47	4	14.0	Day 21	Q5ZM92
Adenosine deaminase	-0.41	17	65.6	Day 35	A0A1D5PDK4
Alpha-actinin-1	-0.41	15	12.7	Day 35	A0A1D5P9P3
Chemerin	-0.41	7	46.2	Day 35	A0A0K0PUH6
Collagen type XVIII alpha 1 chain	-0.40	5	4.7	Day 35	A0A1D5P5M7
Guanine deaminase	-0.38	8	23.5	Day 21	F1NJD6
Macrophage receptor with collagenous structure	-0.33	10	21.4	Day 21	A0A1D5PJZ3
Peroxiredoxin-1	-0.30	8	45.5	Day 35	P0CB50

Protein Description	Fold Change[1]	Peptides	Coverage (%)[2]	Day[3]	Accession
Fibulin-1	-0.16	18	31.1	Day 21	A0A1L1RU28
Heterogeneous nuclear ribonucleoprotein M	-0.15	6	7.7	Day 21	F7B5K7

[1]Fold change refers to the $\log_2$ fold change in protein abundance in response to PT treatment. [2] Coverage (%) refers to the % of protein sequence represented by identified peptides. [3]Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial.

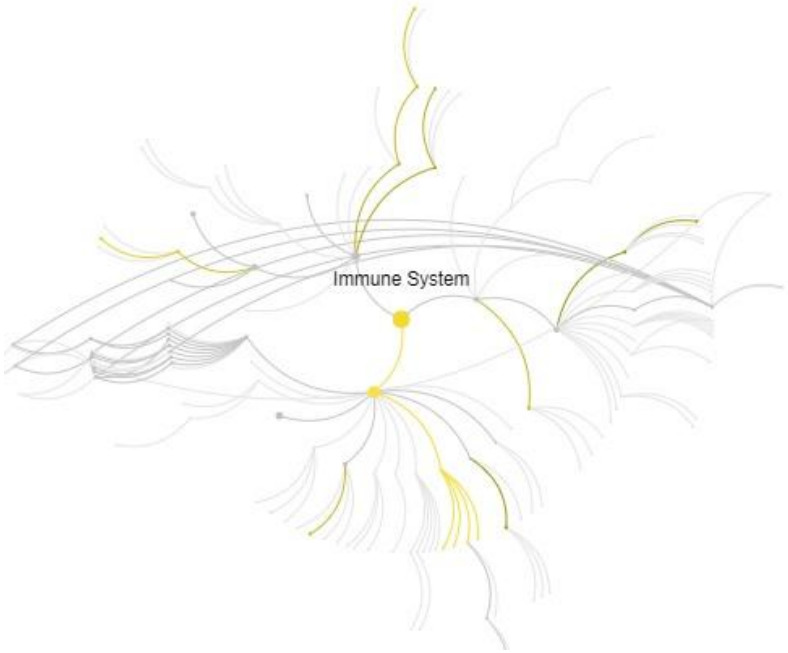

Figure 4.9. Pathway map of 19 proteins involved in the immune system that were significantly increased in abundance or uniquely present in the serum of PT supplemented broiler chickens, obtained using Reactome software.

Table 4.8. Proteins in control samples which were detected to have a SeMet/SeCys substitution.

Protein Description	Sample Pool	Coverage (%)[1]	Peptides	Substitution Site Position	Accession
SEC31 homolog B, COPII coat complex component	Day 7 Pen 7 Pool	3.6	1	4;5	E1BXC8

[1] Coverage (%) refers to the % of protein sequence represented in identified peptides.

Table 4.9. Proteins in PT samples which were detected to have a SeMet/SeCys substitution

Protein Description	Sample Pool	Coverage (%)[1]	Peptides	Substitution Site Position	Accession
Coatomer subunit alpha	Day 7 Pen 9 Pool	0.8	1	719;720	A0A1D5P185
Golgin A4	Day 7 Pen 9 Pool	1.2	2	1320	A0A1D5PNT3
Nuclear factor related to kappaB binding protein	Day 7 Pen 9 Pool	0.8	1	934	E1BZI6
Translocase of outer mitochondrial membrane 34	Day 35 Pen 2 Pool	5.4	2	307	F1P4X4

[1] Coverage (%) refers to the % of protein sequence represented by identified peptides.

Table 4.10. Glutathione Peroxidase (F1NPJ8) Log_2 fold change between control and PT samples on Day 7 Day 21 and Day 35.

Day[1]	**Fold Change**[2]	***p*-value**	**Coverage (%)**[3]	**Peptides**
Day 7	0.099	0.44	45.4	11
Day 21	0.36	0.08	56.0	14
Day 35	0.78	0.09	58.3	15

[1]Day refers to the time point at which Glutathione Peroxidase was detected: Day 7, Day 21 or Day 35 of feed trial.[2]Fold change refers to the log_2 fold change in protein abundance in response to PT treatment. [3]Coverage (%) refers to the % of protein sequence represented by identified peptides.

4.6 Discussion

The supplementation of broiler diets with AP, NS and PT was seen to significantly alter the abundance of many proteins with various functions in the health and immune status of the broiler chicken. Changes in abundance were seen in proteins involved in the innate immune system, nutrient transport, oxidative stress and selenium status - all of which contribute to the overall health of the animal.

4.6.1 Proteomic Results Overview

Supplementation of broiler diet with YCW products had a relatively small but notable effect on the serum proteome with averages of 6.31% - 7.78% of proteins significantly changed in abundance ($p < 0.05$) or unique/absent in the serum of AP-, NS- and PT-treated birds. NS samples showed the largest number of protein changes with 85 proteins significantly increased in abundance ($p < 0.05$) or uniquely present in comparison to control, and 63 proteins significantly decreased in abundance ($p < 0.05$) or absent in comparison to control. PT samples showed the lowest total number of proteins changed with 65 proteins significantly increased in abundance ($p < 0.05$) or uniquely present in comparison to control, and 55 proteins significantly decreased in abundance ($p < 0.05$) or absent in comparison to control. This could suggest that of the three feed diets, NS supplementation has the greatest effect on broilers. However samples from PT supplemented birds showed the greatest number of proteins that were unique to a treatment in inter-treatment analysis and, as will be discussed later in this section, PT showed greater complement activation as well as alterations to the selenium status of the animal which may indicate a larger effect.

There is a small disparity in the protein number between the inter-day and inter-treatment comparators. The total number of proteins identified in inter-treatment analysis is slightly lower ($n = 986$) than the total identified in inter-day analysis ($n =$

1007). This is due to the action of MQ data analysis software, such that there were slight differences in protein annotation when samples were grouped by day and grouped by treatment. This disparity, however, would not affect quantitative results as all samples from the same time point were grouped together in one analytical run in MQ, thereby keeping annotations constant between all feed groups.

PCA was conducted on each comparator group within each treatment group to ensure samples were grouped together and so could be comparable through the LC-MS/MS data obtained. In all PCA plots, treatment and control samples grouped separately. This result was not matched exactly in every comparator group during hierarchical clustering. The cross-over seen in hierarchical clustering is likely a function of the variability of biological replicates combined with the relatively small effects of these feed supplements on the serum proteome.

4.6.2 Immunological Effects

As previously stated, YCW products can have numerous immunological effects on animals (Gao *et al.*, 2003; Chae *et al.*, 2006; Goodridge *et al.*, 2009; Dalonso *et al.*, 2015; Song *et al.*, 2014; Hoving *et al.*, 2018). The increase in abundance of a number of immunological proteins suggests immunostimulatory/immunomodulatory effects by these YCW products.

One likely mediator of this immunostimulation is Surfactant Protein A (Q90XB2). This protein is uniquely present in AP samples on Day 7. This protein is a C-type lectin receptor which is present in mucosal tissues and binds glycan ligands which can result in downstream immunological effects (Haagsman *et al.*, 2008). Carbohydrate recognition domains on this protein bind glycan residues resulting in innate immune stimulation (Turner, 2003) which could explain the increase in abundance of immune-related proteins observed.

Across all treatment groups, there were significant ($p < 0.05$) increases in the level of a number of complement components in the serum of YCW-supplemented broilers harvested on Day 35, which would indicate stimulation of the complement cascade by the YCW feed products. Complement C4 precursor (A0A1D5P4P1), Complement C6 (B8ZX71) and Complement C7 (E1C6U2) were significantly increased ($p < 0.05$) in all treatments samples on Day 35. Complement C5 (E1BRS7) was increased in abundance and approaching significance in AP samples (Table 7.1) and was significantly increased ($p < 0.05$) in abundance in NS and PT samples. Complement C2 was significantly increased ($p < 0.05$) in abundance in PT samples only. PT samples showed the greatest level of complement stimulation. Increases in serum levels of complement components indicate a stimulation of the complement cascade by these YCW feed supplements.

A possible source of this complement stimulation by YCW products is through activation of the complement cascade by mannose binding protein (Q98TA4). This protein was significantly increased ($p < 0.05$) in abundance in PT samples and increased and approaching significance in AP ($p = 0.2$) and NS ($p = 0.162$) samples on Day 35. Mannose Binding protein, also called Mannose Binding Lectin (MBL) (Fraser *et al.*, 1998; Worthley *et al.*, 2005), is a C-type serum lectin (Ulrich-Lynge *et al.*, 2015) that binds mannose residues which results in C1-independent complement activation and binding leads to cleavage of C2 and C4 from their precursors by associated serine proteases (Davis *et al.*, 2004). Both C2 and C4 precursor were significantly increased in abundance ($p < 0.05$) in PT samples. MBL may also interact directly with cell surface receptors which can initiate opsonophagocytosis (Turner, 2003) causing immunostimulation which could also explain the abundance rise in proteins which have been linked with the innate immune system. Interestingly, low serum concentrations of

MBL has been linked to a greater susceptibility to infection (Ulrich-Lynge *et al.*, 2015). There was little data available in the literature regarding stimulation of the chicken complement cascade by YCW feed supplementation. One study by Slawinska *et al.* (2016) reported a downregulation of two genes influencing the complement system after *in ovo* administration of yeast-based prebiotics to broiler chickens followed by transcriptomic analysis. Activation of the complement system by carbohydrate-based feed has been previously seen in aquaculture. Gilthead seabream were fed a diet supplemented with inulin, a branched carbohydrate comparable to β-glucan or MOS (Mensink *et al.*, 2015). Supplementation resulted in significant increases ($p < 0.05$) in serum complement activity (Cerezuela *et al.*, 2012) which is in accordance with results found in the present study.

MOS-based products have been shown to elicit immunological responses without causing acute phase (fever) response (Yang *et al.*, 2009). Yet, the largest fold change was seen (Log_2 Fold change = 2.12) for alpha-1-acid glycoprotein (A1AGP) in LC-MS/MS analyses. This protein is in high abundance in serum and has been previously characterised as an acute phase protein (Horvatić *et al.*, 2018). Large fold changes in A1AGP were also seen between samples from AP and PT supplemented birds and control samples (Log_2 fold change = 3.69 and 1.86, respectively) although the *p*-value for these changes was not significant ($p = 0.37$ and 0.36, respectively).

The high fold changes noted with this protein could suggest the stimulation of the acute phase response. However, non-significant *p*-values ($p <0.05$) seen in AP and PT as well as the lack of significant change ($p <0.05$) in any other acute phase proteins would suggest that increases in the abundance of this protein may not represent an acute phase response but rather the natural variability of the serum level of this protein.

In order to ascertain whether this result could be replicated using another form of analysis, an A1AGP assay was carried out using NS and control samples. Upon first analysis, a high CV% was seen, particularly in two samples (CV% > 30%). This was likely due to familiarisation with the assay. The second assay showed a low CV%, with NS showing slightly higher concentration of A1AGP than control. Though no significant change ($p < 0.05$) was detected using this alternative method for A1AGP quantification, the results did show an increase in mean A1AGP concentration which was in accordance with the results obtained from LC-MS/MS analysis.

4.6.3 Effects on Metabolism

With YCW supplementation, a number of proteins involved in carbohydrate and nucleotide metabolism were significantly altered ($p <0.05$) in abundance across all treatment groups. Glyceraldehyde-3-phosphate dehydrogenase (P00356) was significantly increased ($p < 0.05$) in all treatment groups on Day 7. This protein is involved in glycolysis (Rodacka, 2013) and its increase could indicate a greater level of glycolysis in birds supplemented with these YCW products. Beta-hexosaminidase (F1NTQ2) was significantly increased ($p < 0.05$) in abundance in PT samples and increased and approached significance ($p = 0.08$) (Table 7.5) in NS samples on Day 7. This protein is involved in the release of N-acetylglucosamine and N-acetylgalactosamine from glycoproteins, which are in high concentration in the YCW (Dasgupta, 2015) and its increase in abundance is likely a result of the increased glycoprotein substrate in the diet. Increase in the abundance of these proteins could indicate that the introduction of these feed supplements is stimulating carbohydrate metabolism in broiler chickens. However, individual products had varying effects on the levels of carbohydrate metabolism proteins. NS had a variable effect on carbohydrate metabolism. Proteins ($n = 4$), involved in carbohydrate metabolism were significantly

increased in abundance ($p < 0.05$) or uniquely present in NS samples. However, other proteins involved in carbohydrate metabolism showed a decrease in abundance such as α-Enolase (A0A1D5PSH6) and β-Enolase (P07322). PT also had an inconsistent effect on the abundance of proteins involved in carbohydrate metabolism, with proteins both significantly increased ($p <0.05$) and significantly decreased ($p<0.05$) in abundance. Overall the levels of proteins involved in carbohydrate metabolism were variable and, though significant changes ($p < 0.05$) were seen, no definitive effect on carbohydrate metabolism could be deduced.

Differential effects were also seen on proteins involved in nucleotide metabolism throughout all three products. Significant changes ($p < 0.05$) in the abundance of multiple proteins involved in this process would suggest an effect of YCW supplementation, but with proteins both increased and decreased in abundance, no common trend could be elucidated.

4.6.4 Individual Proteins of Note

A number of individual proteins of note were seen to be significantly altered in abundance ($p < 0.05$) or present/absent in treatment groups with interesting links to a number of aspects in animal health. Gastrokine-2 is secreted by gastric mucosal cells. This protein binds Gastrokine-1 and is involved in regulating homeostasis of gastric mucosa (Menheniott *et al.,* 2016). It has been documented that YCW feed supplementation can have effects on gastric mucosa (Brümmer *et al.,* 2010). Gastokine-2 was seen to be uniquely present in the serum of AP and NS samples on Day 35. One other protein involved in the regulation of gastric mucosa is Gastrin releasing peptide (A0A1D5PXC4). This protein was significantly reduced ($p <0.05$) in PT samples on Day 35. Changes in the abundance of these proteins would indicate that these YCW products have an effect on the regulation of the gut mucosa.

Another protein of interest which was uniquely present in AP and NS samples on Day 21 was osteocalcin (P02822). This protein is a marker for bone turnover and its presence in serum is an indicator of bone resorption (Cepelak & Cvoriscec, 2009). The presence of this protein, only in the serum of birds supplemented with AP and NS, could indicate that these products have an effect on bone formation in the early stages of life. Though no literature could be found linking YCW feeds to bone formation, the formation of bone, particularly in the early stages of life, is of critical importance in chicken husbandry. Leg problems are prevalent in broilers (Sanotra *et al.,* 2001) and can lead to higher culling rates in commercial production systems which have real monetary effects on the producer (Cook, 2000). If these products are benefiting the formation of bone, it could lead to greater efficiencies in flock yield due to lower culling rates.

Iron depletion can be caused by dietary deprivation or malabsorption of iron and can lead to anemia (Huebers *et al.*, 1990; Fernández-Bañares *et al.,* 2009). The receptor responsible for binding and transport of iron is Transferrin receptor protein (TRP) (F1NTM6). TRP found in serum is the truncated form of TRP transmembrane protein that is found on the surface of virtually every cell (Huebers *et al.*, 1990). Iron delivery to cells is mediated by the binding of cell surface TRP by serum TRP (Cook *et al.,* 1993). The level of serum TRP is directly related to the level of TRP on the surface of cells and the level of cell surface TRP has been previously seen to reflect iron requirement (Rao *et al.,* 1985). The levels of serum TRP are comparable to the levels of available iron and increases in serum TRP have been linked with iron deficiency (Huebers *et al.,* 1990). TRP abundance was significantly increased (p <0.05) in NS and PT samples on Day 35 as well as increased and approaching significance (p = 0.054) in AP on Day 7. This increase in serum TRP could be an indicator of reduced iron levels in broiler chickens fed a diet supplemented with AP, NS and PT. Supplementation with

these YCW products may need to be coupled with an additional iron supplement in order to avoid this apparent reduction in iron levels.

4.6.5 Vitamin A transport

Vitamin A is of vital importance in the diet of the broiler chicken as it is not produced naturally by the animals and so must be obtained through the diet (Johnson & Schroeder, 1996). Deficiencies in broiler diets can lead to deterioration of reproductive (Clagett-Dame & DeLuca, 2002) and immunological health (Davis & Sell, 1983; Sklan *et al.*, 1994). Vitamin A is bound by Retinol binding protein 4 (RBP4) (P41263) which is in turn bound to Transthyretin (P27731) (Zabetian-Targhi *et al.*, 2015). These molecules then transport Vitamin A throughout the animal. Retinol binding protein 7 (RBP7) (E1C0M1) is involved in the intracellular binding and transport of Vitamin A in cells (Hu *et al.*, 2017). RBP4 was significantly increased ($p < 0.05$) and RBP7 was uniquely identified on in NS samples on Day 7. Transthyretin was significantly increased on Day 35 in AP samples and RBP7 was uniquely identified on Day 7, RBP4 was also increased in abundance and approached significance ($p = 0.08$) on Day 21 (Appendix 1-Table 7.1). Transthyretin was significantly increased on Day 35 in PT samples. Increases in the abundance of these proteins would indicate a greater abundance or availability of Vitamin A with the supplementation of these YCW products. Vitamin A was part of the basal diet at 1.3g/kg, 1.1g/kg and 1g/kg (starter, grower and finisher basal diet) (Table 2.1) in all feeds, so a greater abundance of Vitamin A would have to be obtained from the YCW products. One common source of Vitamin A is β-carotene which is a precursor to Vitamin A (Li *et al.*, 2017). This molecule is naturally obtained through the consumption of carotenoid-producing organisms such as higher plants or photosynthetic microorganisms (Yamano *et al.*, 1994). A number of species of higher fungi produce carotenoids (Johnson & Schroeder,

1996), which would account for an increase in the abundance of Vitamin A with YCW supplementation. Higher levels of Vitamin A are beneficial as it could reduce the need for inorganic supplementation in the diet.

4.6.6 Effect of Organic Selenium Supplementation

Selenium is an essential element in the diet of broiler chickens and its supplementation has been seen to have beneficial effects on broiler health status and meat quality (Surai, 2002; Choct *et al.,* 2004; Surai & Fisinin, 2014). The dietary source of selenium can have an impact, as inorganic selenium is considered a pro-oxidant and organic selenium has been seen to be have greater bioavailability (Rayman, 2004; Peric *et al.,* 2009; Lönnerdal *et al.,* 2017).

Drip-loss has been linked to GSH-Px level (Choct *et al.,* 2004). GSH-Px was increased in abundance in all treatment groups with the largest fold change noted in PT samples where it was increased and approaching significance on Day 21(Appendix 1 - Table 7.5) and Day 35 (Appendix 1-Table 7.5b). GSH-Px level has been previously shown to be influenced by selenium supplementation and a greater bioavailability of organic selenium from PT would explain this increased GSH-Px level. Additonally, with this increased GSH-Px level, there may be a reduction in drip-loss from the meat obtained from PT supplemented broilers. A reduction in drip-loss and increase in serum GSH-Px has been reported previously in birds supplemented with organic selenium rather than its inorganic form (Wang & Xu, 2008; Wang *et al.,* 2011). This result was also matched in pigs with significantly higher ($p < 0.01$) levels of serum GSH-Px in organic selenium supplemented pigs at 0.2mg/kg and 0.3 mg/kg supplementation (Mahan *et al.,* 1999). In this work, selenium was also supplemented to broiler diets at 0.3 mg/kg.

The effect of the absence of the potentially pro-oxidative inorganic selenium in

the diet of PT supplemented birds can also be seen in the significant reduction or absence of proteins involved in the detoxification of ROS: Catalase (*n* =2), A0A1D5PPU9 and Q5ZL24, which were also absent from PT samples on Day 7 and Day 21, respectively.

The protective effects of selenium have been seen in organs such as the liver and organic forms of selenium have been liked to reduced liver damage (Peric *et al.,* 2009). Glutathione-S-Transferase α (αGST) (Q08392) is an enzyme involved in the detoxification of ROS and its alpha form is present in high concentrations in the cytosol of hepatic cells (Beckett & Hayes, 1993). This enzyme conjugates glutathione to ROS (Federico *et al.,* 1999) and its presence in serum has previously been characterised as a biomarker for acute hepatitis or liver damage (Yukihiko *et al.,* 1980). αGST is absent from the PT samples on Day 7, Day 21 and Day 35 which would indicate that this product is having some hepatoprotective effect on the broiler chicken. This reduction in liver damage could be due to the reduced level of oxidative damage with an organic selenium source, the greater bioavailability of organic selenium leading to increased levels of GSH-Px which is reducing oxidative damage or a dual effect of these two factors.

Evidence suggests that organic selenium has a greater bioavailability than its inorganic forms (Lönnerdal *et al.,* 2017) and this hepatoprotective effect could also be a result of the greater bioavailability of organic selenium allowing greater protective potential of enzymes that make use of the element.

A greater bioavailability of organic selenium from PT is not only supported by the increased levels of GSH-Px on all days, but also by the higher number of proteins detected with SeMet/SeCys substitutions and the unique presence of Selenoprotein F

precursor in the serum of PT treated birds. An increased bioavailability could not only lead to increased benefits to the broiler chicken, but also to the producer and consumer. Increased bioavailability could reduce supplementation cost to the producer and increase levels of selenium produced meat and lead to increased selenium intake in the diet of the consumer.

In NS samples, GSH-Px was also seen to be increased in abundance on Day 7, which was also accompanied by a significant increase ($p < 0.05$) in two other enzymes involved in the detoxification of ROS: Glutaredoxin 3 (A0A1D5NW30) and Peroxiredoxin 6 (F1NBV0). EO, a component of the NS supplement, have been seen to mediate oxidative stress in animals (Zeng *et al.*, 2015). Antioxidative effects of these EO have been achieved by stimulating the expression of a number of proteins which are involved in antioxidant activity (Kang *et al.*, 2015; Zou, et al., 2016). EO have been previously seen to increase the serum concentration of GSH-Px as well as improve the total antioxidant capacity of weaned pigs (Zeng *et al.,* 2015). Therefore, this increase in the abundance of proteins involved in the detoxification of ROS may not be due to an increased oxidative stress, but rather the stimulation of the production of these proteins by EO present in this feed supplement.

4.6.7 Inter-Feed Comparison

The significant alterations in proteins involved in a number of key processes would suggest that these products are having interesting effects on the health status of the broiler chicken that are measurable through proteomic analysis of serum. Overall effects of YCW supplementation include stimulation of the innate immune system, particularly the complement system, alterations in the levels of a number of proteins involved in the detoxification of a number of ROS, stimulation of metabolic processes, mucosal development and transport of iron and Vitamin A.

The three YCW-based products showed some level of similarity regarding their effects, which would be expected from three related products. Individually, results did show that these three products did have product specific effects.

AP supplementation was seen to stimulate the immune system, possibly through the binding of the C-type lectin receptor, Surfactant Protein A, which was significantly increased in abundance on Day 7. This stimulation led to the increase in abundance of proteins involved in a number of processes in the innate immune system. AP showed the lowest level of complement stimulation in comparison to the other products with three complement components significantly increased ($p < 0.05$) on Day 35. Gastrokine-2 and osteocalcin were seen to be uniquely present in AP samples when compared to control which could indicate that this molecule may be playing some role in bone development and gastric mucosal homeostasis. AP supplementation also seemed to play a role in Vitamin A and iron levels of broilers. An increase in the abundance of TRP may indicate a depletion in iron levels with AP supplementation which may indicate a need for iron supplementation.

NS supplementation was also seen to have immunostimulatory effects with a number of proteins involved in innate immune function significantly increased ($p < 0.05$) in abundance throughout the three time points. Complement stimulation was again seen on Day 35 with four complement components significantly increased ($p < 0.05$) in abundance. MBL was increased in abundance and approached significance which would explain the complement stimulation noted. A number of proteins involved in the detoxification of ROS were significantly increased in abundance with NS supplementation, which may indicate impact on the oxidative status of the animal. RBP4 was significantly increased ($p < 0.05$) and RBP7 was uniquely identified in NS samples which may indicate a greater abundance or availability of Vitamin A in birds

supplemented with NS. A significant increase ($p < 0.05$) in the abundance of TRP may indicate a depletion in iron levels with NS supplementation, again iron supplementation may be needed with this product.

PT supplementation seemed to have the greatest effect on the immune status of the animal with a significant increase in abundance of many proteins involved in the innate immune system and five out of six detected complement components significantly increased ($p < 0.05$) on Day 35, Complement C3 precursor (A0A1D5P9F9) exhibited no significant change in any feed treatment. Complement stimulation is likely through the binding of MBL by a mannose residue which resulted in a significant increase ($p < 0.05$) in the abundance of this protein on Day 35. The absence of αGST, a serum biomarker for liver damage, may indicate some hepatoprotective effects of this product which could be explained by the absence of potentially pro-oxidative, inorganic selenium supplementation. This is supported by a concomitant significant reduction ($p <0.05$) in the abundance of two proteins involved in the detoxification of ROS which would again indicate a lower level of oxidative stress. Another explanation for this protective effect is the greater bioavailability of organic selenium which led to an increased ability to protect against ROS, resulting in reduced liver damage. Organic selenium did seem to have an effect on selenium status of PT supplemented broilers with increases in the number of proteins detected with SelMet/SelCys residues, increases in the level of the selenoprotein GSH-Px and the unique presence of selenoprotein F precursor in PT samples with comparison to control. Serum TRP was significantly increased ($p < 0.05$) in PT samples on Day 35 which may indicate greater iron supplementation is required with this product.

Chapter 5

Discussion

5.1 Discussion

The modification applied to the Proteominer protocol facilitated an in-depth serum proteomic investigation into the effects of YCW supplementation on broiler chickens. This analysis revealed a number of interesting effects including (i) stimulation of the innate immune system, particularly the complement cascade, (ii) alterations to the oxidative status of the animal, (iii) stimulation of metabolic processes and (iv) possible alterations in nutrient transport. Similarities amongst the effects of the YCW products were seen, which is to be expected from three YCW-based feed supplements.

The area of animal proteomics is growing, though it still represents a relatively small part of proteomics as a whole (Bili *et al.,* 2018). There is, therefore, no standardised protocol for the preparation of serum samples, from most animal species, for LC-MS/MS analysis. Proteominer™ Technology offers species-independent low abundant protein enrichment, which is tremendously useful in quantitative proteomics. However, proteomic samples enriched using the commercial elution buffer are incompatible with optimum LC-MS/MS analysis. This issue was resolved with this modification applied to the protocol. This modification facilitated a greater depth of proteomic analysis of the broiler serum proteome, in comparison to that achieved using a commercial elution buffer. With the introduction of this reagent, Proteominer™ has the potential to become a useful tool in species-independent protein depletion of proteomic samples with high dynamic range for proteomic analysis using MS technologies. This high dynamic range problem is not specific to serum. Meat proteomics (Bendixen *et al.,* 2011), plant proteomics (Fröhlich *et al.,* 2012) and bacterial proteomics (Ben Mlouka *et al.,* 2015) can all suffer from similar difficulties in the preparation of proteomic samples for LC-MS/MS analysis. For this reason, the implementation of LCR may not only be of use with serum proteomics but could serve as a valuable alternative in the proteomic

investigation of other sample types, particularly when investigating proteins of lower abundance.

Antibiotics, when used sub-therapeutically, have been seen to improve feed efficiencies, reducing cost for both consumer and producer (National Research Council, 1999). The rise in awareness of antibiotic resistance, however, has led to the implementation of a number of bans on their use as growth promoters in animal husbandry (European Commission, 2003; Food and Drug Administration, 2013). These bans, combined with the ever-growing demand for poultry products (Alexandratos & Bruinsma, 2012), has meant that the poultry industry has come under pressure to find viable alternatives to antibiotics. Yeast and its derivatives have emerged as an alternative that possess great potential in both reducing pathogenic invasion (Yang *et al.,* 2009) and beneficially stimulating the host immune response (Gao *et al.,* 2003; Chae *et al.,* 2006; Goodridge *et al.,* 2009; Ganner & Schatzmayr, 2012; Dalonso *et al.,* 2015; Song *et al.,* 2014; Hoving *et al.,* 2018). The stimulation of the complement cascade herein seen in each of the YCW-supplemented avian groups, indicates an immunostimulatory effect which could provide some resistance against pathogenic infection (Samuelsen *et al.,* 2015). This stimulatory effect could be further studied by the examination of the epithelial cells of the intestinal tract for signs of immunostimulation, possibly through qRT-PCR identification of cognate gene expression associated with immunostimulation. Further investigation of markers associated with immunostimulation could also be conducted using ELISA.

This immunostimulation may also be of benefit in the reduction of pathogenic infection. Narrow profit margins in livestock production often mean that preventative, rather than therapeutic measures, are more cost effective and feasible (Layton *et al.,* 2017). Preventative biosecurity measures such as decontamination, culling and

vaccination are all common practice in animal husbandry (Conan *et al.,* 2012). For livestock, vaccination remains one of the most important tools in the prevention of infectious disease (Plotkin, 2005; Stokka & Goldsmith, 2015). Vaccination prevents the spread of infectious disease by generating a sufficient protective immune response to pathogenic microbes and can be in the form of attenuated pathogens, pathogen surface molecules or recombinant pathogen antigens (Li & Wang, 2015). One example of this process in broiler husbandry is Avian Influenza virus (AIV) (Peyr *et al.,* 2009; Spackman & Pantin-Jackwood, 2014), which is one of the most economically important diseases affecting livestock (Layton *et al.,* 2017). Many approaches can be used for combating AIV, however, vaccination is the only sustainable approach (Domenech *et al.,* 2009).

In some cases, vaccines alone do not provide a sufficient immune response to provide protection (Sun *et al.,* 2018). Adjuvants are substances co-administered with a vaccine which strengthen the host immune response to antigens leading to reduced dosage and production cost (Sun *et al.,* 2018), and can lead to improved response magnitude and longevity of the vaccine-mediated protection (Reed *et al.,* 2009). Examples of commonly used adjuvants are aluminium salts, oil emulsions, microparticles and polysaccharides (Sun *et al.,* 2018).

Polysaccharides have previously been defined as excellent vaccine adjuvant candidates as they have low toxicity, are capable of stimulating the immune response and have high biocompatibility (Petrovsky & Cooper, 2011). These molecules are capable of producing immune responses such as macrophage and monocyte stimulation and induce secretion of immune-related proteins such as cytokines and complement molecules (Young *et al.,* 2001; Gantner *et al.,* 2003; Brown, 2006; Levitz, 2010; Song *et al.,* 2014). β-glucans have adjuvant activity and are potent activators of the innate immune

system in both immunocompetent and immunocompromised individuals (Franzusoff *et al.,* 2005; Munson *et al.,* 2007). Yeast-derived β-glucan has been previously used to enhance IgG response to ovalbumin in both intradermal and oral administration (De Smet *et al.,* 2014). Complement C5, which was seen to be significantly increased ($p < 0.05$) in the serum of NS and PT supplemented birds, is the most potent anaphylatoxin generated through complement stimulation and has been seen to have adjuvant effects in teleost fish with higher IgM antibody level produced in response to a soluble antigen (Wu *et al.,* 2014). Mannose Binding Lectin, which was seen to be increased in abundance in serum samples from YCW supplemented broilers, has been shown to participate in the protection of host against viral infection and plays a role in the vaccination of chickens against infectious bronchitis (Kjærup *et al.,* 2014).
With significant increases ($p < 0.05$) in the abundance of a number of proteins involved in innate immune function, proteomic results would suggest that these products could augment the host response to vaccination, reducing the need for intradermal administration of adjuvants such as oil emulsion, which can cause issues at the site of infection (Li & Wang, 2015).

With the greater bioavailability of organic selenium (Rayman, 2004; Lönnerdal *et al.,* 2017) and the potentially pro-oxidative effects of inorganic selenium (Peric *et al.,* 2009), there are a number of advantages to using selenium in its organic form for diet supplementation. Serum from PT supplemented broilers exhibited some effects that were not seen in other samples, such as a possible protective effect on the liver of the animals and alterations in the selenium status of the animal. These effects are possibly due to the organic selenium source available from PT which replaced the inorganic selenium supplement of the basal feed.

Drip-loss can reduce the weight of meat products, affecting its monetary value (Fischer, 2007), and has become synonymous with low meat quality in the last number of decades (Northcutt *et al.,* 1994). Dietary selenium supplementation has been linked to increased levels of glutathione peroxidase in the muscle (Pappas *et al.,* 2005; Wang *et al.,* 2011; Zhou & Wang, 2011; Cai *et al.,* 2012) of chickens. This enzyme has been linked to reduced drip-loss and improved meat quality (Choct *et al.,* 2004). With apparent increases in the levels of selenium and glutathione peroxidase in broiler chickens supplemented with PT, improved meat quality is a possibility.

Several tests for meat quality exist such as water carrying capacity test (Sun & Luo, 1993), filter paper test for drip-loss (Kauffman *et al.,* 1986) and breast hardness test (Sun & Luo, 1993). One possible avenue of future study is to examine the meat quality of broilers supplemented with PT versus a control basal feed, using one or all of these meat quality tests. Improved meat quality which would likely improve the monetary value of meat produced which is of benefit to the producer.

Increases in the selenium levels are not just of benefit to the producer. Selenium is an essential element in the human diet and deficiencies have been found in some socioeconomic groups (Thiry *et al.,* 2013; Hargreaves *et al.,* 2014). Selenium-enriched foods are a helpful supplement in maintaining healthy levels (Thiry *et al.,* 2013) and broilers with increased levels of selenium in the muscle tissue could help to mitigate selenium deficiencies in these economic groups. Testing broiler muscle tissue for selenium content using techniques such as inductively coupled plasma mass spectrometry (ICP-MS) (Bou *et al.,* 2004; Gerber *et al.,* 2009) could provide more information on the selenium levels available to consumers.

Quantitative proteomic results from sera of broilers supplemented with PT were consistent with a hepatoprotective effect on the broiler chicken given the specific

undetectability of αGST, a biomarker for liver damage (Yukihiko *et al.*, 1980; Rees *et al.*, 1995) in treated animals. Interestingly, this is in accordance with observations of Peric *et al.* (2009) which showed that dietary organic selenium led to significant reductions in the levels of both alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are biomarkers of heptotoxicity (e.g., due to oxidative damage), when compared to inorganic supplementation. More investigation is needed here but could reveal an added benefit to supplementation with this product. A αGST Enzyme-Linked Immunosorbent Assay (ELISA) analysis (Rees et al., 1995) could be conducted on serum from PT supplemented birds in order to further evaluate and explore our obervations relating to dietary PT supplementation.

A number of uncharacterised proteins have been identified as significantly changed in abundance (p <0.05) in this study. It is conceivable that some of these may have prognostic or predictive value with respect to feed efficacy. Consequently, cloning and recombinant expression of selected proteins, followed by antibody generation could facilitate immunoblot and/or ELISA development to readily detect alterations in abundance of these antigens in avian serum in response to feed products. It may also aid immunolocalisation studies to provide insight into *in vivo* localisation of proteomic changes. Such work is strongly warranted should the sponsor endeavour to extract all available value from the completed tasks.

To conclude: This thesis has, using a novel sample preparation techonlogy, demonstrated a number of biological effects of three YCW products on the broiler chicken using serum LC-MS/MS analysis. Serum samples were prepared for LC-MS/MS using Proteominer™ technology with the implementation of an alternative buffer, LCR. Quantitative proteomic analysis highlighted a number of effects, namely immunostimulation, alterations to the oxidative status of the animals, stimulation of

metabolic processes and possible hepatoprotection. These results represent areas of possible future investigation which could lead to advancements in the field of animal feed supplementation, further supporting their incorporation in animal diets.

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Chapter 7 Appendix

7.1 Enzyme Linked Immunosorbent Assay to examine Alpha-1-Acid Glycoprotein abundance change in NS samples.

In order to confirm the high fold change (Log_2 Fold Change = 2.12) detected in Alpha 1 Acid Glycoprotein (A1AGP) of NS samples on Day 35, a chicken A1AGP enzyme-linked immunosorbent assay (ELISA) (ab157690) (abcam®) was conducted on pooled Control and NS samples from Day 35. Three pooled serum samples from Day 35 of NS and Control were tested for A1AGP concentration. As high variability was seen in the first assay (Figure 7.1) the assay was repeated (Figure 7.2). High variability was seen in assay results. Results showed a trend that indicated an increase in A1AGP levels though the high fold change detected through LC-MS/MS analysis could not be validated.

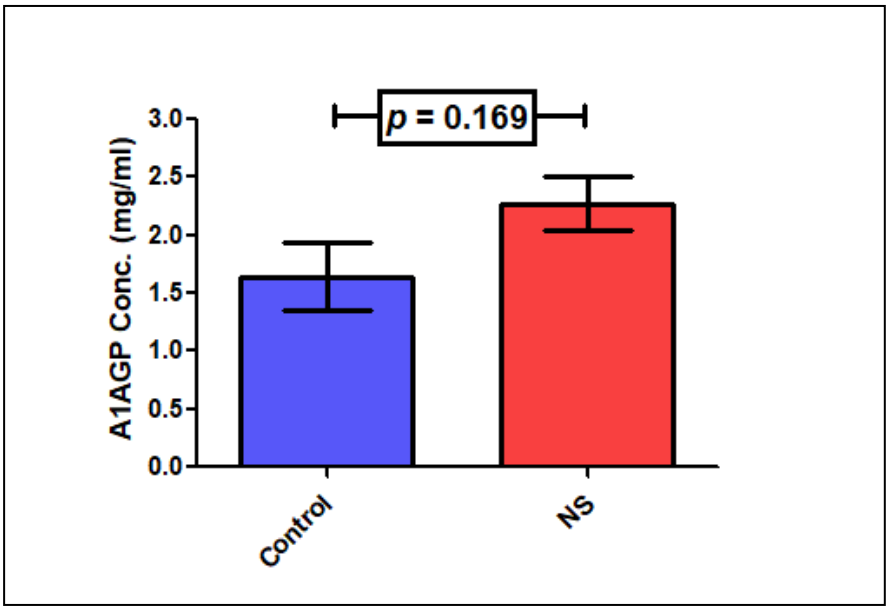

Figure 7.1: Bar chart representing the Alpha-1-Acid Glycoprotein concentration in pooled serum samples from Control and NS fed birds following the first Enzyme Linked Immunosorbent Assay (ELISA). Error bars denote standard error is shown.

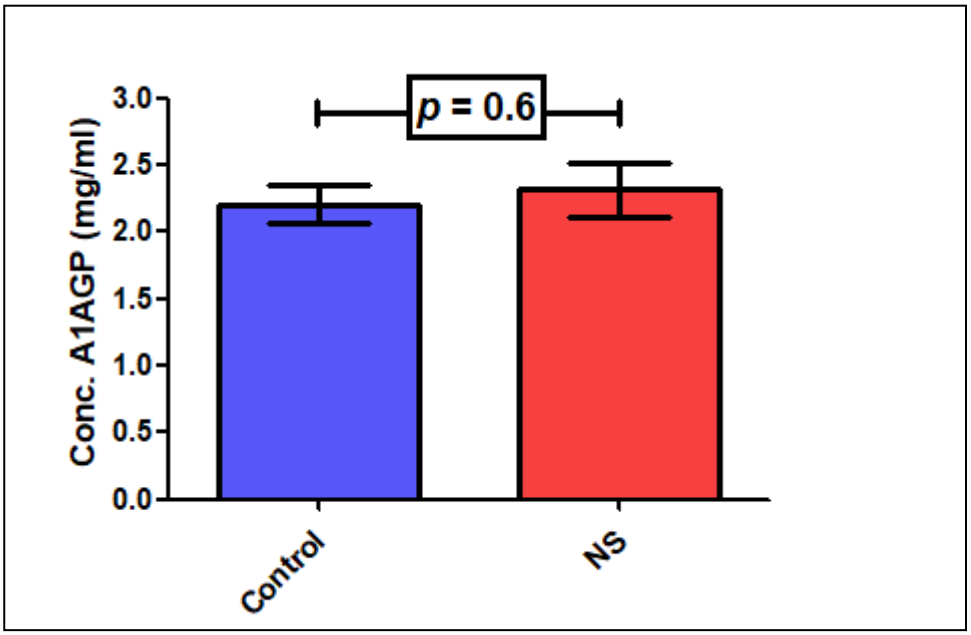

Figure 7.2: Bar chart representing the Alpha-1-Acid Glycoprotein concentration in pooled serum samples from Control and NS fed birds following the second ELISA. Error bars denote standard error is shown.

7.2 Proteins approaching significance and proteins with large fold changes.

Each treatment group was searched for proteins of note with a *p*-value approaching significance ($p < 0.08$). AP had 36 proteins approaching significance, these are listed in Table 7.1. NS had 46 proteins approaching significance, these are listed in Table 7.2. PT had 48 proteins of note approaching significance, these are listed in Table 7.3.

Each treatment group was also searched for proteins of note with high fold changes (Fold Change > 1.8). AP had 10 proteins of note with high fold changes, these are listed in Table 7.4. NS had 2 proteins with high fold changes, these are listed in Table 7.5. PT had 3 proteins of note with high fold changes, these are listed in Table 7.6.

Table 7.1. Proteins approaching significance ($p < 0.08$) in sera from Actigen®-Pak supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Transferrin receptor protein 1	2.01	10	18.3	Day 35	0.06	F1NTM6
Uncharacterized protein	1.36	6	45.3	Day 21	0.05	A0A1D5PUI7
Uncharacterized protein	0.86	6	16.8	Day 35	0.08	E1C206
Uncharacterized protein	0.86	82	63.0	Day 35	0.06	E1BRS7
Eukaryotic translation initiation factor 1	0.72	5	79.6	Day 21	0.06	R4GLE6
Eukaryotic initiation factor 4A-II	0.72	6	24.0	Day 7	0.06	R9PXN1
Alkaline phosphatase	0.71	16	42.8	Day 21	0.07	Q92058
Retinol-binding protein 4	0.66	14	70.9	Day 21	0.08	P41263
Uncharacterized protein	0.64	16	49.4	Day 21	0.06	E1BS56
T-complex protein 1 subunit zeta	0.64	12	36.2	Day 35	0.07	Q5ZJ54
Uncharacterized protein	0.54	2	14.8	Day 7	0.06	F1N9T1
Uncharacterized protein	0.53	32	70.9	Day 21	0.07	F1NVF3
Uncharacterized protein	0.40	8	45.5	Day 7	0.07	A0A1D5PAH2
Glutathione peroxidase	0.38	11	48.2	Day 7	0.06	F1NPJ8
Plasminogen	0.37	50	61.2	Day 21	0.08	F1NWX6
Collagen type XI alpha 1 chain	0.29	7	18.6	Day 21	0.06	A0A1D5PVT6
Uncharacterized protein	0.29	3	30.2	Day 7	0.08	F1N9A3
Uncharacterized protein	0.28	16	31.7	Day 21	0.07	F1P4N9
Apolipoprotein A-I	0.19	55	92.8	Day 21	0.07	P08250
G protein subunit beta 1	-0.19	4	18.2	Day 7	0.07	F1NLV4
Uncharacterized protein	-0.25	30	31.2	Day 7	0.07	A0A1D5PEF7
Fibulin-1	-0.26	18	38.5	Day 21	0.05	A0A1L1RU28
Uncharacterized protein	-0.38	4	52.9	Day 21	0.06	A0A1L1RML6
Uncharacterized protein	-0.39	6	35.7	Day 7	0.08	R4GFI8
MHC class II beta chain 2	-0.46	3	18.6	Day 21	0.06	A5HUL4
Cathepsin B	-0.48	17	60.9	Day 35	0.07	A0A1L1RS19
F-actin-capping protein subunit alpha-1	-0.50	6	33.9	Day 35	0.05	P13127
Chemokine	-0.64	4	48.9	Day 35	0.07	E1C733
Uncharacterized protein	-0.65	2	38.2	Day 7	0.07	F1NSD3
Elastin	-0.65	3	6.1	Day 35	0.06	P07916
Uncharacterized protein	-0.66	27	52.1	Day 35	0.07	F1NAB7
Histone H2A.J	-0.66	4	32.6	Day 7	0.05	P70082
Tubulin beta-7 chain	-0.69	12	46.4	Day 21	0.07	P09244
Myosin light polypeptide 6	-0.74	4	27.2	Day 35	0.06	P02607
Hemoglobin subunit beta	-0.79	9	76.2	Day 7	0.05	P02112
Lamin-A	-0.93	17	28.3	Day 7	0.08	P13648

Table 7.2. Proteins with high fold change (Fold Change >1.8) in sera from Actigen®-Pak supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Alpha-1-acid glycoprotein	3.69	7	36.0	Day 35	0.37	Q8JIG5
Uncharacterized protein	2.61	11	24.4	Day 35	0.31	E1C8N1
Neuronal-glial cell adhesion molecule	2.60	14	21.6	Day 35	0.18	Q03696
Transferrin receptor protein 1	2.01	10	18.3	Day 35	0.06	F1NTM6
Uncharacterized protein	1.98	25	63.0	Day 35	0.53	A0A1L1S0P1
Hemoglobin subunit beta	1.97	15	88.4	Day 35	0.19	P02112
Insulin like growth factor binding protein acid labile subunit	1.86	12	31.1	Day 35	0.17	F1NI07
Ovotransferrin	1.85	50	74.6	Day 35	0.32	A0A1D5P4L7
Hemopexin	1.82	23	81.7	Day 35	0.41	H9L385
Creatine kinase M-type	-1.81	19	51.4	Day 21	0.26	P00565

Table 7.3. Proteins approaching significance ($p < 0.08$) in sera from NS-supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Uncharacterized protein	1.27	10	48.7	Day 35	0.08	A0A1D5PW77
Neuronal-glial cell adhesion molecule	1.26	14	21.6	Day 35	0.05	Q03696
Lamin-A	1.12	21	34.4	Day 35	0.07	P13648
Uncharacterized protein	0.83	8	37.1	Day 35	0.07	F1NXB6
Transthyretin	0.74	9	72.7	Day 35	0.06	P27731
Uncharacterized protein	0.70	7	13.5	Day 35	0.06	E1BYS3
Dihydropyrimidinase-related protein 2	0.62	14	38.3	Day 7	0.07	Q90635
Uncharacterized protein	0.57	4	46.3	Day 7	0.08	A0A1D5P5P6
Uncharacterized protein	0.53	2	38.5	Day 7	0.05	F1NSC8
T-complex protein 1 subunit theta	0.53	8	21.0	Day 7	0.06	F1NEF2
Uncharacterized protein	0.50	11	34.5	Day 21	0.08	E1BS40
Uncharacterized protein	0.49	3	12.1	Day 7	0.07	F1NNV6
Prolyl 4-hydroxylase subunit alpha-2	0.49	6	20.4	Day 7	0.07	Q5ZLK5
Uncharacterized protein	0.49	4	8.3	Day 7	0.08	A0A1D5PQR2
Uncharacterized protein	0.48	13	27.8	Day 7	0.08	A0A1D5P2H3
Uncharacterized protein	0.45	5	52.9	Day 7	0.06	A0A1D5PH37
Uncharacterized protein	0.42	19	48.5	Day 7	0.06	E1BS40
Uncharacterized protein	0.41	26	41.1	Day 35	0.06	A0A1D5PBP6
Uncharacterized protein	0.41	18	29.1	Day 35	0.06	A0A1L1S0T3
Plasminogen	0.38	54	68.9	Day 35	0.06	R4GMH5
Uncharacterized protein	0.35	8	58.2	Day 35	0.06	E1BY93
Uncharacterized protein	0.30	30	70.2	Day 7	0.07	F1NVF3
Uncharacterized protein	0.22	16	30.6	Day 35	0.05	F1P4N9
Collagen alpha-1(X) chain	0.20	5	10.7	Day 35	0.07	P08125
Uncharacterized protein	-0.16	6	10.6	Day 21	0.06	F7B5K7
Uncharacterized protein	-0.22	30	31.2	Day 7	0.06	A0A1D5PEF7
Coatomer subunit epsilon	-0.29	3	18.8	Day 21	0.08	Q5ZIK9
Peroxiredoxin-1	-0.29	8	53.8	Day 35	0.07	P0CB50
Collagen alpha-2(I) chain	-0.30	11	12.7	Day 35	0.07	P02467
Uncharacterized protein	-0.36	18	9.7	Day 7	0.08	A0A1D5NXE0
Uncharacterized protein	-0.37	10	54.9	Day 35	0.05	A0A1D5NTE9
Secreted phosphoprotein 24	-0.40	8	51.0	Day 35	0.06	Q710A0
Collagen alpha-1(VI) chain	-0.44	12	19.0	Day 7	0.07	A0A1D5PWN6
Uncharacterized protein	-0.45	9	33.1	Day 21	0.06	E1C1G8
S-formylglutathione hydrolase	-0.47	6	45.0	Day 21	0.06	A0A1L1RWZ4
Uncharacterized protein	-0.49	3	5.0	Day 7	0.08	A0A1D5NV10
Creatine kinase B-type	-0.51	9	42.0	Day 35	0.07	A0A1L1RVT1
Uncharacterized protein	-0.56	2	15.1	Day 7	0.06	A0A1D5NVS3

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Low molecular weight phosphotyrosine protein phosphatase	-0.60	9	69.0	Day 21	0.05	Q5ZKG5
Uncharacterized protein	-0.65	8	26.2	Day 21	0.06	A0A1D5PAN0
Low molecular weight phosphotyrosine protein phosphatase	-0.65	10	70.3	Day 35	0.05	Q5ZKG5
Dihydropyrimidinase-related protein 2	-0.70	7	22.4	Day 21	0.07	Q90635
Glycerol-3-phosphate dehydrogenase	-0.72	12	51.6	Day 21	0.07	A0A1D5P1Y7
Peroxiredoxin-1	-0.80	6	44.7	Day 21	0.06	P0CB50
Adenylate kinase isoenzyme 1	-0.84	11	63.9	Day 21	0.05	P05081
Creatine kinase M-type	-1.15	17	60.1	Day 35	0.06	P00565

Table 7.3b. Proteins of note approaching significance ($0.16 > p > 0.08$) in sera from NS-supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Mannose-binding protein	0.414	8	32.7	Day 35	0.16	Q98TA4
Beta-hexosaminidase	0.27	20	54.1	Day 7	0.09	F1NTQ2
Beta-enolase	-1.2	12	41	Day 21	0.09	P07322
Alpha-amylase	0.8	19	61.7	Day 7	0.08	A0A1D5PUZ5

Table 7.4. Proteins with high fold change (Fold Change > 1.8) in sera from Natustat® supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
ATP synthase subunit beta	3.68	12	34.1	Day 35	0.43	Q5ZLC5
Uncharacterized protein	-2.75	4	52.9	Day 35	0.35	A0A1L1RML6

Table 7.5. Proteins of note approaching significance in sera from PT-supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Uncharacterized protein	1.34	10	48.7	Day 35	0.07	A0A1D5PW77
Uncharacterized protein	0.93	10	32.9	Day 7	0.05	A0A1D5PAN0
Proteasome subunit alpha type	0.89	6	13.1	Day 35	0.06	E1C7I7
Uncharacterized protein	0.89	6	34.2	Day 7	0.07	E1BQD1
Uncharacterized protein	0.85	8	37.1	Day 35	0.06	F1NXB6
14-3-3 protein zeta	0.83	5	28.1	Day 7	0.08	A0A1L1RRT9
Dihydropyrimidinase-related protein 2	0.76	14	38.3	Day 7	0.08	Q90635
Uncharacterized protein	0.60	24	62.7	Day 21	0.05	F1NMN2
Retinol-binding protein 4	0.51	14	70.9	Day 21	0.06	P41263
Uncharacterized protein	0.48	13	37.1	Day 21	0.08	A0A1D5PU00
Collagen type XI alpha 1 chain	0.46	8	18.7	Day 35	0.07	A0A1D5PVT6
Ribonuclease/angiogenin inhibitor 1	0.43	7	25.4	Day 7	0.07	Q5ZIY8
Uncharacterized protein	0.43	16	31.7	Day 21	0.07	F1P4N9
Uncharacterized protein	0.41	10	23.5	Day 7	0.06	A0A1L1S099
Uncharacterized protein	0.41	5	52.9	Day 7	0.07	A0A1D5PH37
Uncharacterized protein	0.40	4	8.3	Day 7	0.07	A0A1D5PQR2
Proteasome subunit alpha type	0.38	5	41.1	Day 21	0.07	F1NEQ6
Glutathione peroxidase	0.36	14	56.0	Day 21	0.08	F1NPJ8
Ribosomal protein S14	0.34	4	49.7	Day 7	0.08	Q5ZHW8
Tubulin alpha chain	0.34	75	60.2	Day 35	0.06	F1NK40
Collagen type XVIII alpha 1 chain	0.33	5	6.0	Day 21	0.05	A0A1D5P5M7
C-type lectin domain family 3 member B	0.28	11	67.7	Day 21	0.08	Q9DDD4
Peroxiredoxin-6	0.26	12	68.3	Day 7	0.07	F1NBV0
Prolyl 4-hydroxylase subunit alpha-2	0.26	6	20.4	Day 7	0.06	Q5ZLK5
Uncharacterized protein	0.24	15	65.2	Day 35	0.08	F1NHT5
F-actin-capping protein subunit beta isoforms 1 and 2	-0.21	11	57.0	Day 21	0.06	P14315
Coatomer subunit epsilon	-0.35	3	18.8	Day 21	0.06	Q5ZIK9
Coatomer subunit epsilon	-0.40	10	15.5	Day 35	0.06	A0A1D5PWN6
Retinol-binding protein 4	-0.42	19	61.6	Day 35	0.05	A0A1D5P380
Uncharacterized protein	-0.42	15	47.3	Day 35	0.07	A0A1L1RW44
Tubulin alpha chain	-0.43	11	44.6	Day 21	0.07	A0A1D5PC38
Elongation factor 1-alpha 1	-0.46	18	50.0	Day 35	0.07	Q90835
Uncharacterized protein	-0.48	10	9.4	Day 35	0.06	P02457
C-type lectin domain family 3 member B	-0.53	17	62.9	Day 35	0.06	F1NJD6

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Uncharacterized protein	-0.57	3	24.7	Day 35	0.05	A0A1L1RUZ7
Uncharacterized protein	-0.59	5	24.1	Day 21	0.06	A0A1D5PGB2
Ubiquitin-40S ribosomal protein S27a	-0.64	3	28.2	Day 7	0.07	P79781
Uncharacterized protein	-0.64	7	37.3	Day 21	0.06	A0A1D5PKX1
Uncharacterized protein	-0.65	8	42.7	Day 35	0.07	A0A1D5P4K6
Uncharacterized protein	-0.71	18	53.6	Day 35	0.06	P09207
Collagen type XVIII alpha 1 chain	-0.72	18	60.1	Day 35	0.05	Q5ZLJ7
Matrilin-3	-0.75	13	32.5	Day 7	0.06	O42401
Complement C6	-0.77	17	26.5	Day 7	0.07	B8ZX71
Collagen alpha-1(VI) chain	-0.79	12	19.0	Day 7	0.07	A0A1D5PWN6
F-actin-capping protein subunit beta isoforms 1 and 2	-0.89	12	46.8	Day 35	0.06	A0A1D5P342
Glycerol-3-phosphate dehydrogenase	-0.93	12	51.6	Day 21	0.08	A0A1D5P1Y7
Myosin light polypeptide	-1.00	4	27.2	Day 35	0.07	P02607
Glycerol-3-phosphate dehydrogenase	-1.06	2	8.6	Day 35	0.06	Q9PUU8
Uncharacterized protein	1.34	10	48.7	Day 35	0.07	A0A1D5PW77

Table 7.5b. Proteins of note approaching significance ($0.16 > p > 0.08$) in sera from NS-supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Glutathione peroxidase	0.78	15	58.3	Day 35	0.09	F1NPJ8

Table 7.6. Proteins with high fold change in sera from PowerTract® supplemented broilers.

Protein Description	Fold Change[1]	Peptides	Coverage(%)[2]	Day[3]	*p* value	Accession
Creatine kinase M-type	3.12	19	51.4	Day 21	0.13	P00565
Hemoglobin subunit alpha-D	2.11	17	93.6	Day 21	0.41	P02001
Alpha-1-acid glycoprotein	-1.86	7	36.0	Day 35	0.36	Q8JIG5

Table 7.7. List of known selenoproteins obtained from Liu *et al.* (2017).

Glutathione peroxidase 1	Selenoprotein H
Glutathione peroxidase 2	Selenoprotein I
Glutathione peroxidase 3	Selenoprotein M
Glutathione peroxidase 4	Selenoprotein N
Iodothyronine deiodinase 1	Selenoprotein O
Iodothyronine deiodinase 2	Selenoprotein U
Iodothyronine deiodinase 3	Selenoprotein W
Methionine sulfoxide reductase B	Thioredoxin reductase 1
Selenophosphate Synthetase 1	Thioredoxin reductase 2
Selenophosphate Synthetase2	Thioredoxin reductase 3
Selenoprotein 15	