Quantitative Proteomic Analysis Reveals Yeast Cell Wall Products Influence the Serum Proteome Composition of Broiler Chickens

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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 selected 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-based Gut Health Products (GHP) 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, GHP-fed treatment groups versus controls. An average of 7.1% of proteins showed changes in abundance with GHP supplementation. Several effects of these GHPs including immunostimulation (via elevated complement protein detection), potential alterations in the oxidative status of the animal (e.g., glutathione peroxidase and 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 GHP feed supplement, were observed. It is proposed that specific protein detection may be indicative of GHP efficacy to stimulate broiler immune status, i.e., may be biomarkers of GHP 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 GHP supplementation has on the broiler chicken.

Keywords: gut health; poultry; quantitative proteomics; yeast cell wall; immunity; inflammation; intestinal integrity

1. Introduction

Consequent to 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 the mannan-rich fraction (MRF) of the yeast cell wall (YCW), have emerged as viable alternatives to AGP and have been shown to impart several beneficial effects on animals and have application in the field of animal husbandry. Some noted effects of yeast-based products include modulation of the gut microbiome [1], reduced infection by pathogenic bacteria and beneficial stimulation of the host immune system [2–8].

Immunological responses triggered by the YCW are largely due to the action of two molecules, Mannose Oligosaccharide (MOS) and β-glucan which are highly abundant in
YCW. YCW immunostimulation has been reported in a number of species including human [9], fish [7] and mice [10] as well as livestock such as cows [11], pigs [12,13] and broiler chickens [4] and has been shown to induce a range of immunological effects both in vivo and in vitro. These benefits include reducing the levels of pathogenic bacteria in the gut [14], enhancing growth of beneficial bacteria [15] and modulating the immune response [16]. 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 [17] and stimulating the production of mucins by goblet cells found in the digestive tract [14]. Mucins are the glycoprotein constituent of mucus [18] 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 [19]. Thus, intestinal homeostasis depends on complex interactions between microbiota, intestinal epithelium, and host immune system [1].

The physiological effects of MOS can translate into improved performance of broilers. Several 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 [20,21]. A review of the different mechanisms of MOS and their subsequent effect on intestinal microflora, intestinal morphology, immunity, and performance of broilers are discussed in detail elsewhere [14]. With developments in mass spectrometry and bioinformatic technologies in the last decade [22], comparative proteomics has come to the forefront of proteomic research, including a number of reviews published highlighting its potential application in farm animal research [23–25]. However, despite advances in proteomic technologies, only a relatively small number of comparative proteomic studies have been conducted on avian serum in recent years [26–31].

Packialakshmi et al. revealed differential protein profiles among low abundance proteins in chicken plasma, following depletion of high abundant proteins, in an LPS challenge versus control study (n = 9 birds/group) using both LC- and MALDI-MS/MS analyses [32]. Chemokine CCLI10 and alpha 1 acid glycoprotein (A1AGP) were only detected in plasma from LPS-treated birds. Label-free proteomic analysis identified increased abundance of A1AGP, CATH-2 precursor, a heparinase precursor protein and reduced abundance of ISG12-2 in plasma following LPS-only exposure, leading the authors to propose the utility of plasma proteomic analyses to identify biomarkers of innate immunity and inflammation in chickens. Following albumin and IgG depletion from avian plasma, iTRAQ-based LC-MS/MS revealed 468 differentially abundant proteins (increased, 171; decreased, 297) in penguin sera, using an aspergillosis model system to investigate the proteomic, and inflammatory responses to Aspergillus fumigatus exposure [33]. It is notable that although 63 protein families were noted with increased abundance, and 17 individual proteins exhibited >4-fold abundance, while no significant detection of penguin complement proteins was evident. This suggests that complement does not play a role in the avian anti-Aspergillus response, as noted by [33]. Horvatić et al. used a tandem mass tag (TMT) approach linked to subsequent LC-MS/MS to quantitatively investigate the effect of LPS, and exposure time (up to 72 h), on specific protein abundance in chicken plasma (n = 12 chickens in total) [27]. Interestingly, these authors used plasma which had not previously been depleted of high abundance proteins prior to processing and LC-MS/MS. Overall, 87 significantly differentially abundant proteins were evident, of 418 total proteins detected, and identified including serum amyloid A (the major chicken acute phase protein), A1AGP and ovotransferrin in the LPS-treated cohort. Significantly elevated abundance of complement proteins was also observed by Horvatić et al. in the LPS-treated cohort of birds [27]. Using iTRAQ peptide labelling and LC-MS/MS, Shen et al. identified 239 differentially abundant proteins, when comparing laying and brooding hens (n = 6), out of a total of 1235 proteins detected in chicken serum following prior depletion of high abundance protein [34].
Although interest in, and use of, YCW-based products to improve agricultural outputs have grown enormously, to our knowledge no systematic and quantitative analysis of the serum proteome of supplemented animals has been investigated to gain an insight into the metabolic effects of YCW supplementation of animal feed. 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. Here, we use the power of LC-MS/MS to reveal robust alterations in the broiler chicken serum proteome highly indicative of YCW supplement-associated immune priming.

2. Results

2.1. Overview of LC-MS/MS Results.

Use of LC-MS/MS Compatible Reagent; (LCR) for protein re-solubilisation post-ProteoMiner™ treatment was optimal and was used throughout this study (Supplementary Figures S1–S3). Consequently, tryptic digests, from 36 pooled serum samples from each control and GHP treated group, were analysed by LC-MS/MS at three time points, Day 7 (D7), Day 21 (D21) and Day 35 (D35). Within each time point, each treatment group was compared to control which created three comparator groups: GHP1 vs. control, GHP2 vs. control and GHP3 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 identified. In addition, statistically significant alterations ($p < 0.05$) in protein abundance are also expressed as log2fold changes in Tables 1-7, whereby log2(1) = twofold or 100% alteration in abundance, etc.

Between 5% and 12% of the total identified proteins within each comparator group showed changes in abundance (Table 1). The largest change in protein abundance was seen on Day 7, with an average of 9.1% of proteins changed in abundance or unique to/absent from the serum of treated birds when compared to control birds. GHP1-treated birds showed the largest change in protein abundance throughout the three time points with an average of 7.8% of proteins changed in abundance. The largest individual change in abundance was seen in Day 7 GHP1 vs. control samples with 11.1% of proteins changed.

Inter-day and inter-treatment analysis was also conducted on the total protein number 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 (Version 1.5.7.0; http://maxquant.org), first grouped by Day and then, in a separate analysis by treatment. Proteins ($n = 1007$) were identified in at least one pooled serum sample from all time points (Figure 1A). Over half of all 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 ($n = 209$). Shared proteins ($n = 602$) were identified in at least one serum sample from all treatment groups (Figure 1B). Most 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 GHP2 treatment group ($n = 22$), in the GHP1 treatment group ($n = 51$) and the GHP3 treatment group ($n = 150$) (Figure 1B).

| Comparator Group | Total Identified Proteins | Significant changes * | Unique/Absent Changes ** | Total Changes | Total Changes (%) |
|------------------|--------------------------|-----------------------|--------------------------|---------------|------------------|
| D7 GHP1 vs. D7 control | 651                      | 38                    | 26                       | 64            | 9.83             |
| D7 GHP2 vs. D7 control | 383                      | 26                    | 28                       | 54            | 8.22             |
| D7 GHP3 vs. D7 control | 14                      | 28                    | 28                       | 42            | 6.45             |
| D21 GHP1 vs. D21 control | 588                      | 25                    | 9                        | 34            | 5.78             |
| D21 GHP2 vs. D21 control | 23                      | 10                    | 10                       | 33            | 5.61             |
| D21 GHP3 vs. D21 control | 624                      | 21                    | 17                       | 38            | 6.09             |

Table 1. Overview of proteins with significantly changed abundance within comparator groups.
D35 GHP3 vs. D35 control

* Significant changes denote proteins which have changed in abundance with \( p < 0.05 \) in a two-sample *t*-test. In addition, statistically significant alterations \( (p < 0.05) \) in protein abundance are also expressed as log2fold changes in Tables 2–7, whereby log2(1) = twofold or 100% alteration in abundance, etc. ** Unique/Absent Changes denote proteins that were uniquely present in a treatment group or absent from a treatment group when compared to day-specific control.

Figure 1. (A) Shared and distinct proteins among experimental time points. (B) Shared and distinct proteins among control and treatment groups. Figures were generated using the Venn Diagrams tool available at https://vandepeerlab.org/?q=tools/venn-diagrams (accessed on 15 February 2022).

2.2. Quantitative Proteomic Analysis of Serum from Broilers Fed a GHP1 Supplemented Diet vs. Serum from Broilers Fed on a Control Diet.

Proteins \((n = 84)\) were found to be significantly increased \((p < 0.05)\) in abundance or uniquely present in the serum of GHP1 supplemented birds across the three time points (Table 2). Proteins \((n = 62)\) were significantly decreased in abundance \((p < 0.05)\) or not detected from GHP1 samples in comparison to control samples across the three time points (Table 3). Principal component analysis (PCA) (Figure 2A–C) of GHP1 serum samples versus control serum samples shows that GHP1 samples and control samples cluster separately on Day 7, Day 21 and Day 35. Hierarchical 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 GHP1 samples than the control group. Using Reactome pathway mapping software, 19 proteins that were significantly increased \((p < 0.05)\) in abundance or uniquely present in GHP1 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), A1AGP (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 (A0A1D5P3Y8), glutaminyl-peptide cyclotransferase (A0A1D5P3R0), complement C4 precursor (A0A1D5P3V9), complement C5 (E1BR57), complement C6 (B8ZX71) and complement C7 (E1C6U2). These proteins are represented in a Reactome pathway map (Figure 3A). Mannose binding protein (Q98TA4) was also increased in abundance and approaching significance \((p = 0.16)\) on Day 35 in GHP1 samples (Supplementary Table S1). A few
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) (Table 2). Additionally, proteins involved in vitamin A transport and metabolism were increased in abundance in GHP1 samples. Retinol binding protein 4 (P41263) level was significantly increased and retinol binding protein 7 (E1C0M1) was uniquely present in GHP1 samples on Day 7. The largest significant \((p < 0.05)\) Log2 fold-change detected across all serum samples was observed in the Day 35 GHP1 vs. control comparator group. A1AGP (Q8JIG5) had a Log2 fold change of 2.12 on Day 35. Complementary and replicate ELISA analysis of pooled sera from Day 35 control versus GHP1-treated confirmed direction of elevated A1AGP levels ( assay 1: 1.64 v 2.27 mg/mL and assay 2: 2.2 v 2.3 mg/mL) by previous LC-MS/MS approach, albeit not at statistical significance (Supplementary Figure S4).

Several notable proteins showed changes in abundance in GHP1 samples. Osteocalcin (P02822) was uniquely detected in GHP1 samples on Day 21. Gastrokine-2 (A0A1D5PFM9) was uniquely present in GHP1 samples on Day 35. Transferrin receptor protein was significantly increased \((p < 0.05)\) with a relatively large Log2 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 GHP1 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 GHP1 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 (Supplementary Table S1). 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 (Supplementary Table S1), also noted by McCaffrey et al. to be significantly increased in intestinal lumen of GHP supplemented broilers. β-enolase (P07322) showed decrease in abundance and approaching significance \((p = 0.09)\) on Day 21 (Supplementary Table S1) and significantly decreased \((p < 0.05)\) in abundance on Day 35 (Table 3) [8]. Proteins involved in nucleotide metabolism were significantly altered in abundance \((p < 0.05)\) or unique to/absent from, GHP1 samples. Nucleoside diphosphate kinase (O57535) and cytidine/uridine monophosphate kinase 2 (R4GJC4) were uniquely present in GHP1 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 2. GHP1 Principal Component Analysis (PCA) comparing GHP1 (Blue) vs. day-specific control (Black) pooled serum samples for (A) Day 7, (B) Day 21, and (C) Day 35. GHP2 Principal Component Analysis (PCA) comparing GHP2 serum samples (Red) vs. day-specific control serum samples (Black) for (D) Day 7, (E) Day 21, and (F) Day 35. GHP3 Principal Component Analysis (PCA) comparing GHP3 (Green) vs. day-specific control (Black) pooled serum samples for (G) Day 7, (H) Day 21, and (I) Day 35.
Figure 3. Reactome pathway map analysis of proteomic data following broiler dietary GHP supplementation over 35-day period. This data reveals consistent elevation of immune-function associated proteins indicative of an activated innate immune response associated with improved capacity to ameliorate infection. (A) Pathway map of 19 proteins involved in the immune system that were significantly increased in abundance or uniquely present in the serum of GHP1 supplemented broiler chickens. (B) Reactome pathway map of 19 proteins involved in the immune system that were significantly increased in abundance in the serum of GHP2 supplemented broiler chickens. Highlighted lines and dots represent proteomic pathways within the immune system that contain proteins which have been increased in abundance with GHP2 supplementation. (C) Pathway map of 19 proteins involved in the immune system that were significantly increased in abundance or uniquely present in the serum of GHP3 supplemented broiler chickens. All data obtained using Reactome.org [35].
| Protein Description                                      | Fold Change | Peptides | Coverage (%) | Day      | 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    | A0A1D5PQG9 |
| Uncharacterized protein                                  | Unique      | 5        | 41.4         | Day 7    | A0A1D5PQ15 |
| Proteasome endopeptidase complex                         | Unique      | 3        | 43.7         | Day 7    | A0A1L1R5U8 |
| Proteasome subunit beta 1                                | Unique      | 3        | 36.4         | Day 7    | A0A1L1RYK5 |
| Retinol binding protein 7                                | Unique      | 3        | 32.1         | Day 7    | E1C0M1     |
| Uncharacterized protein                                  | Unique      | 4        | 30.8         | Day 7    | FIN8Y3     |
| Elongation factor 1-alpha                                | Unique      | 15       | 49.9         | Day 7    | FIN9H4     |
| Succinyl-CoA:3-ketoacid-coenzyme A transferase           | Unique      | 3        | 11           | Day 7    | FIN9Z7     |
| Endoplasmic reticulum lectin 1                          | Unique      | 3        | 30.5         | Day 7    | FINI6      |
| Small nuclear ribonucleoprotein 13                       | Unique      | 3        | 12           | Day 7    | FINC8      |
| PDZ and LIM domain 5                                     | Unique      | 4        | 8.2          | Day 7    | FINTC8     |
| Proteasome 26S subunit, ATPase 5                         | Unique      | 3        | 12.7         | Day 7    | FINU79     |
| 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    | R4GJ4      |
| Nucleoside diphosphate kinase                            | Unique      | 2        | 20.3         | Day 21   | O57535     |
| Osteocalcin                                              | Unique      | 3        | 45.4         | Day 21   | P02822     |
| Tenasin                                                  | Unique      | 8        | 8.7          | Day 21   | P1I0039    |
| Gastrokinase 2                                           | Unique      | 2        | 18.6         | Day 35   | A0A1D5PFM9 |
| N-acetylglucosamine-1-phosphate transferase γ subunit    | Unique      | 2        | 16           | Day 35   | E1B568     |
| N-acetylglucosamine-1-phosphate transferase γ subunit    | 0.34        | 5        | 27.2         | Day 35   | E1B568     |
| Adhesion G protein-coupled receptor G6                   | Unique      | 2        | 2.5          | Day 35   | E1C3C2     |
| Alpha-1-anti-ase                                         | Unique      | 9        | 27.8         | Day 35   | FINX5      |
| Aggrecan core protein                                    | Unique      | 3        | 2.3          | Day 35   | FINZX1     |
| Alpha-1-acid glycoprotein                                | 2.12        | 7        | 13.9         | Day 35   | Q8I1G5     |
| Transferrin receptor protein 1                           | 1.7         | 10       | 10.5         | Day 35   | Q90997     |
| Heat shock protein beta-I                                | 1.3         | 18       | 77.9         | Day 7    | F1P953     |
| Myosin light chain 1, skeletal muscle isoform            | 1.23        | 9        | 54.9         | Day 7    | F02604     |
| Myosin regulatory light chain 2, skeletal muscle isoform | 1.22        | 9        | 57.7         | Day 7    | F02609     |
| Adenylate kinase isoenzyme 1                            | 1.15        | 11       | 58.1         | Day 7    | F05681     |
| 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 35   | A0A1D5PAN0 |
| Glycerol-3-phosphate dehydrogenase                       | 1.11        | 9        | 24           | Day 7    | A0A1D5PY7  |
| Proteasome subunit alpha type                            | 1.08        | 7        | 36.2         | Day 7    | FINC02     |
| Uncharacterized protein                                  | 1.08        | 7        | 21.1         | Day 7    | FINP5      |
| N-Phosphoglycerate kinase                               | 0.94        | 17       | 51.9         | Day 7    | FINU17     |
| Complement C5                                            | 0.93        | 82       | 54.8         | Day 35   | E1B5R7     |
| Dynactin subunit 3                                       | 0.89        | 6        | 32.9         | Day 7    | A0A1D6U5U1 |
| Glutaredoxin 3                                           | 0.88        | 8        | 29.8         | Day 7    | A0A1D5NW30 |
| Uncharacterized protein                                  | 0.86        | 7        | 15.2         | Day 7    | A0A1L1RQM3 |
| Low mol. weight phosphoryl osine protein phosphatase     | 0.84        | 10       | 58.5         | Day 7    | A0A1D5PZ21 |
| Proteasome subunit alpha type                            | 0.84        | 9        | 46.3         | Day 7    | A0A1L15J99 |
| Protein/nucleic acid deglycase DJ-1                      | 0.84        | 5        | 51.4         | Day 7    | A0A1D5PN39 |
| Complement C6                                            | 0.83        | 29       | 33.4         | Day 35   | B5ZX71     |
| Proteasome subunit beta type                             | 0.79        | 13       | 56.6         | Day 7    | A0A1L1RUE7 |
| Proteasome subunit alpha type                            | 0.79        | 5        | 36.1         | Day 7    | A0A1D5PHL0 |
| Peristin                                                 | 0.77        | 16       | 24.5         | Day 21   | F1P4N9     |
| Receptor of-activated protein C kinase 1                 | 0.76        | 9        | 40.9         | Day 7    | A0A177Q3Y2 |
| Proteasome subunit alpha type                            | 0.76        | 6        | 23.9         | Day 7    | F1N9Q6     |
| Serpin H1                                                | 0.76        | 12       | 34.9         | Day 7    | P13731     |
| Vascular cell adhesion molecule 1                        | 0.75        | 6        | 13           | Day 21   | F1P201     |
| Ribosomal protein S14                                     | 0.69        | 4        | 34.9         | Day 7    | Q5ZHW8     |
| T-complex 1                                               | 0.68        | 9        | 17.6         | Day 7    | Q5ZMC9     |
| Uncharacterized protein                                  | 0.67        | 8        | 38.3         | Day 7    | A0A1D5PAH2 |
| Complement C7                                            | 0.66        | 28       | 52.3         | Day 35   | E1C6U2     |
Table 3. Proteins with significantly ($p < 0.05$) decreased abundance or absent in GHP1 vs. control serum samples. Proteins are listed in order of change of abundance.

| Protein Description | Fold Change | Peptides | Coverage (%) | Day | 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 | A0A1D5PPS57 |
| Splicing factor proline and glutamine rich| Absent      | 3        | 18.4         | Day 21 | A0A1D5PPW4 |
| Glutathione S-transferase| Absent      | 2        | 9.5          | Day 21 | Q8392     |
| 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    |
| Serpin family F member 2| −2.17       | 13       | 24.2         | Day 7  | FINAR5    |
| Beta-enolase| −1.65       | 12       | 40.9         | Day 35 | P07322    |
| Receptor of-activated protein C kinase 1| −1.33       | 6        | 21.9         | Day 21 | A0A1D7QYY2 |
| Serpin family G member 1| −1.24       | 12       | 15.8         | Day 7  | FINAS8    |
| 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 | A0A1L1RP91 |
| Phosphoglycerate kinase| −1.03       | 11       | 27.8         | Day 21 | A0A1D5NZWW9 |
| 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 PM| −0.83       | 26       | 45.6         | Day 35 | P00548    |
| Adenosine deaminase| −0.8        | 17       | 60.4         | Day 35 | Q5ZKPP6   |
| DAZ associated protein 1| −0.79       | 4        | 15.4         | Day 21 | Q5ZM92    |

1 Fold change refers to the log fold change in protein abundance in response to GHP1 treatment; Unique refers to proteins only identified in GHP1 serum compared to control serum. 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. Proteins referred to in text are highlighted in (red).
| Alpha-enolase          | -0.74 | 14 | 34.3 | Day 21 | A0A1D5PSH6 |
|-----------------------|-------|----|------|--------|------------|
| Chaperonin containing TCP1 subunit 5 | -0.73 | 9  | 22.25 | Day 21 | Q5F411     |
| Macrophage receptor with collagenous structure | -0.71 | 10 | 21.4 | Day 21 | A0A1D5PZ3 |
| Adenosine deaminase   | -0.7  | 20 | 69.8 | Day 21 | A0A1D5PDK4 |
| Tubulin alpha chain   | -0.68 | 11 | 30.3 | Day 21 | A0A1D5PC8 |
| 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 | P90207     |
| LSMB 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 | A0A1D5PP3 |
| Uncharacterized protein | -0.54 | 7  | 18.5 | Day 21 | F1NIS5     |
| Complement Clq C chain | -0.52 | 5  | 24   | Day 21 | A0A1D5PB2 |
| Kininogen 1           | -0.52 | 15 | 28.1 | Day 7  | A0A1L1RN4 |
| Complement Clq B chain | -0.51 | 6  | 33.6 | Day 21 | F1NH19     |
| Insulin-like growth factor binding protein 5 | -0.5 | 3  | 19.3 | Day 35 | F1NDD8     |
| Vitronecin            | -0.48 | 20 | 88.8 | Day 35 | Q5F424     |
| Fibulin-1             | -0.46 | 23 | 39.3 | Day 35 | Q5ZLN1     |
| Ubiquilin 4           | -0.46 | 5  | 12.4 | Day 21 | A0A1D5P624 |
| Methylthioribose-1-phosphate isomerase 1 | -0.46 | 9  | 46.6 | Day 21 | A0A1D5PN7 |
| 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 | A0A1D5PH3 |
| Apolipoprotein H       | -0.44 | 14 | 42.7 | Day 35 | A0A1L1RTQ4 |
| Epiphycan             | -0.43 | 5  | 13.7 | Day 7  | Q06944     |
| Ig lambda chain C region | -0.42 | 9  | 53.3 | Day 35 | P20763     |
| Agggren core protein  | -0.38 | 16 | 7.7  | Day 7  | Q06944     |
| 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 | Q5F424     |
| F-actin-capping protein subunit beta isoforms 1 and 2 | -0.29 | 11 | 36.2 | Day 21 | P14315     |
| Uncharacterized protein | -0.28 | 2  | 38.5 | Day 21 | F1NSC8     |
| Vimentin              | -0.27 | 30 | 64.6 | Day 21 | A0A1L1XL9 |
| Fibrinogen gamma chain | -0.18 | 9  | 33.7 | Day 21 | E1BV78     |

1 Fold change refers to the log2 fold change in protein abundance in response to GHP1 treatment. 2 Coverage (%) refers to the % of protein sequence represented by identified peptides. 3 Day refers to the point at which the differentially abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial. Proteins referred to in text are highlighted in (red).

2.3. Quantitative Proteomic Analysis of Serum from Broilers Fed a GHP2 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 sera from GHP2 fed birds compared to controls (Table 4). Proteins (n =55) were found to be significantly decreased (p < 0.05) in abundance or not detected in serum of GHP2 samples (Table 5). PCA of proteomic data from GHP2 and control sera (Figure 2D–F) showed that GHP2 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. 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 GHP 2 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 (F1NP5N), 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 (Q08635), proteasome subunit alpha type (F1NEQ6), metalloproteinase inhibitor 2 (R4GL5), cathepsin D (Q05744), complement C4 Precursor (A0A1D5P5V5), complement C6 (B8Z71) and...
complement C7 (E1C6U2). These are represented in a Reactome pathway map (Figure 3B). Mannose-binding protein (Q98TA4) was increased in abundance and approaching significance ($p = 0.2$) on Day 35 in GHP 2 samples.

Several proteins involved in carbohydrate metabolism were also significantly increased in abundance in GHP 2 samples. Specifically, glyceraldehyde-3-phosphate dehydrogenase (P00356) and phosphoglycerate kinase (F1NU17) were significantly increased ($p < 0.05$) in GHP 2 samples on Day 7. Individual proteins of note also showed changes in abundance in GHP 2 samples. Osteocalcin (P02822), a marker for bone resorption, was uniquely identified on Day 21. Gastrokine-2 (A0A1D5PFM9) was uniquely present in the serum of GHP 2 treated birds on Day 35. Insulin-like growth factor II was significantly decreased ($p < 0.05$) in abundance on Day 35 (Table 5). Transferrin receptor protein 1 (F1NTM6) was increased in abundance and approaching significance ($p = 0.06$) (Supplementary Table S2) on Day 35. Proteins ($n = 3$) involved in vitamin A transport [36,37] were increased in abundance in GHP 2 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 (Supplementary Table S2). Several proteins involved in nucleotide metabolism were significantly altered in abundance ($p < 0.05$) in GHP 2 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 (Table 4). 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 (Table 5). Finally, A1AGP was increased in abundance on Day 35 in samples from GHP 2 supplemented birds with a large fold change (Supplementary Table S3). However, this change was not significant ($p = 0.37$).
Table 4. Proteins with significantly (p < 0.05) increased abundance or unique in GHP2 vs. control serum samples. Proteins are listed in order of change of abundance.

| Protein Description                                                                 | Fold change | Peptides | Coverage (%) | 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 | A0A1D5PQ9  |
| Legumain                                                                            | Unique      | 2        | 11.8         | Day 7 | A0A1L1RX51 |
| Proteasome subunit beta 1                                                            | Unique      | 3        | 26.4         | 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 I-alpha                                                            | Unique      | 15       | 49.9         | Day 7 | F1N9H4     |
| Succinyl-CoA3-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     |
| mall nuclear ribonucleoprotein 13                                                    | Unique      | 3        | 30.5         | Day 7 | F1NI6      |
| 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 | F21566     |
| 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 | Q6AXV0     |
| Surfactant protein A                                                                 | Unique      | 3        | 16.2         | Day 7 | Q6QX82     |
| Seryl-tRNA synthetase                                                               | Unique      | 2        | 4.3          | Day 7 | R5G5J9     |
| 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| F02822     |
| Tenascin                                                                            | Unique      | 8        | 8.7          | Day 21| P10039     |
| Tenasin                                                                            | Unique      | 10       | 10.8         | Day 35| P10039     |
| Gastrokine-2                                                                        | Unique      | 2        | 18.6         | Day 35| A0A1D5PFM9 |
| N-acetylglucosamine-1-phosphate transferase γ subunit                              | Unique      | 2        | 16          | Day 35| E1B6S6     |
| 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 | F1F930     |
| Myosin light chain 1, skeletal muscle isoform                                        | 1.08        | 9        | 60.9         | Day 7 | P02604     |
| 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/nuclease acid deglycase DJ-1                                                | 0.91        | 5        | 59           | Day 7 | A0A1D5PN39 |
| Actin, alpha skeletal muscle                                                      | 0.89        | 13       | 24.4         | Day 21| A0A1H7Q14  |
| Uncharacterized protein                                                             | 0.84        | 2        | 3.7          | Day 21| F1NNM2     |
| Carbonic anhydrase 2                                                                | 0.83        | 7        | 44.6         | Day 35| P07630     |
| Alpha-1,4 glucan phosphorylase                                                      | 0.81        | 5        | 7.8          | Day 35| E1BN57     |
| Uncharacterized protein                                                             | 0.8         | 54       | 24           | Day 21| A0A1D5PW77 |
| Receptor-of-activated protein C kinase 1                                             | 0.75        | 9        | 56.9         | Day 7 | A0A1FQ3Y2  |
| Complement C7                                                                   | 0.73        | 28       | 54.3         | Day 35| E1C6U2     |
| Low mol. 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 |
| Transfhyretin                                                                        | 0.64        | 9        | 72.7         | Day 35| P27731     |
| Cystatin A                                                                         | 0.62        | 4        | 18.3         | Day 21| F1NHG8     |
| Protein Description                                                                 | Fold Change | Peptides | Coverage (%) | Day     | Accession   |
|-----------------------------------------------------------------------------------|-------------|----------|--------------|---------|-------------|
| Growth differentiation factor 11                                                 | Absent      | 2        | 6.4          | Day 7   | A0A1D5P7V6  |
| Uncharacterized protein                                                           | Absent      | 5        | 10.3         | Day 7   | F1NCV8     |
| Uncharacterized protein                                                           | Absent      | 3        | 9            | Day 21  | A0A1D5PFS2  |
| Glutathione S-transferase                                                         | Absent      | 2        | 9.5          | Day 21  | Q08392     |
| Catalase                                                                          | Absent      | 2        | 8.9          | Day 21  | Q52L24     |
| 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  | Q08392     |
| Nuclear transport factor 2                                                        | Absent      | 2        | 63           | Day 35  | FINLL4     |
| Nuclear transport factor 2                                                        | -0.6        | 4        | 40.7         | Day 21  | FINLL4     |
| TAR DNA-binding protein 43 (TDP-43)                                               | Absent      | 4        | 14.5         | Day 35  | Q5ZL5      |
| Heterogeneous nuclear ribonucleoprotein A2/B1                                     | Absent      | 3        | 20.1         | Day 35  | Q5ZM51     |
| Junction plakoglobin                                                              | -1.45       | 33       | 7.1          | Day 7   | E1CVV3     |
| Uncharacterized protein                                                           | -1.23       | 12       | 0.7          | Day 7   | A0A1L1RLW1  |
| Insulin like growth factor binding protein 5                                      | -1.17       | 3        | 15.9         | Day 35  | FIN88      |
| 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  | A0A1D5PZL2  |
| Uncharacterized protein                                                           | -0.93       | 2        | 37.5         | Day 21  | FINSC7     |
| Uncharacterized protein                                                           | -0.33       | 3        | 37.5         | Day 21  | FINSC7     |
| Olfactomedin-like protein 3                                                       | -0.92       | 20       | 50.5         | Day 35  | Q5SC36     |
| Ovothihibitor                                                                    | -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   | P32001     |
| Fibrinogen beta chain                                                             | -0.65       | 8        | 26.9         | Day 7   | Q02020     |
| Carboxypeptidase                                                                  | -0.62       | 7        | 16           | Day 21  | A0A1L1RNXB2 |
| Fibrinogen gamma chain                                                            | -0.61       | 6        | 34.1         | Day 7   | E1BV78     |
| **Insulin-like growth factor II**                                                 | -0.59       | 2        | 11           | Day 35  | F33717     |
| Uncharacterized protein                                                           | -0.59       | 21       | 23.5         | Day 7   | A0A1D5PSJ4  |
| Histone H2B 8                                                                     | -0.58       | 11       | 19           | Day 7   | Q9PSW9     |

1 Fold change refers to the log2 fold change in protein abundance in response to GHP2 treatment; Unique refers to proteins only identified in GHP2 serum compared to control serum. Coverage (%) refers to the % of protein sequence represented by identified peptides. Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial. Proteins referred to in text are highlighted in (red).

Table 5. Proteins with significantly (p < 0.05) decreased abundance or absent in GHP2 vs. control serum samples. Proteins are listed in order of change of abundance.
Uncharacterized protein  
-0.53  3  36.4  Day 21  F1NC22
Chemerin  
-0.52  2  38.5  Day 21  F1NSC8
Uncharacterized protein  
-0.52  7  36.4  Day 21  A0A0K0PUH6
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  R9PM45
Hyaluronan binding protein 2  
-0.52  18  31.4  Day 21  F1NEB3
Uncharacterized protein  
-0.47  8  58.7  Day 21  A0A1D5P4U0
Ig lambda chain C region  
-0.46  22  50.7  Day 35  A0A1D5P1U0
Protein-lysine 6-oxidase  
-0.43  17  31.4  Day 21  F1NEB3
Ig lambda chain C region  
-0.4  9  28  Day 21  A0A1D5P4U0
Adenosine deaminase  
-0.4  17  66  Day 35  P20763
HGF activator  
-0.37  11  15.6  Day 7  F1NLX8
Collagen alpha 1(VI) chain  
-0.34  10  12.7  Day 35  A0A1D5PWM6
Apolipoprotein A-I  
-0.25  2  90.3  Day 7  A0A1D5NX81
CD74 molecule  
-0.21  4  20.8  Day 35  F1NLX8
ADAM metallopeptidase with thrombospondin type 1 motif 13  
-0.12  29  27.1  Day 35  A0A1D5P6FE7

1 Fold change refers to the log 2 fold change in protein abundance in response to GHP2 treatment.  
2 Coverage (%) refers to the % of protein sequence represented by identified peptides.  
3 Day refers to the point in time at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial. Proteins referred to in text are highlighted in (red).

2.4. Quantitative Proteomic Analysis of Serum from Broilers Fed a GHP3 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 GHP3 samples when compared to control, across all time points (Table 6). Proteins (n = 54) were significantly decreased (p < 0.05) or absent from GHP3 samples across all time points (Table 7). Figure 2G–I shows PCA of GHP3 serum samples versus control serum samples and reveals that GHP3 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. Using the Reactome pathway mapping software[35], 19 proteins that were found to be significantly increased (p < 0.05) in abundance or uniquely present in GHP3 samples, were identified as involved in the innate immune response (Figure 3C). 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), β-2-microglobulin (P21611), β-hexosaminidase (F1NTQ2), complement C2 (A0A1D5P4P1), complement C4 precursor (A0A1D5P5V5), complement C5 (E1BR57), complement C6 (B8ZX71), complement C7 (E1C6U2) and mannose-binding protein (Q98TA4) (Table 6).

Proteins (n = 4) involved in carbohydrate metabolism showed changes in abundance in GHP3 samples. Glyceraldehyde-3-phosphate dehydrogenase (P00356) was significantly increased (p < 0.05) on Day 7 (Table 6). Glutamate dehydrogenase 1 and β-1,4-galactosyltransferase 4 (E1C9B0) were absent from GHP3 samples on Day 7 (Table 7). Proteins (n = 4) involved in nucleotide metabolism showed significant (p < 0.05) alterations in abundance in GHP3 samples. Adenylate kinase isoenzyme significantly increased (p < 0.05) on Day 7 (Table 6). Guanine deaminase (F1NJ06) significantly decreased (p <0.05) on Day 21. Adenosine deaminase (A0A1D5PDF4) significantly decreased (p < 0.05) on Day 35. Deoxythymidylate kinase (A0A1D5PKC2) was absent from GHP3 samples on Day 7 (Table 7).

Proteins (n = 2) involved in vitamin A transport increased in abundance in GHP3 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 (Table 6). Many key proteins involved in the detoxification of ROS were significantly decreased (p < 0.05) in abundance or absent from GHP3 samples.
Two catalases were absent from GHP3 samples on Day 7 and Day 21, respectively, catalase (A0A1D5PPU9) and catalase (Q5ZL24) (Table 7). 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 GHP3 samples on Day 21 and Day 35 (Supplementary Table S5). Glutathione S-transferase (GST) (Q08392) was absent from GHP3 samples in all time points, and this protein was identified as GST alpha-class. A few proteins showed changes in abundance in GHP3 samples whereby transferrin receptor protein was significantly increased ($p < 0.05$) on Day 35 and gastrin releasing peptide (A0A1D5PXC4) was significantly decreased ($p < 0.05$) in abundance on Day 35.

GHP3-supplemented birds were supplemented an organic source of selenium in their diet instead of the inorganic source, selenium selenite, which was used for GHP1, GHP2 and control diets. To examine any potential effects of this change in selenium source, control and GHP3 samples were examined for a SeMet/SeCys substitution for methionine/cysteine using MAXQUANT [38]. Control and GHP3 samples were also searched for known selenoproteins. There was one protein identified to have SeMet/SeCys substitution in control samples (Supplementary Table S8A) which 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 GHP3 samples (Supplementary Table S8B), coatomer subunit alpha (A0A1D5P185), golgin A4 (A0A1D5PN73), nuclear factor related to kappaB binding protein (E1BZI6) and translocase of outer mitochondrial membrane 34 (F1P4X4). The sequence coverage of proteins identified was low, at 0.8–5.4%. Of the four proteins identified with a SeMet/SeCys substitution, three were identified in one GHP3 pooled serum sample obtained on Day 7. Using a list of previously identified selenoproteins obtained from Liu et al. [39] (Supplementary Table S7), one selenoprotein was identified in GHP3 and control samples, glutathione peroxidase (F1NPJ8) [39]. This protein was increased in abundance with $p$-value approaching significance on Day 21 ($p = 0.08$) and Day 35 ($p = 0.09$) (Supplementary Table S9). Selenoprotein F precursor (A0A1D5PF6) was uniquely detected in GHP3 samples, but was not detected in GHP2, GHP1 and control samples. AIAGP was increased in abundance on Day 35 in samples from GHP3 supplemented birds with a large fold change (Supplementary Table S6), however, this was not significant ($p = 0.36$).

Table 6. Proteins with significantly ($p < 0.05$) increased abundance or unique in GHP3 vs. control serum samples. Proteins are listed in order of change of abundance.

| Protein Description                          | Fold Change | Peptides | Coverage (%) | Day   | Accession  |
|---------------------------------------------|-------------|----------|--------------|-------|------------|
| Barrier to autointegration factor 1         | Unique      | 2        | 42.2         | Day 7 | A0A1D5NX4  |
| 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 | E1CIQ6     |
| Plexin domain containing 2                 | Unique      | 3        | 5.9          | Day 7 | E1C468     |
| 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 | FIN0H4     |
| Four and a half LIM domains 1              | Unique      | 4        | 30.5         | Day 7 | FINJ16     |
| Chromogranin A                             | Unique      | 3        | 10.7         | Day 7 | FINLZ2     |
| PDZ and LIM domain 5                       | Unique      | 3        | 8.2          | Day 7 | FINTC8     |
| Proteasome 26S subunit, ATPase 5           | Unique      | 2        | 12.7         | Day 7 | FINU79     |
| Uncharacterized protein                    | Unique      | 2        | 14.1         | Day 7 | F1NW82     |
| 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 |
| Protein Description | Fold Change | Peptides | Coverage (%) | Day | Accession |
|---------------------|-------------|----------|--------------|-----|-----------|
| Glutamate dehydrogenase 1, mitochondrial | Absent | 4 | 9.9 | Day 7 | A0A1D5SN761 |
| Growth differentiation factor 11 | Absent | 2 | 6.4 | Day 35 | A0A1D5SFTV6 |
| Catalase | Absent | 3 | 5.3 | Day 7 | A0A1D5FPL79 |
| 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 | A0A1D5R9U7 |
| Beta-1,4-galactosyltransferase 4 | Absent | 5 | 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 |

1 Fold change refers to the log2 fold change in protein abundance in response to GHP3 treatment; Unique refers to proteins only identified in GHP3 serum compared to control serum; Coverage (%) refers to the % of protein sequence represented by identified peptides. 2 Day refers to the time point at which the differently abundant protein was detected: Day 7, Day 21 or Day 35 of feed trial. Proteins referred to in text are highlighted in (red).

Table 7. Proteins with significant (p < 0.05) decreased abundance or absent in GHP3 vs. control serum samples. Proteins are listed in order of change of abundance.
Uncharacterized protein | Absent | 3  | 9  | Day 21 | A0A1D5PF52
Deoxynucleoside kinase | Absent | 3  | 22.6 | Day 21 | A0A1D5PKC2
Lysozyme | Absent | 2  | 19 | Day 21 | E1BRI5
Small nuclear ribonucleoprotein 13 | Absent | 3  | 36.7 | Day 21 | F1NI6
Proteasome 26S subunit, ATPase 5 | Absent | 3  | 12.7 | Day 21 | F1NU79
Glutathione S-transferase | Absent | 2  | 9.5 | Day 21 | Q8392
Catalase | Absent | 2  | 8.9 | Day 21 | Q5ZL24
Carbohydrate sulfotransferase 3 | Absent | 4  | 13.3 | Day 21 | Q9179
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 | A0A1D5TW4
Uncharacterized protein | Absent | 3  | 29.6 | Day 35 | A0A1D5PW9
Glutathione S-transferase | Absent | 2  | 9.5 | Day 35 | Q8392
TAR DNA-binding protein 43 | Absent | 3  | 14.5 | Day 35 | Q5ZL5
Heterogeneous nuclear ribonucleoprotein A2/B1 | Absent | 5  | 20.1 | Day 35 | Q5ZME1
Cytidine/uridine monophosphate kinase 2 | Absent | 5  | 25.7 | Day 35 | R4GC4
Gastrin-releasing peptide | –1.30 | 3  | 19.7 | Day 35 | A0A1D5PC4
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
Tropomyosin alpha-1 chain | –0.89 | 6  | 20.3 | Day 21 | Q5ZM91
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 | Q60835
Beta-tropomyosin | –0.71 | 22 | 61.3 | Day 21 | Q5ZM91
Hyaluronan binding protein 2 | –0.70 | 15 | 32.5 | Day 21 | F1NEB3
Phosphatidylinositol-sterol acyltransferase | –0.68 | 13 | 37.4 | Day 35 | P57360
Heat shock protein beta-1 | –0.67 | 17 | 79.4 | Day 35 | F1P993
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 | A0A1D5PF3
DAZ associated protein 1 | –0.47 | 4  | 14.0 | Day 21 | Q5ZM92
Adenosine deaminase | –0.41 | 17 | 65.6 | Day 35 | A0A1D5PDF4
Alpha-actinin-1 | –0.41 | 15 | 12.7 | Day 35 | A0A1D5PFN3
Cheimerin | –0.41 | 7  | 46.2 | Day 35 | A0A0K0PUH6
Collagen type XVIII alpha 1 chain | –0.40 | 5  | 4.7 | Day 35 | A0A1D5PM7
Guanine deaminase | –0.38 | 8  | 23.5 | Day 21 | F1NJ6
Macrophage receptor with collagenous structure | –0.33 | 10 | 21.4 | Day 21 | A0A1D5P23Z
Peroxiredoxin-1 | –0.30 | 8  | 45.5 | Day 35 | P0CB50
Fibulin-1 | –0.16 | 18 | 31.1 | Day 21 | A0A1L1RQ28
Heterogeneous nuclear ribonucleoprotein M | –0.15 | 6  | 7.7 | Day 21 | F7BSK7

1 Fold change refers to the log2 fold change in protein abundance in response to GHP3 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. Proteins referred to in text are highlighted in (red).

3. Discussion

3.1. Overview

Quantitative proteomic analysis has revealed that diet supplementation with GHP1, 2 and 3 significantly alters the abundance of specific serum proteins which contribute to the immune status and health of the broiler chicken. Consistent alterations in protein abundance were seen for those involved in the complement component of the innate immune system, nutrient transport, oxidative stress, and selenium status—all of which contribute to the overall animal health and provide evidential support for the supplemental effects of GHP products. Supplementation of broiler diet with GHP products had a detectable effect on the avian 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 GHP 1-, 2- and 3-, treated birds.
GHP1 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. GHP3 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. However, sera from GHP3 supplemented birds showed the greatest number of proteins that were unique to a treatment in inter-treatment analysis and, as will be discussed, GHP3 showed greater complement activation as well as alterations to serum seleno-protein abundance which may indicate a substantial impact on broiler health status.

### 3.2. Individual Proteins of Note

Several 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 several 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 [40]. It has been documented that GHP feed supplementation can have effects on gastric mucosa [41]. Gastrokine-2 was uniquely present in the serum of GHP1 and GHP2 samples on Day 35. One other protein involved in the regulation of gastric mucosa is gastrin releasing peptide (A0A1D5PXC4). Abundance of this protein was significantly reduced ($p < 0.05$) in GHP3 samples on Day 35. Changes in the abundance of these proteins would indicate that these GHPs may have an effect on the regulation of the gastric mucosa.

Osteocalcin (P02822), uniquely present in GHP1 and GHP2 samples on Day 21, is a marker for bone turnover and its presence in serum is an indicator of bone resorption [36]. The presence of osteocalcin, only in the serum of birds supplemented with GHP1 and GHP2, could indicate that these products affect bone formation in the early stages of life which is of critical importance in chicken husbandry. Leg problems are prevalent in broilers [37] and can lead to higher culling rates in commercial production systems which have real monetary effects on the producer [38].

In GHP1 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). This increase in the abundance of proteins involved in ROS detoxification may not be due to increased oxidative stress, but rather the stimulation of the production of these proteins by feed constituents. However, elucidating the molecular mechanisms of redox homeostasis maintenance in poultry and farm animals has received limited attention and are poorly characterized. Nevertheless, determining effective means of mitigating redox stress through nutritional modulation are exciting areas of research gaining momentum [42].

### 3.3. Immunological Effects

As noted, GHP products can have numerous immunological effects on animals [2–7]. Across all treatment components, there were significant ($p < 0.05$) increases in the level of a number of complement components in the serum of GHP-supplemented broilers harvested on Day 35, which would indicate stimulation of the complement cascade by the GHP feed constituents. 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 (E1BR57) was increased in abundance and approaching significance in GHP2 samples and was significantly increased ($p < 0.05$) in abundance in GHP1 and GHP3 samples. Complement C2 was significantly increased ($p < 0.05$) in abundance in GHP3 samples only. Indeed, GHP3 samples showed the greatest level of complement stimulation. Increases in serum levels of complement components indicate a stimulation of the complement cascade by these GHP feed supplements.
A possible source of this complement stimulation by GHP products is through activation of the complement cascade by mannose binding protein (Q98TA4). This protein was significantly increased ($p < 0.05$) in abundance in GHP3 samples and increased and approaching significance in GHP2 ($p = 0.2$) and GHP1 ($p = 0.162$) samples on Day 35. Mannose binding protein, also called Mannose binding lectin (MBL) [43,44] is a C-type serum lectin [45] 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 [46]. Both C2 and C4 precursor were significantly increased in abundance ($p < 0.05$) in GHP3 samples. MBL may also interact directly with cell surface receptors which can initiate opsonophagocytosis [47] 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 [45]. There is little data available in the literature regarding stimulation of the chicken complement cascade by GHP feed supplementation. Slawinska et al. 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 [48]. 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 $\beta$-glucan or MOS [49]. Supplementation resulted in significant increases ($p < 0.05$) in serum complement activity [50] which is in accordance with results found in the present study.

The increase in abundance of additional immunologically relevant proteins suggests immunostimulatory/immunomodulatory effects by these GHPs. One likely mediator of this immunostimulation is Surfactant Protein A (Q90XB2). This protein was uniquely present in GHP3 samples on Day 7, and is a C-type lectin receptor which is present in mucosal tissues and binds glycan ligands which can result in downstream immunological effects [51]. Carbohydrate recognition domains on this protein bind glycan residues resulting in innate immune stimulation [47] which could explain the increase in abundance of immune-related proteins observed. MOS-based products have been shown to elicit immunological responses without causing acute phase (fever) response [1]. Yet, the largest fold change was seen (Log2 Fold change = 2.12) for A1AGP in LC-MS/MS analyses. This protein is in high abundance in serum and has been previously characterised as an acute phase protein [26]. Large fold changes in A1AGP were also seen between samples from GHP2 and GHP3 supplemented birds and control samples (Log2 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). It is unlikely that the high fold changes noted for A1AGP indicate stimulation of the acute phase response because of the non-significant $p$-values ($p < 0.05$) seen in GHP2 and GHP3 as well as the lack of significant change ($p < 0.05$) in any other acute phase proteins (e.g., serum amyloid A). The data 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. Indeed, complementary A1AGP ELISA analysis of GHP1-treated and control samples revealed non-significant elevation in A1AGP in sera from GHP1-treated birds, in accordance with the results obtained from LC-MS/MS analysis.

### 3.4. Effects on Metabolism

With GHP 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 [52] and its increase could indicate a greater level of glycolysis in birds supplemented with these GHP products. Beta-hexosaminidase (F1NTQ2) was significantly increased ($p < 0.05$) in abundance in GHP3 samples and increased and approached significance ($p = 0.08$) in GHP1 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 GHP [53] 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 stimulated carbohydrate metabolism in broiler chickens. However, individual products had varying effects on the levels of carbohydrate metabolism proteins. GHP1 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 GHP1 samples. However, other proteins involved in carbohydrate metabolism showed a decrease in abundance such as \(\alpha\)-Enolase (A0A1D5PSH6) and \(\beta\)-Enolase (P07322). GHP3 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 GHP supplementation, but with proteins both increased and decreased in abundance, no common trend could be elucidated.

3.5. 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 [54]. Deficiencies in broiler diets can lead to deterioration of reproductive [55] and immunological health [56,57]. Vitamin A is bound by Retinol binding protein 4 (RBP4) (P41263) which is in turn bound to Transthyretin (P27731) [58]. These proteins 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 [59]. RBP4 was significantly increased \((p < 0.05)\) and RBP7 was uniquely identified in GHP1 samples on Day 7. Transthyretin was significantly increased on Day 35 in GHP2 samples and RBP7 was uniquely identified on Day 7, RBP4 was also increased in abundance and approached significance \((p = 0.08)\) on Day 21. Transthyretin was significantly increased on Day 35 in GHP3 samples. Increases in the abundance of these proteins may indicate a greater abundance or availability of Vitamin A with the supplementation of these GHP products, however this requires further investigation. One common source of Vitamin A is \(\beta\)-carotene, a precursor to Vitamin A [60] that is naturally obtained through the consumption of carotenoid-producing organisms such as higher plants or photosynthetic microorganisms [61].

3.6. 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 [62–64]. The dietary source of selenium can have an impact, as inorganic selenium is considered a pro-oxidant and organic selenium has been seen to have greater bioavailability [65–67]. Drip-loss has been linked to GSH-Px level [62]. GSH-Px was increased in abundance in all treatment groups with the largest fold-change noted in GHP3 samples where it was increased and approaching significance on Day 21 and Day 35. GSH-Px level has been previously shown to be influenced by selenium supplementation and a greater bioavailability of organic selenium from GHP3 would explain this increased GSH-Px level. 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 [68,69]. 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 [70]. In this work, selenium was also supplemented to broiler diets at 0.3 mg/kg (inorganic selenium selenite for GHP1 and GHP2, Se yeast for GHP3).

The absence of the potentially pro-oxidative inorganic selenium in the diet of GHP3 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 GHP3 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 linked to reduced liver damage [66]. Glutathione S-transferase α (αGST) (Q08392) is an enzyme involved in the detoxification of reactive xenobiotics and is present in high concentrations in the cytosol of hepatocytes [71–73]. This enzyme conjugates glutathione to xenobiotics [74] and its presence in serum has previously been characterised as an indicative biomarker for acute hepatitis or liver damage [75]. αGST is absent from the GHP3 samples on Day 7, Day 21 and Day 35 which would indicate that this product may confer some hepatoprotective effect on the broiler chicken. This absence 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 reduces oxidative damage or a dual effect of these two factors. Evidence suggests that organic selenium has a greater bioavailability than its inorganic forms [65] 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 GHP3 was 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 GHP3 treated birds. An increased bioavailability might not only lead to increased benefits to the broiler chicken, but also to the producer and consumer.

4. Materials and Methods

4.1. 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 (Figure 4A). The pens were divided into four groups: group 1, fed a basal diet; group 2–4, fed a basal diet which included supplements GHP1, 2 and 3, respectively, at 1 kg/tonne in starter, grower and finisher diets. These supplements were mannan-rich fractions extracted from the yeast cell wall of Saccharomyces cerevisiae. Additional individual GHP details and the manufacturers recommended inclusion levels are published elsewhere (Alltech Inc., Lexington, KY, USA) [30]. Basal diets were prepared by a commercial feed mill and consisted primarily of wheat and soybean meal which met full dietary nutritional requirements [76]. 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 in fresh litter for bedding from day zero. The temperature was initially set at 30 °C per day up to day 10 and then decreased linearly by 1 °C every second day. 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.
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 [76].

Figure 4. (A) Overview of serum sample collection, preparation, and analysis from sample collection to serum pooling. (B) Serum analysis from ProteoMiner™ enrichment to Bioinformatic Analysis. * B1 denotes Bird 1.

4.2. ProteoMiner™ Serum Enrichment Evaluation

Individual and pooled avian sera were first cleared of precipitate by centrifugation (10,000× g, 10 min). Sera (200 μL) were then applied to the ProteoMiner™ Protein Enrichment kit (#1633006; BioRad) according to manufacturer’s instructions. After loading and washing, 20 μL of either the provided ProteoMiner elution buffer or 0.1 M Tris-HCL, 6 M Urea and 2 M Thiourea pH 8.0 (LC-MS/MS Compatible Reagent; LCR) 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 LC-MS/MS analysis (Supplementary Figures S1–S3) which showed LCR gave improved protein detection.

4.3. Upgraded ProteoMiner™ Processing for Serum Enrichment

All pooled serum samples were first cleared of precipitate by centrifugation (10,000× g, 10 min). Sera (200 μL) were then applied to the ProteoMiner™ Protein Enrichment kit according to manufacturer’s instructions. After washing, 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 LCR 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 at −20 °C until further processing.

4.4. Protein Digestion and 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 [77]. 10 μL of the enriched sample was then added to 10 mM Dithiothreitol (DTT) using 0.05 M DTT and incubated at 56 °C for 30 min. Samples were then brought to 25 mM Iodoacetamide (IAA) using 0.11 M IAA. Lys-C (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 (0.8 μL) was then added to samples at 1:25 ratio (Protease: Protein). Protease Max (0.45 μL) was then added. Samples were incubated at 37 °C overnight. Using Sample Buffer (0.33 volumes), samples were then diluted. Peptide samples were evaporated to dryness in a SpeedVac™ (DNA 120; Thermo Scientific, Waltham, MA, USA) and stored at −20 °C prior to ZipTip treatment and transfer to Q Exactive mass spectrometer vials for injection (Figure 4B). Peptide samples were then analysed using a Thermo Scientific™ Q Exactive connected to a Dionex Ultimate™ 2000 (RSLCnano) chromatography system. Each sample was loaded onto an EASY-Spray™ PepMap RSLC C18 column (75 μm × 500 mm) and separated by an increasing acetonitrile gradient over 120 min at a flow rate of 250 nL/min [78,79]. The mass spectrometer was operated in positive mode with MS carried out on the 15 most abundant precursor ions at each time point. Singly charged ions were excluded from analysis.

4.5. 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), accessed on 9 April 2021 [80]. 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, accessed on 9 April 2021). Search parameters included: peptide tolerance (ppm) of 20 for first search and 4.5 for main search, carbamidomethylation 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://coxedocs.org/doku.php?id=perseus:start; accessed on 15 April 2021) for data filtering [81]. 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 log2 transformed.

4.6. A1AGP Immunoassay

A chicken A1AGP enzyme-linked immunosorbent assay (ELISA) (ab157690) (abcam®) was used for complementary analysis of serum A1AGP levels accordingly to the manufacturer’s instructions.
4.7. Software and Statistical Analysis

Overall, 36 pooled serum samples were prepared, extracted, and analysed by LFQ proteomics. These comprised of Control pool (Day-specific) and 3 Test pools at day 7, 21 and 35 (4 × 3 × 3 = 36). Thus, at each time point (Day 7, Day 21, and Day 35), treatment groups were compared to each day-specific control group creating three comparator groups (GHP1 vs. Control, GHP2 vs. Control and GHP3 vs. Control). A 2-sample t-test was performed to identify proteins with significant differences (p < 0.05) within each comparator group. Proteins uniquely detected in a treatment group and not detected in control, or proteins not detected in treatment and detected in control groups, were identified and tabulated. Pathway mapping was carried out using Reactome Pathway Database (https://reactome.org; accessed on 30 April 2021). Proteins were specifically analysed for features and/or functions in UniProt (http://uniprot.org).

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

5. Conclusions

The significant alterations in proteins involved in several key processes would suggest that these products had effects on the systemic health status of the broiler chicken that were measurable through serum quantitative proteomic analysis. In this first study of its type, the multifaceted effects of GHP supplementation including stimulation of the innate immune system, particularly the complement system, alterations in the levels of a few proteins involved in the detoxification of ROS, stimulation of metabolic processes, gastric mucosal development and transport of iron and Vitamin A, were noted. The three YCW-based products showed similarity regarding their effects, which would be expected from three related products. We recognise that the use of sample pooling may influence the precise nature of the observed changes in protein abundance. However, individual results also show that these three products have product-specific effects which may be elucidated with further study. Future investigations should ideally deploy the use of smaller pools or individual serum samples to detect alterations in discrete protein abundance.

Future animal challenge studies, using bacterial (e.g., *Escherichia coli* or *Campylobacter jejuni*) or fungal pathogens (e.g., *Aspergillus fumigatus*) will ultimately be required to definitively elucidate the protective effects of the GHP diets studied herein.

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