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Effect of an immune challenge and two feed supplements on broiler chicken individual breast muscle protein synthesis rate

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ABSTRACT

Optimization of broiler chicken breast muscle protein accretion is key for the efficient production of poultry meat, whose demand is steadily increasing. In a context where antimicrobial growth promoters use is being restricted, it is important to find alternatives as well as to characterize the effect of immunological stress on broiler chicken's growth. Despite its importance, research on broiler chicken muscle protein dynamics has mostly been limited to the study of mixed protein turnover. The present study aims to characterize the effect of a bacterial challenge and the feed supplementation of citrus and cucumber extracts on broiler chicken individual breast muscle proteins fractional synthesis rates (FSR) using a recently developed dynamic proteomics pipeline. Twenty-one day-old broiler chickens were administered a single $^{2}H_2O$ dose before being culled at different timepoints. A total of 60 breast muscle protein extracts from five experimental groups (Unchallenged, Challenged, Control Diet, Diet 1 and Diet 2) were analysed using a DDA proteomics approach. Proteomics data was filtered in order to reliably calculate multiple proteins FSR making use of a newly developed bioinformatics pipeline. Broiler breast muscle proteins FSR uniformly decreased following a bacterial challenge, this change was judged significant for 15 individual proteins, the two major functional clusters identified as well as for mixed breast muscle protein. Citrus or cucumber extract feed supplementation did not show any effect on the breast muscle protein FSR of immunologically challenged broilers. The present study has identified potential predictive markers of breast muscle growth and provided new information on broiler chicken breast muscle protein synthesis which could be essential for improving the efficiency of broiler chicken meat production.

Significance: The present study constitutes the first dynamic proteomics study conducted in a farm animal species which has characterized FSR in a large number of proteins, establishing a precedent for biomarker discovery and assessment of health and growth status. Moreover, it has been evidenced that the decrease in broiler chicken breast muscle protein following an immune challenge is a coordinated event which seems to be the main cause of the decreased growth observed in these animals.

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1. Introduction

Global meat production is expected to keep increasing over the forthcoming decades reaching 377Mt by 2031 [1] as a result of an increasing global population, which is expected to reach 8.6 billion in 2030 [2], who aim to satisfy their protein and amino acid requirements. Meat consumption will keep shifting towards poultry meat due to its efficient production, acceptance by most cultures and low price [1]. This high demand can be satisfied due to the selection of chicken strains that show accelerated growth, particularly in the pectoralis (breast) muscle [3]. Muscle protein accretion is the result of the difference between protein synthesis and breakdown. Improving our understanding of broiler chicken muscle synthesis would contribute towards the enhancement of poultry meat production efficiency and feeding an increasing human population.

Despite its importance, research on chicken muscle protein dynamics has been mostly limited to the study of mixed muscle protein turnover [4–8]. The recent introduction of proteomics methods to study protein synthesis in mammalian species allows calculation of individual protein synthesis rates at a proteome scale [9]. In recent years, Omics methods have been used to broaden our current understanding of broiler chicken breast muscle proteome [10], transcriptome [11] and metabolome [12]. The importance of integrating multi-omics approaches to obtain a full picture of biological processes influencing animal performance and disease resistance has been highlighted [13]. However, only one study, conducted during the early developmental stages of the dynamic proteomics field, has aimed to characterize the turnover of individual chicken breast muscle proteins [14]. Moreover, studies on broiler mixed muscle protein synthesis have mainly revolved around the comparison of different broiler strains' growth [5,15–17] and the effect of dietary modifications on muscle synthesis [18–22], with only one study aiming to characterize the effect of an acute immunological challenge on broiler muscle protein synthesis [23]. Restrictions imposed on the use of antimicrobial growth promoters (AGPs) in animal production around the globe [24] increase the necessity to characterize the effects of immunological stress on broiler chicken growth in order to better identify and prevent it. Dynamic proteomics studies could prove extremely valuable to find early biomarkers of immune stress due to the fact that protein synthesis rates are modified before significant changes in protein concentration can be measured [25].

Feed additives have been identified as a promising alternative to the use of AGPs in poultry production [26–28], enhancing chicken growth performance and immune status, with a good number of candidates having been proposed [26]. Citrus extracts are one of these candidates. Citrus extracts are rich in flavonoids, carotenoids, soluble fiber (including pectins) and insoluble fiber [29]. Citrus pectin has been reported to improve chicken energy utilization and nutrient digestibility [30] when added to broiler's diet as well as having immunomodulatory effects on broiler monocytes [31]. Citrus flavonoids have been reported to improve feed efficiency of broilers under heat stress [32] as well as showing immunomodulatory properties in chickens challenged with *E. coli* LPS [33]. Extracts derived from cucumber have shown anti-inflammatory properties in rodents [34] and in porcine aortic endothelial cells challenged with *E.coli* LPS [35] as well as antioxidant properties in human blood cells [36]. Hence, citrus and cucumber extracts are two feed supplements with potential to positively impact broiler chicken's growth and health status in the absence of AGPs.

Recently, we developed a new dynamic proteomics approach for the calculation of protein synthesis rates following a single bolus dose of $^{2}H_2O$, this approach was validated for its use on the broiler chicken species [37]. A simple experimental design makes this approach especially convenient for its use in free living animals. Additionally, the aforementioned study presented a list of *n(AA)* values (which describes the apparent number of deuterium atoms incorporated by free amino acids from body water) specific for its use in broiler chicken dynamic proteomics studies. However, this approach was applied to the calculation of a reduced number of handpicked peptides fractional synthesis rate (FSR). The application of this approach to analyze large proteomics datasets has yet to be accomplished and requires the methodology to be expanded with a robust bioinformatics pipeline capable of filtering peptides and calculating their ^{2}H enrichment as well as rate constant (*k1*) and FSR. Different bioinformatics tools for the analysis of ^{2}H labelled proteomics data for the calculation of protein synthesis rates are freely available, such as ProTurn [38], DeuteRater [39] and d2ome [40]. However, these software solutions have been developed for use in studies following a different experimental design other than a single $^{2}H_2O$ bolus, making it necessary to develop a bioinformatics pipeline for this purpose. Furthermore, some of the available tools embed an amino acid ^{2}H labelling pattern which may not correspond with the one recently characterized in the broiler chicken species [37].

The present study aimed to calculate the FSR of individual broiler chicken breast muscle proteins using our recently developed $^{2}H_2O$ labelling approach complemented with a newly developed bioinformatics pipeline. Estimation of protein FSR was performed on healthy broilers as well as broilers immunologically challenged with *E.coli* LPS in order to characterize the effect of an acute immune challenge in broilers breast muscle protein synthesis. Additionally, the effect of citrus and cucumber extracts on the rates of synthesis of breast muscle proteins of immunologically challenged chickens was also assessed.

2. Materials and methods

2.1. Experimental design and in vivo animal procedures

All chemicals were supplied by Sigma-Aldrich (Gillingham, UK) unless otherwise stated.

In vivo animal trials have been described in detail elsewhere [37]. Briefly, two different animal trials were held at the University of Glasgow Cochno Farm and Research Centre. The aim of the first trial (conducted in June 2018) was to characterize the effect of an immune challenge in broiler chicken muscle protein synthesis by comparing an experimental group of healthy broiler chicken with a group of immunologically compromised chicks. The aim of the second trial (conducted in August 2018) was to investigate the effect of supplementing two different feed additives into the diet consumed by immunologically challenged broiler chickens under the literature-based hypothesis that these extracts may be able to counteract the effect of an LPS-induced inflammatory response. Therefore, the first trial comprised two different experimental groups ("Unchallenged" and "Challenged") while the second trial featured three experimental groups ("Control Diet", "Diet 1" and "Diet 2"). It is worth highlighting that experimental conditions for the "Challenged" experimental group in the first trial and the "Control Diet" group in the second trial were identical (*E.coli* LPS injected and basal diet provided), however these groups should not be considered biological replicates (and directly compared) as it is not possible to guarantee that they were exposed to the same stimuli from hatching to the time they were culled. The fact that is not recommended to draw comparisons between experimental groups which are part of trials carried out at different points in time was the reason why the "Control Diet" group was included in the second trial. While it would have been appropriate to carry out a single trial comprising four experimental groups ("Unchallenged", "Challenged", "Diet 1 and "Diet 2) limitations related with the available staff and experimental facilities made this option unviable. Experimental groups were comprised of four replicate pens in which 7 one day old, male Ross 308 broiler chicks (PD Hook Hatcheries Ltd., UK) were allocated, making the total number of birds 140 (56 in the first trial, 84 in the second trial). Access to water and a corn-soybean meal-based feed (Nuscience, Ghent, Belgium) was provided *ad libitum*. Diet energy and digestible AA content was slightly lower (97%) than commercially available diets. Birds in the "Diet 1"″ experimental group had their basal diet supplemented with a citrus extract (300 g/ton) while birds in the group "Diet 2"″ had their basal diet

supplemented with a cucumber extract (75 g/ton).

The experiment started on day 21 of the birds life (0 h). At time 0 h, five birds per pen were administered an oral dose of $^{2}H_2O$ (10 g/kg 99.8 atom percent $^{2}H_2O$) dropwise using a 10 mL syringe while its movement was restrained. One hour after $^{2}H_2O$ administration, all birds in the pen were weighed and injected subcutaneously a dose of *Escherichia coli* lipopolysaccharide (LPS from *E.coli* O111:B4; 2 mg/kg of body weight) or an equal volume of sterile saline (0.9% sodium chloride) if birds belonged to the "Unchallenged" experimental group. One $^{2}H_2O$ dosed bird from each pen was weighed, blood sampled and culled by barbiturate (Euthatal 200 mg/mL, Merial, UK) overdose 4 h, 24 h, 48 h and 96 h post $^{2}H_2O$ administration with an extra dosed bird being culled at the 96 h timepoint. Undosed birds were culled at the 48 h and 96 h timepoints. A visual summary of the procedures carried out from the 0 h timepoint to broiler chickens in one exemplary pen can be observed in Fig. 1. Blood samples up to 2 mL volume were collected from the bird's wing vein and placed into EDTA coated tubes (Midmeds, Hertford, UK). Blood samples were centrifuged at 3000 *g* for 15 min at 4 °C and the resulting plasma was collected, aliquoted and immediately frozen at − 20 °C. Breast muscle samples were collected immediately after the birds were culled and were snap-frozen using a dry ice bath before being transferred to a − 80 °C freezer.

The *in vivo* trial and all its associated animal procedures were approved by the University of Glasgow MVLS College Ethics Committee and conducted under UK Home Office license (N° P4A4CA831).

2.2. Plasma FTIR analysis

All birds used in the trial had their body water (BW) ^{2}H abundance measured directly on small plasma samples without further processing using a portable Fourier-Transform Infrared Spectroscopy (FTIR) instrument (model 4500, Agilent, Cheadle, UK) as previously described [37]. Briefly, D—O bonds absorbance was measured around a wavenumber of 2504 cm^{-1} in duplicate using a 20 μL plasma volume. Two water standards were measured in duplicate before and after each analysis session. BW ^{2}H abundance (mg/kg) was calculated using an internal calibration model and exported from the manufacturer's Microlab software to Microsoft Excel where molar abundance was obtained before calculation of enrichment values expressed in atom % excess (APE) deuterium.

2.3. Breast muscle protein extraction

Three ^{2}H labelled breast muscle samples (collected at 24 h, 48 h and 96 h) and an unlabelled sample, from three different pens per each of our five experimental groups (for a total of 60 samples), had their protein content extracted following a protocol adapted from the work of Almeida et al. [41], which has been shown to allow the extraction of both sarcoplasmic and myofibrillar proteins. Two hundred mg of tissue were blended with 1 mL 50 mM ammonium bicarbonate, 8 M urea, 2 M thiourea and 10 μL Halt™ protease inhibitor cocktail (100×) (Thermo Fisher Scientific, Renfrew, UK). A 5 mm diameter stainless steel milling ball was added to the tube before the mixture was homogeneised using a Retsch MM400 oscillating mill (Retsch GmbH, Haan, Germany) for 2 min at a frequency of 30 Hz. Samples were then centrifuged for 6 min at 12,500 g and the supernatant was collected. One hundred μL of the collected supernatant were mixed with nine hundred μL of sodium dodecyl sulphate (SDS) buffer (4% SDS, 100 mM Tris/HCL pH 8.2, 100 mM DTT) before being heated for 5 min at 95 °C. Lastly, samples were placed for 10 min on an ultrasonic bath (U100, Ultrawave, Cardiff, UK) with the aim of disrupting protein aggregates. Breast muscle extracts had their protein concentration measured by using a Pierce 660 nm protein assay kit (Thermo Fisher Scientific, Renfrew, UK).

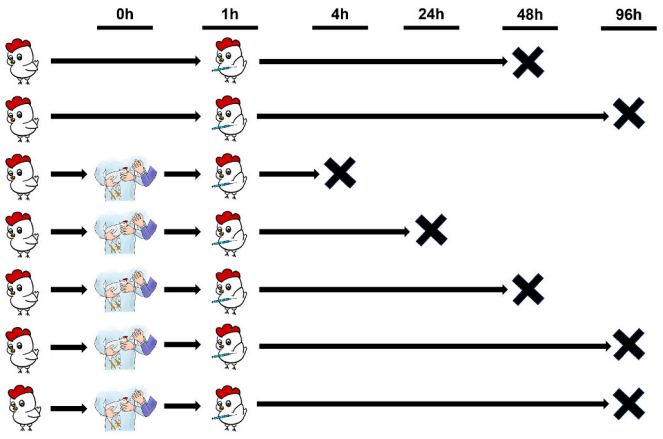

Fig. 1. Experimental design visual summary detailing the procedures carried out on broiler chickens allocated in the same experimental pen from the 0 h timepoint to the 96 h timepoint.

2.4. LC-MS/MS analysis and protein identification

Muscle protein extracts (50 μg protein) were diluted in 2% SDS, 100 mM triethylammonium bicarbonate (TEAB) pH 7.55, then were reduced and alkylated (15 mM tris(2-carboxyethyl)phosphine, 25 mM chloroacetamide) at 45 °C for 60 min. Protein digestion was performed following the single-pot solid-phase-enhanced sample preparation (SP3) protocol [42]. Briefly, ethanol was added to the samples to a final concentration of 70% and proteins were incubated for 15 min with SP3 beads at a bead/protein ratio of 10:1 (weight/weight). Beads were then rinsed using 80% ethanol and proteins were digested with trypsin (Promega, Wisconsin, USA) and Lys-C (Wako, Osaka, Japan) in 50 mM TEAB pH 7.55 (protein: enzyme ratio 1:50, 16 h at 37 °C).

Samples were analysed using an UltiMate 3000 RSLnano LC system coupled to a Q Exactive HF-X mass spectrometer equipped with an EASY-Spray source (Thermo Fisher Scientific, Renfrew, UK). Peptides were loaded onto a trap column (Acclaim™ PepMap™ 100 C18 LC Columns, 5 μm particle size, 100 μm diameter, 20 mm length) (Thermo Fisher Scientific, Renfrew, UK) for 3 min at a flow rate of 10 μL/min in 0.1% FA. Then, peptides were transferred to an EASY-Spray PepMap RSLC C18 column (2 μm particle size, 75 μm diameter, 500 mm length) (Thermo Fisher Scientific, Renfrew, UK) operated at 45 °C and separated using a 60 min effective gradient (buffer A: 0.1% formic acid; buffer B: 100% ACN, 0.1% formic acid) at a flow rate of 250 nL/min. The gradient used was, from 2% to 6% of buffer B in 2 min, from 6% to 33% B in 58 min, from 33% to 45% in 2 min, plus 10 additional minutes at 98% B. Peptides were sprayed at 1.5 kV into the mass spectrometer *via* the EASY-Spray source and the capillary temperature was set to 300 °C. The mass spectrometer was operated in a data-dependent acquisition (DDA) mode, with an automatic switch between MS and MS/MS scans using a top 12 method. Intensity threshold was set to $\geq 3.5e^{5}$. Dynamic exclusion was set to 20 s and charges +1 and > +6 were excluded. MS spectra were acquired from 350 to 1400 *m/z* with a resolution of 60,000 FWHM (200 m/z). Ion peptides were isolated either using a 1.6 Th window and fragmented using higher-energy collisional dissociation (HCD) with a normalized collision energy of 27. MS/MS spectral resolution was set to 15,000 (200 m/z). The ion target values were $3e^{6}$ for MS (maximum IT of 25 ms) and $1e^{5}$ for MS/MS (maximum IT of 22 ms).

Protein identification from Thermo raw files was performed using MaxQuant 2.0.1.0 (Andromeda search engine) [43] against a *Gallus gallus* database (Swiss-Prot/TrEMBL, 34,808 sequences, accessed 23/06/2021). Main search peptide tolerance was set at 4.5 ppm and MS/MS match tolerance at 20 ppm. Carbamidomethylation of cysteines was set as a fixed modification whereas oxidation of methionine and protein N-terminal acetylation were set as variable modifications. Minimal peptide length was set to 6 AA and a maximum of 2 tryptic missed cleavages per peptide were allowed. Results at the peptide and protein level were

filtered at a 0.01 false discovery rate (FDR). For shared peptides, only an instance assigned to the leading razor protein was considered for analysis. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium *via* the PRIDE [44] partner repository with the dataset identifier PXD044325.

2.5. Peptide ^2H enrichment and protein FSR calculation

The experimental design utilized in this study is based on the administration of a single ^2H_2O bolus. In this kinetic model, precursor (assumed to be BW) ^2H enrichment will steadily decline after reaching its maximum enrichment once mixed in BW shortly after ^2H_2O dosing. An equation (Eq. (1)) has been developed for the calculation of protein FSR when following this experimental approach. This equation has been adapted to the calculation of FSR at the peptide level following product ^2H enrichment calculation from proteomics data.

$$E = p \text{ x } n(pep) \text{ x } e^{k2 \text{ x } t} \text{ x } \left(1\text{–}e^{-k1 \text{ x } t}\right) \quad (1)$$

Where E represents peptide ^2H enrichment calculated from proteomics mass isotopologue intensity data (see below), p represents maximum precursor enrichment expressed in APE and derived as the exponent of the intercept of the regression line of the natural logarithm of BW ^2H enrichment against time for each individual pen, k_2 represents precursor enrichment elimination rate expressed as the slope of the logarithmic regression line and *n(pep)* represents the maximum apparent number of ^2H atoms that can be incorporated by each peptide, which is the sum of its component AA *n(AA)* values. Theoretical considerations for the use of the present formula as well as the calculation of *n(AA)* values used in this study have been explained elsewhere [37].

Eq. (1) allows the calculation of a protein fractional synthesis rate constant (k_1, in units of per hour) which can then be multiplied by 100 to express protein FSR in % hour^{-1}, which in turn can be converted to % day^{-1} when multiplied by 24. K_1 is calculated by the least squares method by fitting modelled data to the product enrichment data collected at different timepoints (t) post ^2H_2O administration. Product enrichment data from four birds from the same pen (with equal p and k_2), culled at different timepoints (24 h, 48 h, 96 h and an undosed bird), was used in the present study to calculate protein FSR values at the broiler pen level. The decision to use only four timepoints despite having a fifth one available (4 h) was informed by a pilot experiment in which a set of five breast muscle samples (4 h, 24 h, 48 h, 96 h and baseline) was analysed in duplicate. This pilot revealed that the amount of ^2H incorporated by muscle proteins at 4 h (indicated by the ratio between the peak area of the M_1 and M_0 isotopologues) was very low (Fig. S1B). As a result, it was judged adequate to optimize time and expense by not analysing breast muscle samples collected at the 4 h timepoint.

For the calculation of peptide enrichment (E) expressed in mole percent excess (MPE) at each timepoint, baseline peptide ^2H abundance (MP; Mole percent) needs to be subtracted from a peptide ^2H abundance at the given timepoint. Peptide MP is calculated using the formula:

$$\text{Peptide Mole\%(MP)} = \text{MPM}_1 \text{ x } 1 + \text{MPM}_2 \text{ x } 2 + \text{MPM}_3 \text{ x } 3 \quad (2)$$

Where MPM_i is determined as:

$$\text{MPM}_\text{I} = \left[\text{M}_\text{I} \Big/ \sum (\text{M}_0 \ldots \text{M}_3)\right] \text{ x } 100\% \quad (3)$$

Peptide mass isotopologue intensities were mined using Skyline (version 21.1.0.146) [45], where a spectral library was built using the results from the peptide search and proteomics raw data. The number of transitions to be displayed was set to 4 (M_0 to M_3). Mass isotopologue intensities were exported from Skyline, allowing the calculation of peptide MPE and protein FSR using a script developed in Rstudio (version 2022.7.1.554, R version 3.6) software [46]. This script, which has been named SB-FSR (Single Bolus Fractional Synthesis Rate), has been made available in Github under a CC BY-SA-4.0 license (https://github.com/Jorge-Peinado-Izaguerri/SB-FSR) along with the original metadata that was used to obtain the protein FSR values described in the Results section.

The net change of a protein pool over a particular interval is defined as the difference between its FSR and FBR (Fractional breakdown rate). On the assumption that broiler chicken weight changes are in proportion to breast muscle protein changes, we can derive breast muscle protein FBR (%, day-1) from the equation: FBR = FSR – FGR. 21 day old chicken's FGR (Fractional growth rate; %, day-1) was estimated from weight gain (body mass change).

2.6. Peptide selection and data processing

Accurate peptide ^2H enrichment, and therefore protein FSR, assessment relies on the presence of accurate and consistent raw mass isotopologue intensity data across multiple samples. As a result, it is crucial to establish a functioning quality control (QC) system capable of filtering out those peptides whose acquired signal should not be relied upon for accurate FSR calculation. Optimal raw data signals should show symmetrical and consistent Gaussian peak shape in the absence of interfering transitions, Fig. S1A displays an example of the raw data signal profile that is expected from peptides used for FSR calculation. With this aim, a number of QC parameters were used to filter peptide data using SB-FSR based on both, chromatographic and non-chromatographic properties, as described below. The general approach applied to the selection of QC parameters values was that peptides eliminated by a given parameter would consist of a higher percentage of peptides with an improperly characterized signal, acknowledging that all QC checks are likely to remove some peptides with a proper signal if they filter data effectively. Whenever an instance of a peptide, acquired at a certain timepoint, failed to fulfill any QC condition, all instances of that peptide across the sample timecourse (24 h, 48 h, 96 h and baseline), for that specific pen, were removed from the dataset. Different charge states of a peptide were treated independently.

The first QC parameter applied to the dataset was a 0.95 isotope dot product (idotp) cut-off. The idotp parameter is determined in the Skyline software and was used to compare the measured isotopic distribution of a peptide signal with its theoretical profile. This filter was set purposely coarse with the aim to effectively remove just noisy signals present in the dataset, which are likely to represent a peptide present in the spectral library but not in every sample acquired raw data. Subsequent QC filters were applied by the SB-FSR script after data was exported from Skyline. Peptides that did not include an M_3 transition were deleted from the dataset in order to make the number of transitions consistent across all peptides used for MPE calculation. It has been reported that the number of mass isotopologue peaks used affects calculation results [47], this phenomenon has also been demonstrated using data from the present study (data not shown). Peptides with an M_0 peak area intensity lower than 2×10^9 were eliminated from the dataset in an effort to remove peptides with inconsistent/noisy signal. Peptides whose coefficient of variation (CV) between the retention time (RT) of its different mass isotopologues was $>5 \times 10^{-4}$ were eliminated, aiming to remove those peptides with interfering signals or those which may have skewed peak shapes, causing response errors. At this point *n(pep)* values were calculated for each peptide. All peptides with an *n(pep)* value lower than 5 were eliminated as they were unlikely to incorporate sufficient ^2H to allow accurate FSR calculation.

Peptide ^2H enrichment and then FSR were calculated at this stage by the SB-FSR script. Peptides with calculated FSR value higher than 240% day^{-1} were eliminated from the dataset as the experimental design in this study was unlikely to allow an accurate calculation of greater protein FSR and the fact that such high values for breast muscle proteins were more likely to be the result of inaccurate mass isotopologue intensity data than of any biological phenomenon. Peptides with negative FSR values were also removed. Relative root mean square error (RRMSE) was calculated for every peptide by dividing the root mean square error (RMSE) between calculated and measured MPE values

across the sampling timecourse by the mean calculated MPE value and then multiplying the result by 100 as described in Despotovic et al. study [48]. Peptides whose RRMSE was higher than 60% (80% for Diet 1 experimental group, details in Section 3.2) were eliminated from the dataset. Lastly, baseline peptide MP was compared with a theoretical estimation of the peptide MP using natural isotopic abundances, which was facilitated by the R package enviPat. Peptides whose baseline MP was 3% higher or 5% lower than its theoretical MP were eliminated.

At this point in the analysis, peptide annotation was manually inspected and corrected in Microsoft Excel using the Uniprot database and its BLAST tool [49]. All changes made to the dataset annotation are detailed in Table S1. The final list of peptides which fulfilled all the QC criteria is shown in Table S2. An individual pen protein FSR was determined by averaging all the FSR values estimated for peptides associated with that protein. The mean FSR for each experimental group was determined by averaging protein FSR from all individual pens belonging to that group.

2.7. Statistical and functional analysis

Unpaired two-tailed *t*-tests were performed in GraphPad Prism version 10.1.1 for Windows (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) on mean protein FSR estimates between the Unchallenged and Challenged experimental groups values in Experiment 1, as well as between the Control Diet and Diet1 and Control Diet and Diet 2 in Experiment 2. Equal variance was assumed for all proteins mean FSR estimations after observing that only 7.5% of the estimations showed a significant F-test *p*-value (<0.05). Mean protein FSR was assumed to follow a normal distribution. This assumption was supported by frequency distribution analysis of individual pens and experimental group peptide FSR estimations (Fig. S2). In the instances were multiple t-tests were performed on the same dataset, q-values (*p*-values adjusted for multiple comparisons) were calculated using the False Discovery Rate (FDR) method described by Benjamini and Hochberg [50].

Protein functional classification was performed using STRING online tool version 12 [51].

3. Results

3.1. BW ^2H enrichment

One of the main differences between this study's experimental design and that of previous studies was the fact that FSR was calculated at the broiler pen level and not at the level of an individual bird. The fact that ^2H enrichment measurements (precursor and product) were collected from different individuals at each timepoint, which received a similar but not exactly equal ^2H_2O dose per unit of weight due to practical issues with administering the dose, may have introduced a degree of imprecision into the final FSR values. This issue would unlikely be a concern in larger animals whose tissues can be sampled serially. Precursor ^2H enrichment elimination plots, which are needed for the calculation of protein FSR, allow us to assess differences in dosing and ^2H_2O metabolism between individual birds and its impact on the overall pen values as well as to identify pens which were likely to produce more accurate FSR values because of a more even dose administration. For data inspection, calculated BW ^2H enrichment values (APE) were multiplied by a normalization value before drawing semi logarithmic elimination plots (Fig. 2). Normalization values for each bird were calculated by dividing each pen's mean ^2H_2O dose (g) per unit of bird body weight (kg) by each individual bird ^2H_2O dose per body weight. Regression lines derived from logarithmic elimination plots goodness of fit indicated by the square of Pearson's correlation coefficient (R^2) and the normalization values coefficient of variation (CV) were used to judge the pens in which birds had ingested similar ^2H_2O doses and were more likely to produce accurate FSR results, pens were ranked accordingly (Table S3). The pen from each experimental group

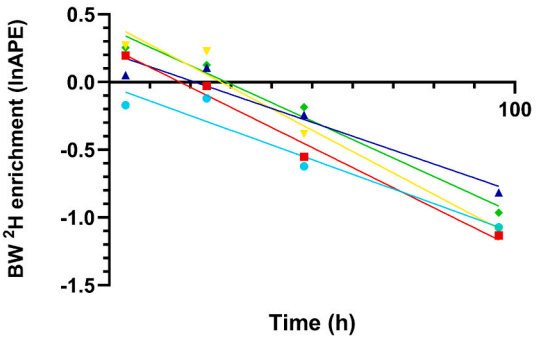

Fig. 2. BW ^2H enrichment (lnAPE) elimination plots and their resulting regression line from which p and k_2 values were derived for the best ranked pen among each experimental group. Light Blue = Unchallenged, Red = Challenged, Dark Blue = Control Diet. Yellow = Diet 1, Green = Diet 2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that received the lowest rank did not have its product enrichment analysed for protein FSR calculation. R^2 values were also used to decide which of the two available 96 h samples would be selected for proteomics analysis and FSR calculation, being chosen the one that maximised R^2 for that particular pen.

Normalized BW ^2H enrichment values were only used to select birds for proteomics analysis and not for the purpose of protein FSR calculation. Here, logarithmic BW ^2H elimination plots were constructed using raw data. The exponent of the intercept (p) and the slope (k_2) of the logarithmic regression lines derived from these plots were used for protein FSR calculation (Fig. 2).

3.2. Effect of QC parameters

MaxQuant protein search identified a total of 14,611 non-redundant peptides across the 60 analysed muscle samples. Given the fact that 15 groups (corresponding to pens) of 4 samples each (corresponding to different timepoints) were loaded into separated Skyline instances, the count of starting peptides was 219,165. 74% of these peptides were eliminated by the idotp Skyline filter. The final number of peptides exported from Skyline was 56,526. Skyline software counts different charge state instances of a peptide as the same peptide, however these were treated as individual peptides for the purpose of data filtering and FSR calculation, making the total number of peptides loaded into SB-FSR 73,394. The percentage of peptides eliminated by each QC parameter (referring to the number of remaining peptides at that stage) was: M_3 signal = 0.25%; M_0 peak area = 85%; RT CV = 5.8%; *n(pep)* = 0.01%; Minimum FSR = 2.64%; Maximum FSR = 0.65%; RRMSE = 10.59%; baseline MP high = 2.43%; baseline MP low = 3.31%; The total number of peptides that fulfilled all the QC criteria was 8442, an average of 563 peptides per pen.

Fig. 3 shows that the goodness of fit (indicated by RRMSE) between measured and calculated peptide MPE was worse than the average for two out of three pens in the Diet 1 experimental groups. If the general RRMSE cut off value of 60 would have been applied to this experimental group, it would have severely limited the number of peptides from this experimental group in the final dataset and therefore the comparison between this experimental group results with others. This observation motivated the use of a coarser RRMSE filter for this experimental group, however data interpretation needs to take into account this phenomenon. The mean percentage of peptides eliminated by the RRMSE filter (80) in Diet 1 pens (3 pens) was 12.35% which, even with a higher cut off value, was higher than the mean percentage of peptides eliminated from the rest of the dataset (12 pens) at 10.16%.

Despite the baseline MP filter being skewed, the number of peptides eliminated with a MP value below the limit was still higher than the

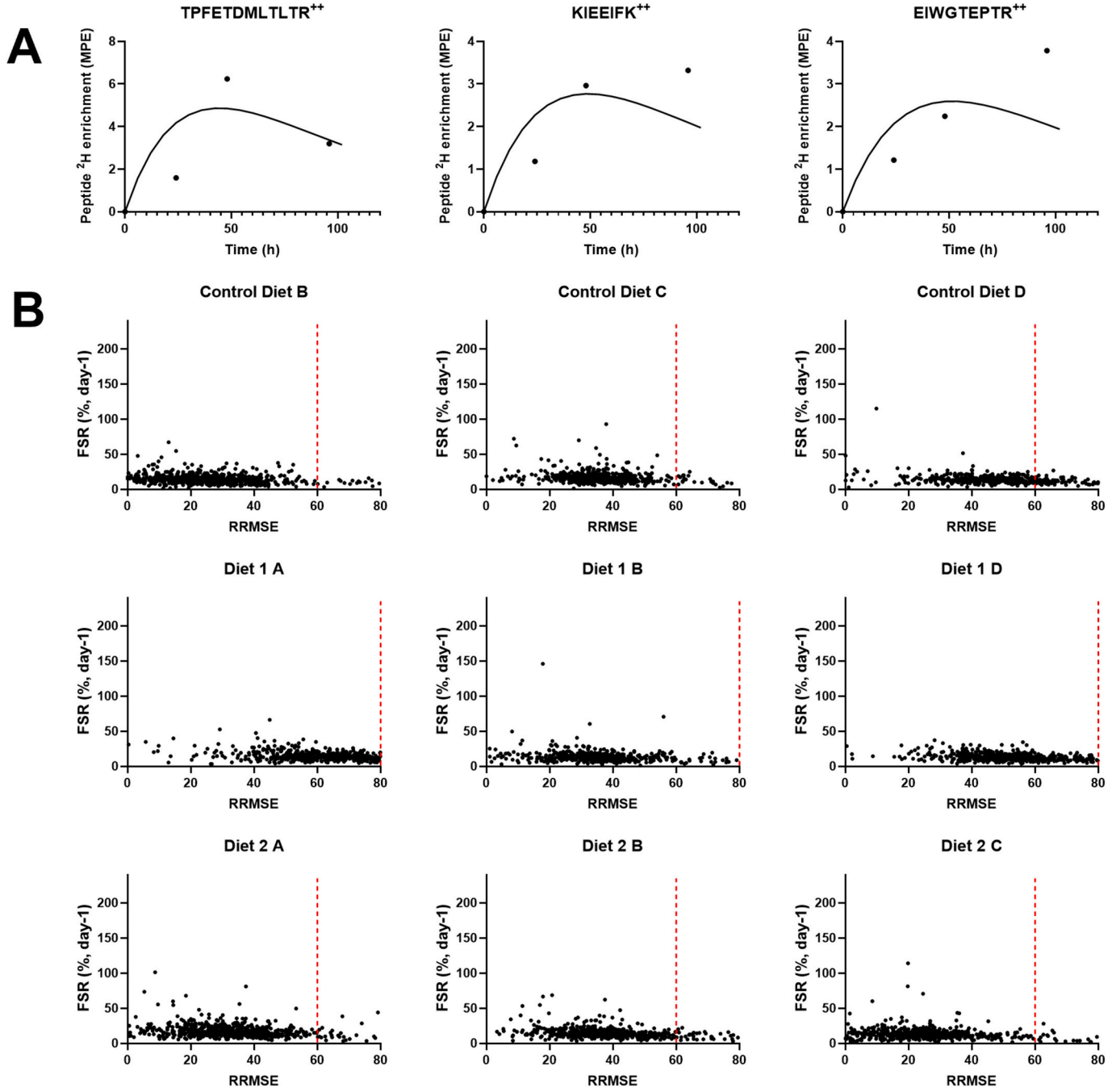

Fig. 3. RRMSE filter effect. A) Calculated (line) and measured (dots) peptide ^{2}H enrichment for three different peptides from pen "Diet 1 A". Peptide RRMSE was (left to right): 20.86, 43.22, 58.11. B) Peptide RRMSE plotted against FSR for all peptides from pens in experiment 2 which fulfilled all the QC criteria (before RRMSE filter was applied). Red dotted line represents the RRMSE cut off applied to each pen dataset. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

number of peptides eliminated above this filter. This provides support for the decision to use an unequal filter as well as evidence that the Orbitrap MS may tend to underestimate the natural ^{2}H abundance in peptides [47].

3.3. *Effect of an immune challenge in breast muscle protein FSR*

Table 1 shows the mean protein FSR calculated for breast muscle proteins in Experiment 1 groups ordered by the total number of peptides which had their FSR estimated across all available pens (sum of the peptides which had their FSR calculated from each pen). Proteins whose FSR was not estimated in all 6 analysed pens have not been reported in Table 1. Every mean protein FSR value calculated at the individual pen level in experiment 1 can be found in Table S4.

Exemplar mass spectrometry data, showing mass isotopomer distribution, from three peptides that had their FSR estimated is provided on Fig. S3. One peptide belonging to "Myosin heavy chain, skeletal muscle, adult (1F)" (protein with the higher number of peptide FSR estimates in the dataset), one peptide belonging to "Actin, alpha skeletal muscle" (protein with the lowest FSR value in the Unchallenged group) and one additional peptide belonging to Albumin (protein with the highest FSR value in the Unchallenged group). Mass spectrometry data from the four samples (Baseline, 24 h, 48 h and 96 h) that were used for FSR calculation for each of the three biological replicates in experimental groups

Table 1
List of breast muscle proteins FSR estimated for each experimental group in experiment 1. FSR estimates are reported as the mean ± standard deviation of that protein FSR value estimated for three replicate pens. FSR $\log_2$ fold change between Unchallenged and Challenged group is also reported as well as the p-value and q-value resulting from an FDR adjusted unpaired t-test ($n = 3$) performed between both experimental groups FSR estimations.

Protein Description	Protein Accession	Gene Name	Total peptides	Unchallenged FSR (%, day^{-1})	Challenged FSR (%, day^{-1})	Fold Change ($\log_2$)	p_value	q_value
Myosin heavy chain, skeletal muscle, adult (1F)	A0A1D5P603	MYH1F	683	15.5 ± 2.2	12.0 ± 1.5	−0.36	0.088	0.164
Myosin heavy chain, skeletal muscle, adult (1E)	A0A1D5NYC2	MYH1E	385	21.3 ± 3.2	15.7 ± 1.2	−0.43	0.048	0.137
Actin, alpha skeletal muscle	P68139	ACTA1	224	12.8 ± 1.4	9.7 ± 1.4	−0.42	0.049	0.137
Pyruvate kinase	A0A1D5P9V0	PKLR	135	17.5 ± 2.2	13.2 ± 1.8	−0.4	0.06	0.141
Alpha-actinin-2	P20111	ACTN2	129	23.2 ± 3	16 ± 2.2	−0.54	0.028	0.132
Glyceraldehyde-3-phosphate dehydrogenase	P00356	GAPDH	122	15.4 ± 1.6	11.2 ± 0.8	−0.45	0.014	0.105
L-lactate dehydrogenase	E1BTT8	LDHA	108	18.3 ± 5.6	11.6 ± 1.5	−0.67	0.114	0.195
Triosephosphate isomerase	P00940	TPI1	106	17.2 ± 2	13 ± 1.4	−0.42	0.037	0.137
Tropomyosin alpha-1 chain	P04268	TPM1	104	16.1 ± 2.5	10.7 ± 2.1	−0.6	0.045	0.137
Beta-enolase	P07322	ENO3	103	16.5 ± 2.1	11.1 ± 0.5	−0.58	0.013	0.105
Creatine kinase M-type	P00565	CKM	85	17.9 ± 2.7	14.6 ± 0.5	−0.3	0.105	0.188
Phosphoglycerate kinase	F1NU17	PGK2	80	18 ± 2.1	12.8 ± 1	−0.49	0.019	0.105
Phosphoglycerate mutase 1	Q5ZLN1	PGAM1	75	16.5 ± 2.7	11.9 ± 1.2	−0.47	0.053	0.137
Myosin regulatory light chain 2, skeletal muscle isoform	P02609	MYLPF	74	16.1 ± 1.2	11.8 ± 0.7	−0.45	0.007	0.105
Glucose-6-phosphate isomerase	A0A1L1RQ91	GPI	68	16.6 ± 3.3	13.4 ± 3	−0.3	0.286	0.340
Phosphoglucomutase 1	F1NN63	PGM1	62	20.6 ± 5.4	14.3 ± 1.5	−0.54	0.121	0.199
Myosin light chain 1, skeletal muscle isoform	P02604	MYL1	60	18.8 ± 1.2	13.1 ± 1.5	−0.54	0.007	0.105
Fast skeletal muscle troponin T isoform	Q7ZZH5	TNNT3	55	25.4 ± 3.7	17.4 ± 2.4	−0.56	0.035	0.137
Alpha-1,4 glucan phosphorylase	A0A3Q3AC33	LOC107049660	47	21.4 ± 3.4	18.7 ± 1.3	−0.2	0.267	0.332
Myosin-binding protein C, fast-type	P16419	MYBPC2	43	19.6 ± 1.6	17.1 ± 0.4	−0.12	0.158	0.242
Troponin C, skeletal muscle	P02588	TNNC2	38	20 ± 2.6	17.5 ± 4	−0.18	0.424	0.479
Albumin	P19121	ALB	36	46.5 ± 12.4	37.2 ± 1.8	−0.32	0.268	0.332
Troponin I, fast skeletal muscle	P68246	TNNI2	34	20.5 ± 1.5	16.9 ± 2.2	−0.27	0.08	0.163
ATP-dependent 6-phosphofructokinase	Q90YA3	pfk	29	38.6 ± 9.6	30.2 ± 5.1	−0.36	0.253	0.332
Fructose-bisphosphate aldolase	R4GM10	ALDOC	29	17.9 ± 2.1	14.5 ± 1.5	−0.3	0.088	0.164
Glycerol-3-phosphate dehydrogenase [NAD(+)]	A0A3Q8WI14	GPD1	29	29.7 ± 14.4	19.03 ± 1.1	−0.64	0.27	0.332
Elongation factor 1-alpha	F1N9H4	EEF1A2	23	27.1 ± 6.7	17 ± 1.5	−0.67	0.064	0.141
Alpha-1,4 glucan phosphorylase	E1BSN7	PYGB	22	26.2 ± 5.6	18.9 ± 5.5	−0.47	0.183	0.262
Calsequestrin	A0A3Q2TXF6	CASQ2	21	19.7 ± 5.5	19.8 ± 5.9	0.01	0.977	0.976
Fructose-bisphosphate aldolase (Fragment)	Q92007	ALDOA	18	16.2 ± 5.1	14.3 ± 0.6	−0.18	0.563	0.605
Fructose-bisphosphatase	A0A1D5PN46	FBP2	17	36.7 ± 11.3	16.3 ± 4.9	−1.18	0.046	0.137
Myosin heavy chain, skeletal muscle, adult	P13538	MYH1A	15	24.9 ± 4	18.3 ± 1.6	−0.45	0.054	0.137
Adenylate kinase isoenzyme 1	P05081	AK1	14	15.4 ± 6.7	12.6 ± 4.4	−0.29	0.582	0.610
Fructose-bisphosphate aldolase (Fragment)	Q7LZE8	LOC107050559	12	25.2 ± 3.6	14.9 ± 3.1	−0.76	0.02	0.105
Myosin heavy chain, skeletal muscle, adult (1G)	F1P3X1	MYH1G	11	17.3 ± 5	14.6 ± 4.8	−0.25	0.535	0.589
Nebulin (Fragment)	Q9DEG4	NEB	10	17.5 ± 2.4	15.1 ± 1.2	−0.22	0.183	0.262
Myosin, heavy chain 7B, cardiac muscle, beta	A0A1D5NZY9	MYH7B	9	18.2 ± 1.5	13.5 ± 0.6	−0.43	0.008	0.105
2-phospho-D-glycerate hydro-lyase	A0A1L1RQQ0	ENO1	6	16.8 ± 1.5	10.3 ± 4.2	−0.71	0.066	0.141
GOLD domain-containing protein	Q5ZIR6	TMED10	6	23.1 ± 9.4	12.9 ± 8.1	−0.84	0.228	0.316
Heat shock cognate 71 kDa protein	A0A1D5PYK0	HSPA8	6	21.4 ± 1.8	24 ± 8.7	0.16	0.636	0.651
Myosin light chain 3, skeletal muscle isoform	P02605	MYL1	6	26.1 ± 5.2	21.4 ± 6.6	−0.29	0.387	0.449
Tropomyosin 3	A0A1D5PV56	TPM3	6	19.9 ± 5.6	13.7 ± 1	−0.54	0.132	0.210
Tropomyosin 4	F1NK75	TPM4	6	14.3 ± 1.9	10 ± 0.1	−0.51	0.019	0.105

"Unchallenged" and "Challenged" is provided. Visual representation of the MPE values calculated from proteomics data at each measured timepoint as well as the continuous MPE values calculated using the fitted K_1 value for the same peptides has been generated by the SB-FSR script and can be inspected in Fig. S4.

Among the 43 proteins whose FSR was estimated, 41 (95%) proteins showed a decrease in their FSR in the presence of an LPS challenge. This negative change in protein FSR was judged significant by an unpaired *t*-test (p-value <0.05) for 15 proteins. Fig. 4 visually summarises this information in a volcano plot.

STRING online tool identified two major local network clusters in which most proteins whose FSR was estimated in this experiment, and whose identifier was recognised by STRING, were grouped (Fig. 5). 14 proteins were assigned to the "Mixed incl. Contractile fiber and Muscle protein" (Cluster 1) cluster while 14 proteins were assigned to the "Carbon metabolism and Starch and sucrose metabolism" (Cluster 2). Mean FSR for Cluster 2 was 21.26 (%, day^{-1}) for the Unchallenged group and 15.01 (%, day^{-1}) for the Challenged group, as calculated by averaging the mean Cluster 2 FSR from the three pens in each group. The calculation of a mean FSR for Cluster 1 needs to take into account the notable difference in concentration between some of the proteins present in this cluster. First a mean myosin heavy chain (MYHC) FSR was

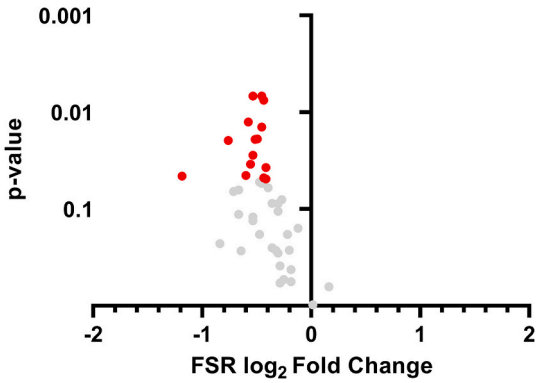

Fig. 4. Differences in breast muscle proteins FSR (%, day^{-1}) expressed as log_2(Fold change) between the Unchallenged and Challenged experimental groups. *p*-values were obtained from an unpaired two-tailed *t*-test ($n = 3$) and values below 0.05 are labelled in red as significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

calculated by averaging the FSR of MYHC 1F, 1E, 1G and 7B, then a weighed mean was performed were MYHC had 35.9% proportion, α-actin had 21.9% proportion and the rest of the proteins in the cluster had 42.2% [52], this calculation was performed at the pen level. Mean Cluster 1 FSR was 17.17 (%, day^{-1}) for the Unchallenged group and 13.10 (%, day^{-1}) for the Challenged group. The change in both clusters mean FSR between both groups was judged significant by a *t*-test with *p*-values of 0.034 for Cluster 1 and 0.005 for Cluster 2 (Fig. 6).

Broiler FGR was determined for each pen at each timepoint from the bird's weight recorded at the time of ^2H_2O dosing and at the time they were culled. Cluster 1 mean protein FSR was assumed to represent mean breast muscle protein FSR for the calculation of broiler breast muscle protein FBR. Results from these calculations are showed in Table 3.

3.4. Effect of two novel feed compounds in breast muscle protein FSR

Breast muscle proteins mean FSR values calculated for experimental groups in Experiment 2 using are shown in Table 2. Proteins which did not have its FSR estimated in all experimental pens are not reported in Table 2. Individual pen's mean protein FSR values are showed in Table S5.

Out of 38 proteins, only 1 protein showed a significant change in FSR compared to the Control Diet value in the Diet 1 and Diet 2 groups. Only 7 proteins showed a positive protein FSR fold change in Diet 1 and 14 proteins in Diet 2, however fold change values were modest overall. Proteins were assigned to the same STRING functional local network clusters described in Section 3.3 (Fig. 5). 11 proteins were assigned to Cluster 1 while 13 proteins were assigned to Cluster 2. Mean FSR for Cluster 1 was calculated as previously described but including only MYHC 1F and 1E for MYHC FSR calculation. Cluster 1 mean FSR values were 14.54 (%, day^{-1}) for the Control Diet group, 13.53 (%, day^{-1}) for the Diet1 and 14.01 (%, day^{-1}) for Diet2. Mean FSR for Cluster 2 was 17.31 (%, day^{-1}) for Control Diet, 15.87 (%, day^{-1}) for Diet 1 and 16.84 (%, day^{-1}) for Diet 2. These cluster mean FSR showed similar values and were not judged significant upon performing a *t*-test (Fig. 6). Broiler FGR values as well as mean breast muscle protein FBR were calculated as previously described and are shown in Table 3.

4. Discussion

A panel of broiler chicken breast muscle protein FSRs has been reliably estimated in the present study following a recently developed single ^2H_2O bolus labelling approach optimised for the study of free-living animals. The calculation of these FSR values has been facilitated by a newly developed dynamic proteomics bioinformatic pipeline integrating freely available software. The study has shown that an acute *E.coli* LPS immunological challenge causes breast muscle protein's FSR to uniformly decrease over a period of 96 h. Neither citrus nor cucumber extracts supplemented in the feed were shown capable of alleviating the effect of the immune challenge on breast muscle protein FSR at the concentration used in this study.

The ^2H_2O labelling methodology and experimental design used in this study contains some limitations which have been described elsewhere **[37]**, results from the present study provide new information which help further characterising these limitations. Timepoints selected for data collection in the present study were not fully optimised for the calculation of muscle protein FSR, as shown by the lack of significant tracer incorporation in muscle samples collected 4 h post ^2H_2O administration. However, the lack of data collected at an early timepoint is unlikely to have impacted muscle protein FSR estimation, due to their relatively slow turnover. The absence of early timepoints mostly limits the accuracy of fast turnover protein's FSR (FSR $\geq$ 240% day^{-1}), which was the rationale for the inclusion of a maximum FSR QC criterion. The maximum FSR QC deleted only 0.65% of the remaining peptides at that stage of the QC process, a small number of peptides whose high FSR estimates were most likely derived from mathematical errors caused by inaccuracy in their isotopic abundance measurements and not representative of broiler muscle biology. A different characteristic of the experimental design used in this study was the decision to estimate FSR at pen level instead of at an individual bird level. This feature is a necessary compromise that facilitates the study of protein dynamics in certain small free living animal tissues maximizing animal welfare by avoiding the collection of serial biopsies and is facilitated by broiler chicken's flock homogeneity. A consistent pattern was identified among peptides whose calculated and measured MPE differed the most (evaluated by the RRMSE QC parameter), many of these peptides showed a higher measured MPE compared to that calculated for the 96 h timepoint (Fig. 3A). This phenomenon may be the result of the fitted pen BW ^2H_2O enrichment dropping below the precursor enrichment of the bird culled at the 96 h timepoint. Alternatively, tracer recycling, which may become noticeable 96 h post ^2H_2O administration **[37]**, could also explain this observation. Poor overall peptide RRMSE observed in two of the pens from the Diet 1 group might be due to these phenomena which could have impacted the FSR values calculated from these pens. While this observation should be considered when interpreting the data, the overall MPE fit was regarded to be robust and FSR values calculated for these pens were regarded close to reality. The fact that this phenomenon was noticeable in two pens from the same experimental group is unlikely to be linked with the experimental group itself and more likely to have happened by chance. Future studies in muscle dynamic proteomics performed in larger animals may involve taking serial samples from the same individual, these studies should not observe this phenomenon occurring while studies similar to the one described here could ameliorate this phenomenon by collecting additional samples at earlier timepoints.

The bioinformatics pipeline applied in this study was originally developed using the freely available software Skyline paired with the SB-FSR script. This pipeline implements a strict QC process to ensure the reliability of protein FSR estimates, evidenced by the overall goodness of fit observed in our peptide calculated MPE estimates (Fig. 3B). This bioinformatic pipeline could be easily replicated in future studies aiming to study dynamic proteomics using a single ^2H_2O bolus experimental design, it could also be readily adapted to be applied in experiments following a plateau labelling approach. The necessity to ensure FSR estimate's reliability by implementing a QC process that warrants a peptide MPE close to that fitted across multiple samples is a reason for the number of proteins reported in this study being lower than conventional chicken breast muscle proteomics studies **[10]**. The fact that chicken breast muscle shows a great dynamic range solely in its sarcoplasmic fraction **[3]** and the fact that the present study aimed to characterize protein FSR in both fractions, myofibrillar and sarcoplasmic,

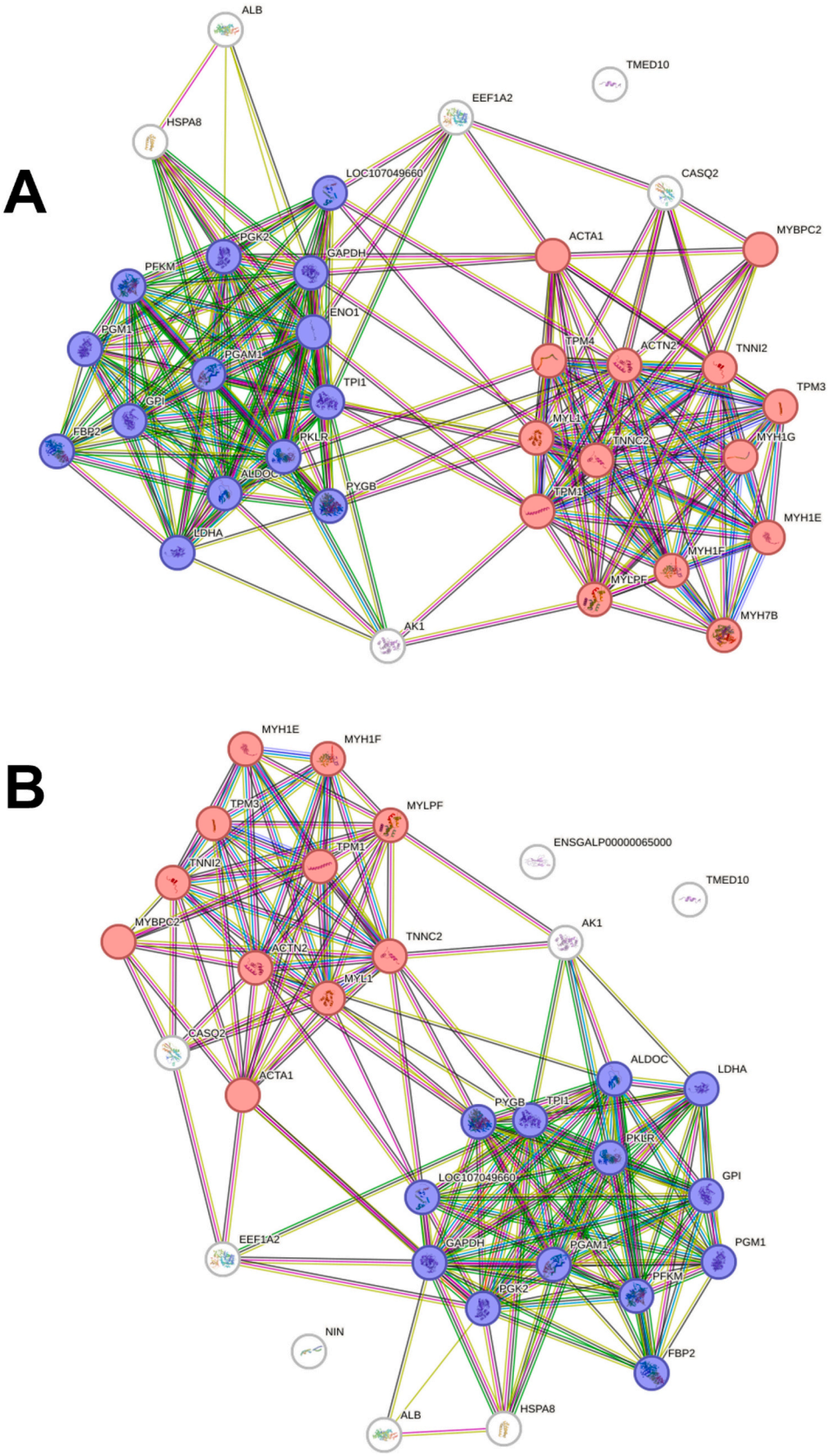

Fig. 5. Experiment 1 (A) and experiment 2 (B) STRING protein-protein interaction network. Nodes colored in red were assigned to the local network cluster "Mixed incl. Contractile fiber and Muscle protein" (Cluster 1) while nodes colored in blue were assigned to the local network cluster "Carbon metabolism and Starch and sucrose metabolism" (Cluster 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

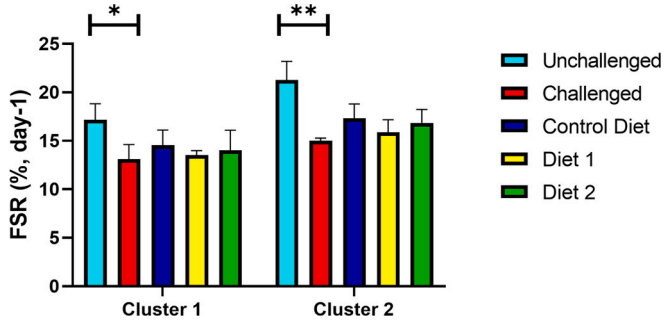

Fig. 6. Cluster 1 (Mixed incl. Contractile fiber and Muscle protein) and cluster 2 (Carbon metabolism and Starch and sucrose metabolism) mean protein FSR (%, day^{-1}) for each experimental group. t-test (n = 3) were performed between Unchallenged and Challenged data as well as between Control Diet and Diet 1 and Control Diet and Diet 2* = 0.05 > p-value >0.01; ** = 0.01 > p-value.

contribute to the reduced number of reported FSR values. Nevertheless, the most abundant enzyme proteins in the sarcoplasmic fraction reported by Doherty and coworkers [3], β-enolase, glyceraldehyde 3-phosphate dehydrogenase, lactate dehydrogenase, triosephosphate isomerase, phosphoglycerate mutase, creatine kinase, pyruvate kinase and phosphoglycerate kinase, had their FSR calculated in the present study. Future studies could improve the number of protein FSR estimates by focusing on the sarcoplasmic fraction of breast muscle or by depleting its most abundant proteins, improving the likelihood to estimate FSR for less abundant proteins such as those present in the mitochondria.

More than >43 breast muscle proteins had their FSR calculated in this study. Calculated FSR values were very similar across all breast muscle proteins with the vast majority of the proteins in the Unchallenged group (76.74%) showing FSR values between 15 and 25%, day^{-1} and proteins in the groups challenged with LPS (88.37%) showing lower values between 10 and 20%, day^{-1}. These FSR values are in line with values reported in the literature for growing broiler chickens (21 days-old) mixed breast muscle protein FSR [5,18,20,53,54], usually between 10 and 30% day^{-1}, consistency across different breast muscle protein FSR values may be unsurprising when accounting for broiler breast muscle tissue specialization [3]. Moreover, it has been shown that an acute immune challenge causes a coordinated and uniformed decrease in breast muscle protein FSR as evidenced here by the asymmetrical Volcano plot displayed in Fig. 4. When interpreting the statistical significance of individual proteins FSR reduction reported in this study following an immune challenge, it is necessary to consider that q-values for these proteins showed values between 0.105 and 0.141. Correcting for multiple test comparisons is good statistical practice when 43 different *t*-tests are being conducted in the same dataset [55], as 2.15 of the *p*-values calculated by those t-tests are expected to be below 0.05 just by chance. Individual proteins that showed a significant reduction in their FSR would be considered a discovery by an FDR corrected t-test if the FDR would be set at a minimum of 15%, meaning that at most 15% of the significant findings (2.25 out of 15) are mistakenly concluded to be true positives. Therefore, it is important to point out some degree of uncertainty regarding the statistical significance of these individual FSR changes and that none of them would be considered a discovery shall a more conservative 5% FDR cut-off be applied.

Proteins whose FSR values have been reported in this study have been functionally classified in two different clusters that correspond closely with myofibrillar (Cluster 1) and sarcoplasmic (Cluster 2) proteins, both clusters showed a similar reduction in their mean FSR in response to an acute LPS challenge. These observations contradict the results reported by Barnes and coworkers [23] who showed a (non-significant) increase in broiler chicken mixed breast muscle protein FSR following an *E.coli* LPS challenge, attributing the muscle wasting observed during the acute phase response (APR) to an increase in muscle protein FBR. Moreover, estimates reported in this study show that mixed breast muscle protein FSR is significantly reduced during an immune challenge while the reduction in protein FBR is not significant. These results suggest that broiler chicken muscle wasting observed during stress conditions can be attributed mainly to a reduction of protein synthesis rather than an increased breakdown. It has been hypothesised that muscle wasting associated with infection in mammals was due to an increased demand of aromatic AAs for the purpose of APP synthesis [56,57], a recent study on heat-stressed broiler chickens breast muscle metabolome suggested that breast muscle AAs can be reprioritized away from muscle growth to provide substrates to support amino acid and energy metabolism during periods of reduced nutrient intake or disease [12]. The results presented in this study support these observations and provide new information that could serve future studies aiming to develop new strategies to maximize chicken growth performance or to characterize and minimize the economic cost of disease and inflammation in broiler chicken production systems.

The citrus and cucumber extracts which were added to broiler chicken diets in groups Diet 1 and Diet 2 respectively, did not show any effect on breast muscle protein FSR of immunologically challenged chickens at the concentration used in this study. Individual protein FSR fold changes were low and non-significant, moreover mean FSR calculated for Cluster 1 and Cluster 2 proteins reinforced this observation as well as the calculated FGR values. However, the immunological challenge assayed in the present study is an acute insult which was triggered by an injection of *E.coli* LPS, hence avoiding the gut, a location where feed supplements may exert a good portion of their effect. Citrus and cucumber extracts may be able to show a protective effect against different stresses such as viable pathogens or when added to chicken diets at a higher concentration. Future studies might be able to observe anti-inflammatory effects derived from the supplementation of these extracts following the analysis of chicken plasma or intestinal dynamic proteome.

The coordinated decrease in breast muscle protein FSR following an immune challenge observed in the present study suggest that many of the proteins which have shown a significant decrease in their FSR are good candidates as markers of breast muscle status. Myosin regulatory light chain 2 and myosin light chain 1 were the two myofibrillar proteins with the most significant decrease in their FSR (p value = 0.007) while β-enolase showed the most significant FSR reduction among the sarcoplasmic proteins. A good biomarker should be readily measured, as a result, the best candidate for a breast muscle biomarker of disease should ideally be measurable in an accessible pool such as plasma, hence avoiding the necessity to collect less-accessible muscle biopsies. Shankaran and coworkers [58] proposed creatine kinase M and carbonic anhydrase 3 as markers of muscle status from human and mouse plasma samples. Carbonic anhydrase FSR was not estimated in the current study while creatine kinase M showed a non-significant FSR reduction following an immune challenge. However, β-enolase is a glycolytic enzyme which plays an important role in glycolytic fast-twitch breast muscle fibers energy metabolism. Future studies aiming to characterize broiler chicken's plasma dynamic proteome have the potential to estimate β-enolase FSR, to confirm the hypothesis that β-enolase is a dynamic marker of muscle status in broiler chickens. Albumin is a negative APP in broiler chickens [59] whose FSR has been estimated in the present study from muscle biopsies despite of it being a plasma protein synthetized in the liver. Its 20% FSR reduction following an immune challenge is in line with the 23.7% FSR reduction observed in mixed breast muscle protein. Estimations of albumin FSR in future chicken plasma dynamic proteomics studies could be compared with albumin FSR values reported in this study. If a good agreement is found, albumin FSR could be established as an accessible and readily measurable marker of broiler chicken muscle metabolism.

Table 2
List of breast muscle proteins FSR estimated for each experimental group in experiment 2. FSR estimates are reported as mean ± standard deviation of that protein FSR value estimated for three replicate pens. FSR $\log_2$ fold change as well as the *p*-value and q-value resulting from an FDR adjusted unpaired t-test ($n = 3$) are reported for Diet 1 and Diet 2 experimental groups compared with the Control Diet group.

				Control Diet	*Diet 1*				*Diet 2*			
Protein Description	Protein Accession	Gene Name	Total peptides	FSR (%, day^{-1})	FSR (%, day^{-1})	Fold Change (log_2)	p_value	q_value	FSR (%, day^{-1})	Fold Change (log_2)	p_value	q_value
Myosin heavy chain, skeletal muscle, adult (1F)	A0A1D5P603	MYH1F	1053	14.1 ± 2	12.1 ± 0.4	−0.22	0.167	0.679	12.5 ± 2.5	−0.17	0.438	0.986
Myosin heavy chain, skeletal muscle, adult (1E)	A0A1D5NYC2	MYH1E	597	16.3 ± 1.3	15.5 ± 0.7	−0.07	0.398	0.799	16.2 ± 1.9	0	0.98	0.996
Actin, alpha skeletal muscle	P68139	ACTA1	339	10.6 ± 0.6	9.7 ± 0.3	−0.12	0.07	0.674	10.1 ± 1.4	−0.07	0.594	0.986
Pyruvate kinase	A0A1D5P9V0	PKLR	205	14.2 ± 0.8	14.5 ± 3.3	0.03	0.888	0.914	13.4 ± 1.3	−0.07	0.429	0.986
Alpha-actinin-2	P20111	ACTN2	198	17.5 ± 2.7	17.1 ± 0.9	−0.04	0.785	0.914	17 ± 2.3	−0.04	0.806	0.986
Glyceraldehyde-3-phosphate dehydrogenase	P00356	GAPDH	186	11.7 ± 1.7	11.5 ± 1.3	−0.03	0.835	0.914	12 ± 0.8	0.03	0.853	0.986
L-lactate dehydrogenase	E1BTT8	LDHA	161	14.6 ± 0.9	12.5 ± 1.5	−0.23	0.095	0.679	13 ± 0.9	−0.17	0.093	0.986
Triosephosphate isomerase	P00940	TPI1	159	14.7 ± 0.2	13.8 ± 3	−0.09	0.651	0.884	13.9 ± 1.2	−0.07	0.325	0.986
Tropomyosin alpha-1 chain	P04268	TPM1	158	12.8 ± 1.8	11 ± 0.6	−0.22	0.179	0.679	12.1 ± 2.1	−0.09	0.664	0.986
Beta-enolase	P07322	ENO3	146	12.6 ± 1	12.9 ± 0.7	0.03	0.678	0.884	13.6 ± 1.6	0.11	0.42	0.986
Creatine kinase M-type	P00565	CKM	123	15.5 ± 1.9	14.6 ± 3.4	−0.09	0.717	0.908	14.9 ± 1.4	−0.06	0.672	0.986
Myosin regulatory light chain 2, skeletal muscle isoform	P02609	MYLPF	116	13.8 ± 2.4	12.8 ± 1.4	−0.1	0.56	0.875	12.8 ± 2.3	−0.1	0.657	0.986
Phosphoglycerate mutase 1	Q5ZLN1	PGAM1	110	13.9 ± 2.4	14.1 ± 1	0.03	0.891	0.914	14.0 ± 2	0.01	0.953	0.996
Phosphoglycerate kinase	F1NU17	PGK2	103	13.3 ± 1.6	12.5 ± 2.4	−0.09	0.659	0.884	14.1 ± 2.2	0.08	0.629	0.986
Glucose-6-phosphate isomerase	A0A1L1RQ91	GPI	96	15.5 ± 2.2	13.7 ± 2.2	−0.17	0.378	0.799	14.5 ± 2.1	−0.1	0.587	0.986
Myosin light chain 1, skeletal muscle isoform	P02604	MYL1	91	14.8 ± 1.4	13.3 ± 0.7	−0.15	0.16	0.679	14.6 ± 1.6	−0.03	0.842	0.986
Myosin-binding protein C, fast-type	P16419	MYBPC2	81	17.2 ± 1.2	17.9 ± 1.6	0.06	0.576	0.875	17.2 ± 3.7	0	0.981	0.996
Fast skeletal muscle troponin T isoform	Q7ZZH5	TNNT3	79	19.4 ± 2.2	18.4 ± 0.8	−0.07	0.495	0.817	21.2 ± 2.7	0.12	0.438	0.986
Phosphoglucomutase 1	F1NN63	PGM1	73	15.1 ± 3	14.2 ± 1.6	−0.09	0.669	0.884	16.9 ± 1.5	0.16	0.413	0.986
Troponin C, skeletal muscle	P02588	TNNC2	62	19.5 ± 3.5	17.3 ± 0.9	−0.17	0.367	0.799	20.5 ± 3.3	0.07	0.744	0.986
ATP-dependent 6-phosphofructokinase	Q90YA3	pfk	60	32.8 ± 19.3	23.3 ± 8	−0.49	0.476	0.817	29.6 ± 4.8	−0.15	0.795	0.986
Alpha-1,4 glucan phosphorylase	A0A3Q3AC33	LOC107049660	55	24.5 ± 6.3	17.5 ± 2.8	−0.49	0.151	0.679	20.6 ± 5.9	−0.25	0.474	0.986
Titin	A0A3Q2TS15	TTN	50	32.4 ± 16.9	17.2 ± 7.9	−0.92	0.231	0.729	17.1 ± 2.5	−0.92	0.197	0.986
Fructose-bisphosphate aldolase	R4GM10	ALDOC	48	14.6 ± 1.8	14.3 ± 0.9	−0.03	0.823	0.914	15.1 ± 0.6	0.04	0.675	0.986
Glycerol-3-phosphate dehydrogenase [NAD(+)]	A0A3Q8WI14	GPD1	47	21.2 ± 5.8	18.5 ± 1.4	−0.2	0.484	0.817	22.9 ± 5.7	0.11	0.732	0.986
Troponin I, fast skeletal muscle	P68246	TNNI2	47	18.1 ± 2.7	17.9 ± 1.3	−0.01	0.914	0.914	17.7 ± 2.8	−0.03	0.883	0.986
Calsequestrin	A0A3Q2TXF6	CASQ2	38	12.2 ± 1.5	15.3 ± 2.2	0.33	0.113	0.679	15.5 ± 6.1	0.36	0.407	0.986
Elongation factor 1-alpha	F1N9H4	EEF1A2	33	15.7 ± 2.8	13.3 ± 2.1	−0.23	0.303	0.799	15.4 ± 2.4	−0.03	0.881	0.986
Alpha-1,4 glucan phosphorylase	E1BSN7	PYGB	32	20.2 ± 4.6	17.9 ± 2.4	−0.17	0.486	0.817	20.2 ± 5.4	0	0.996	0.996
Fructose-bisphosphatase	A0A1D5PN46	FBP2	27	20 ± 6.1	26.5 ± 8.4	0.4	0.34	0.799	21.8 ± 2.2	0.12	0.653	0.986
Fructose-bisphosphate aldolase (Fragment)	Q92007	ALDOA	27	16.9 ± 0.5	15.7 ± 2	−0.1	0.36	0.799	15 ± 0.5	−0.17	0.01	0.393

(*continued on next page*)

Table 2 (*continued*)

				Control Diet	*Diet 1*				*Diet 2*			
Protein Description	Protein Accession	Gene Name	Total peptides	FSR (%, day^{-1})	FSR (%, day^{-1})	Fold Change (log_2)	p_value	q_value	FSR (%, day^{-1})	Fold Change (log_2)	p_value	q_value
Adenylate kinase isoenzyme 1	P05081	AK1	23	13.8 ± 2.3	11.2 ± 2.7	−0.3	0.282	0.799	15.5 ± 3	0.18	0.462	0.986
Myosin heavy chain, skeletal muscle, adult	P13538	MYH1A	23	20.3 ± 2.2	18.3 ± 1	−0.15	0.217	0.729	21.4 ± 3.1	0.08	0.622	0.986
Fructose-bisphosphate aldolase (Fragment)	Q7LZE8	LOC107050559	17	20.4 ± 1.4	17.9 ± 0.8	−0.2	0.053	0.674	19.1 ± 0.9	−0.09	0.25	0.986
EF-hand domain-containing protein	A0A3Q2UDH6	NIN	9	21.5 ± 0.7	18.1 ± 1.2	−0.25	0.014	0.534	20.6 ± 7.1	−0.06	0.838	0.986
Heat shock cognate 71 kDa protein	A0A1D5PYK0	HSPA8	9	16.5 ± 2.6	15.7 ± 3.1	−0.07	0.751	0.914	17.3 ± 4	0.06	0.8	0.986
Myosin light chain 3, skeletal muscle isoform	P02605	MYL1	9	28.4 ± 5.6	20.1 ± 0.6	−0.49	0.064	0.674	21.9 ± 3	−0.38	0.154	0.986
Tropomyosin 3	A0A1D5PV56	TPM3	9	14.9 ± 3.2	15.2 ± 2	0.03	0.891	0.914	14.3 ± 2.1	−0.06	0.801	0.986

Table 3
Experimental group FGR, muscle protein FSR and muscle protein FBR (all expressed in %, day^{-1}) mean ± standard deviation. T-test (n = 3) were performed between Unchallenged and Challenged data as well as between Control Diet and Diet 1 and Control Diet and Diet 2. * = 0.05 > p-value >0.01; ** = 0.01 > p-value.

	Experiment 1		*Experiment 2*		
	Unchallenged	Challenged	Control Diet	Diet 1	Diet 2
FGR (4 h)	11.7 ± 4.5	−15 ± 3.5 (**)	−14.4 ± 5.8	−9.5 ± 2.9	−10.3 ± 6.4
FGR (24 h)	8.4 ± 0.5	−0.1 ± 2.2 (**)	0. ± 2.3	−0.4 ± 0.7	−1.3 ± 1.5
FGR (48 h)	9 ± 0.7	5.6 ± 0.7 (**)	6.4 ± 1	5.5 ± 0.6	4.6 ± 1.3
FGR (96 h)	8.4 ± 0.5	6.2 ± 0.8 (*)	7.2 ± 0.4	7.4 ± 0.1	6.2 ± 0.9
FSR	17.2 ± 1.6	13.1 ± 1.5 (*)	14.5 ± 1.6	13.5 ± 0.5	14 ± 2
FBR	8.8 ± 1.7	6.9 ± 0.8	7.3 ± 1.9	6.1 ± 0.4	7.8 ± 1.6

4.1. Conclusion

The present study constitutes the first dynamic proteomics study to produce a large panel of protein FSR among farm animal species, establishing a precedent for new studies which aim to gain insights into animal health and growth status, providing an innovative approach for the assessment of the effect of feed supplements and contributing towards the general application of Omics approaches in veterinary medicine and animal production. Furthermore, this study represents a step forward in our understanding of broiler chicken reaction to an acute immunological challenge. Results presented in this study can contribute towards the refinement of broiler chicken heath status monitoring methods and feeding programmes aiming to maximize broiler chicken muscle growth, economic profitability and, ultimately, efficient human food production.

CRediT authorship contribution statement

Jorge Peinado-Izaguerri: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Alexander Corbishley:** Data curation, Methodology, Resources, Software, Validation, Visualization, Writing – review & editing. **Eduardo Zarzuela:** Investigation, Methodology, Resources, Writing – review & editing, Data curation. **Blanca Pina-Beltrán:** Formal analysis, Resources, Software, Visualization, Writing – review & editing. **Francesca Riva:** Investigation, Resources, Writing – review & editing. **Dorothy E.F. McKeegan:** Investigation, Resources, Writing – review & editing. **Maureen Bain:** Investigation, Resources, Writing – review & editing. **Javier Muñoz:** Data curation, Investigation, Resources, Writing – review & editing. **Mangesh Bhide:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Mark McLaughlin:** Funding acquisition, Investigation, Resources, Supervision, Writing – review & editing. **Tom Preston:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

Authors declare no conflicts of interests.

Data availability

Yes

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Appendix A. Supplementary data

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References

[1] OECD-FAO, OECD-FAO Agricultural Outlook 2022–2031, 2022.
[2] United Nations, World Population Prospects: The 2017 Revision, Key Findings and Advance Tables, 2017.
[3] M.K. Doherty, L. McLean, J.R. Hayter, J.M. Pratt, D.H.L. Robertson, A. El-Shafei, S. J. Gaskell, R.J. Beynon, The proteome of chicken skeletal muscle: changes in soluble protein expression during growth in a layer strain, Proteomics 4 (2004) 2082–2093.
[4] K. Maruyama, M. Sunde, R. Swick, Growth and muscle protein turnover in the chick, Biochem. J. 176 (1978).
[5] S. Tesseraud, A.M. Chagneau, J. Grizard, Muscle protein turnover during early development in chickens divergently selected for growth rate, Poult. Sci. 79 (2000) 1465–1471.
[6] S. Dänicke, W. Böttcher, O. Simon, H. Jeroch, The measurement of muscle protein synthesis in broilers with a flooding dose technique: use of 15N-labelled phenylalanine, GC-MS and GC-C-IRMS, Isot. Environ. Health Stud. 37 (2001) 213–225.
[7] K. Vignale, J. Caldas, J. England, N. Boonsinchai, P. Sodsee, M. Putsakum, E. Pollock, S. Dridi, C. Coon, The effect of four different feeding regimens from rearing period to sexual maturity on breast muscle protein turnover in broiler breeder parent stock, Poult. Sci. 96 (2017) 1219–1227.
[8] P. Maharjan, G. Mullenix, K. Hilton, J. Weil, A. Beitia, J. Caldas, V.D.N. Haro, C. Coon, Effects of dietary energy levels on pectoralis major mixed muscle protein turnover and body composition in two broiler lines housed in different grow-out environments, J. Anim. Physiol. Anim. Nutr. (Berl) 105 (2021) 535–548.
[9] T. Kasumov, S. Ilchenko, L. Li, N. Rachdaoui, R.G. Sadygov, B. Willard, A. J. McCullough, S. Previs, Measuring protein synthesis using metabolic (2)H labeling, high-resolution mass spectrometry, and an algorithm, Anal. Biochem. 412 (2011) 47–55.
[10] B.-W. Kong, K. Lassiter, A. Piekarski-Welsher, S. Dridi, A. Reverter-Gomez, N. J. Hudson, W.G. Bottje, Proteomics of breast muscle tissue associated with the phenotypic expression of feed efficiency within a pedigree male broiler line: I. Highlight on mitochondria, PLoS One 11 (2016) e0155679.
[11] N. Zhou, W.R. Lee, B. Abasht, Messenger RNA sequencing and pathway analysis provide novel insights into the biological basis of chickens' feed efficiency, BMC Genomics 16 (2015) 195.
[12] M. Zampiga, L. Laghi, C. Zhu, A. Cartoni Mancinelli, S. Mattioli, F. Sirri, Breast muscle and plasma metabolomics profile of broiler chickens exposed to chronic heat stress conditions, Animal 15 (2021) 100275.
[13] T. Dehau, R. Ducatelle, F. Van Immerseel, E. Goossens, Omics technologies in poultry health and productivity - part 1: current use in poultry research, Avian Pathol. 51 (2022) 407–417.
[14] M.K. Doherty, C. Whitehead, H. McCormack, S.J. Gaskell, R.J. Beynon, Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates, Proteomics 5 (2005) 522–533.
[15] Y. Maeda, K. Hayashi, S. Toyohara, T. Hashiguchi, Variation among chicken stocks in the fractional rates of muscle protein synthesis and degradation, Biochem. Genet. 22 (1984) 687–700.
[16] S.J. Jones, M.D. Judge, E.D. Aberle, Muscle protein turnover in sex-linked dwarf and normal broiler chickens, Poult. Sci. 65 (1986) 2082–2089.
[17] C.L. Saunderson, S. Leslie, Muscle growth and protein degradation during early development in chicks of fast and slow growing strains, Comp. Biochem. Physiol. A Physiol. 89 (1988) 333–337.
[18] S. Tesseraud, R. Peresson, J. Lopes, A.M. Chagneau, Dietary lysine deficiency greatly affects muscle and liver protein turnover in growing chickens, Br. J. Nutr. 75 (1996) 853–865.
[19] S. Dänicke, O. Simon, H. Jeroch, Effects of dietary fat type and non-starch-polysaccharide-hydrolysing enzyme addition to rye-based diets on muscle protein turnover in broilers, Br. Poult. Sci. 44 (2003) 245–255.
[20] M. Urdaneta-Rincon, S. Leeson, Muscle (pectoralis major) protein turnover in young broiler chickens fed graded levels of lysine and crude protein, Poult. Sci. 83 (2004) 1897–1903.
[21] R.D. Ekmay, C. Salas, J. England, S. Cerrate, C.N. Coon, The effects of age, energy and protein intake on protein turnover and the expression of proteolysis-related genes in the broiler breeder hen, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 164 (2013) 38–43.
[22] P. Maharjan, G. Mullenix, K. Hilton, A. Beitia, J. Weil, N. Suesuttajit, D. Martinez, C. Umberson, J. England, J. Caldas, V.D.N. Haro, C. Coon, Effects of dietary amino acid levels and ambient temperature on mixed muscle protein turnover in Pectoralis major during finisher feeding period in two broiler lines, J. Anim. Physiol. Anim. Nutr. (Berl) 104 (2020) 1351–1364.
[23] D.M. Barnes, Z. Song, K.C. Klasing, W. Bottje, Protein metabolism during an acute phase response in chickens, Amino Acids 22 (2002) 15–26.
[24] K. Tiseo, L. Huber, M. Gilbert, T.P. Robinson, T.P. Van Boeckel, Global trends in antimicrobial use in food animals from 2017 to 2030, Antibiotics (Basel) 9 (2020).
[25] M. Hellerstein, W. Evans, Recent advances for measurement of protein synthesis rates, use of the "virtual biopsy" approach, and measurement of muscle mass, Curr. Opin. Clin. Nutr. Metab. Care 20 (2017) 191–200.
[26] U. Gadde, W.H. Kim, S.T. Oh, H.S. Lillehoj, Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review, Anim. Health Res. Rev. 18 (2017) 26–45.
[27] Y. Mehdi, M.-P. Létourneau-Montminy, M.-L. Gaucher, Y. Chorfi, G. Suresh, T. Rouissi, S.K. Brar, C. Côté, A.A. Ramirez, S. Godbout, Use of antibiotics in broiler production: global impacts and alternatives, Anim. Nutr. 4 (2018) 170–178.
[28] H. Ayalew, H. Zhang, J. Wang, S. Wu, K. Qiu, G. Qi, A. Tekeste, T. Wassie, D. Chanie, Potential feed additives as antibiotic alternatives in broiler production, Front. Vet. Sci. 9 (2022) 916473.
[29] S. Rafiq, R. Kaul, S.A. Sofi, N. Bashir, F. Nazir, G. Ahmad Nayik, Citrus peel as a source of functional ingredient: a review, J. Saudi Soc. Agric. Sci. 17 (2018) 351–358.
[30] V. Silva, V. Morita, I. Boleli, Effect of pectin extracted from citrus pulp on digesta characteristics and nutrient digestibility in broilers chickens, Rev. Bras. Zootec. 42 (2013) 575–583.
[31] G. Ávila, D. De Leonardis, G. Grilli, C. Lecchi, F. Ceciliani, Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response, Vet. Immunol. Immunopathol. 237 (2021) 110269.
[32] J. Peña, S. Vieira, J. López, R. Reis, R. Barros, F. Furtado, P. Silva, Ascorbic acid and citric flavonoids for broilers under heat stress: effects on performance and meat quality, Braz. J. Poult. Sci. 10 (2008).
[33] A.A. Kamboh, S.-Q. Hang, M.A. Khan, W.-Y. Zhu, In vivo immunomodulatory effects of plant flavonoids in lipopolysaccharide-challenged broilers, Animal 10 (2016) 1619–1625.
[34] U.M.-M. Agatemor, O.F.C. Nwodo, C.A. Anosike, Anti-inflammatory activity of Cucumis sativus L. J. Pharm. Res. Int. (2015) 1–8.
[35] C. Bernardini, A. Zannoni, M. Bertocchi, I. Tubon, M. Fernandez, M. Forni, Water/ethanol extract of Cucumis sativus L. fruit attenuates lipopolysaccharide-induced inflammatory response in endothelial cells, BMC Complement. Altern. Med. 18 (2018) 194.
[36] N. Muruganantham, S. Solomon, M.M. Senthamilselvi, Anti-oxidant and anti-inflammatory activity of Cucumis sativas (Cucumber) flowers, Int. J. Pharm. Sci. Res. 1740–1745 (2016).
[37] J. Peinado-Izaguerri, E. Zarzuela, M. McLaughlin, A.C. Small, F. Riva, D.E. F. McKeegan, M. Bain, J. Muñoz, M. Bhide, T. Preston, A novel dynamic proteomics approach for the measurement of broiler chicken protein fractional synthesis rate, Rapid Commun. Mass Spectrom. 37 (2023) e9497.
[38] E. Lau, Q. Cao, D.C.M. Ng, B.J. Bleakley, T.U. Dincer, B.M. Bot, D. Wang, D. A. Liem, M.P.Y. Lam, J. Ge, P. Ping, A large dataset of protein dynamics in the mammalian heart proteome, Sci. Data 3 (2016) 160015.
[39] B.C. Naylor, M.T. Porter, E. Wilson, A. Herring, S. Lofthouse, A. Hannemann, S. R. Piccolo, A.L. Rockwood, J.C. Price, DeuteRater: a tool for quantifying peptide isotope precision and kinetic proteomics, Bioinformatics 33 (2017) 1514–1520.
[40] R.G. Sadygov, J. Avva, M. Rahman, K. Lee, S. Ilchenko, T. Kasumov, A. Borzou, d2ome, software for in vivo protein turnover analysis using heavy water labeling and LC-MS, reveals alterations of hepatic proteome dynamics in a mouse model of NAFLD, J. Proteome Res. 17 (2018) 3740–3748.
[41] A.M. Almeida, P. Nanni, A.M. Ferreira, C. Fortes, J. Grossmann, R.J.B. Bessa, P. Costa, The longissimus thoracis muscle proteome in Alentejana bulls as affected by growth path, J. Proteome 152 (2017) 206–215.
[42] C.S. Hughes, S. Moggridge, T. Müller, P.H. Sorensen, G.B. Morin, J. Krijgsveld, Single-pot, solid-phase-enhanced sample preparation for proteomics experiments, Nat. Protoc. 14 (2019) 68–85.
[43] J. Cox, N. Neuhauser, A. Michalski, R.A. Scheltema, J.V. Olsen, M. Mann, Andromeda: a peptide search engine integrated into the MaxQuant environment, J. Proteome Res. 10 (2011) 1794–1805.
[44] Y. Perez-Riverol, A. Csordas, J. Bai, M. Bernal-Llinares, S. Hewapathirana, D. J. Kundu, A. Inuganti, J. Griss, G. Mayer, M. Eisenacher, E. Pérez, J. Uszkoreit, J. Pfeuffer, T. Sachsenberg, Ş. Yılmaz, S. Tiwary, J. Cox, E. Audain, M. Walzer, A. F. Jarnuczak, T. Ternent, A. Brazma, J.A. Vizcaíno, The PRIDE database and related tools and resources in 2019: improving support for quantification data, Nucleic Acids Res. 47 (2019) D442–D450.
[45] L.K. Pino, B.C. Searle, J.G. Bollinger, B. Nunn, B. MacLean, M.J. MacCoss, The skyline ecosystem: informatics for quantitative mass spectrometry proteomics, Mass Spectrom. Rev. 39 (2020) 229–244.
[46] RStudio Team, RStudio: Integrated Development for R, 2020.
[47] T. Kasumov, B. Willard, L. Li, R.G. Sadygov, S. Previs, Proteome dynamics with heavy water — Instrumentations, data analysis, and biological applications, in: Recent Advances in Proteomics Research, IntechOpen, 2015.
[48] M. Despotovic, V. Nedic, D. Despotovic, S. Cvetanovic, Evaluation of empirical models for predicting monthly mean horizontal diffuse solar radiation, Renew. Sust. Energ. Rev. 56 (2016) 246–260.
[49] UniProt Consortium, UniProt: the universal protein knowledgebase in 2023, Nucleic Acids Res. 51 (2023) D523–D531.
[50] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. R. Stat. Soc. Ser. B Methodol. 57 (1995) 289–300.
[51] D. Szklarczyk, R. Kirsch, M. Koutrouli, K. Nastou, F. Mehryary, R. Hachilif, A. L. Gable, T. Fang, N.T. Doncheva, S. Pyysalo, P. Bork, L.J. Jensen, C. von Mering, The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest, Nucleic Acids Res. 51 (2023) D638–D646.
[52] J.C. Acton, R.L. Dick, Thermal transitions of natural actomyosin from poultry breast and thigh Tissues1, Poult. Sci. 65 (1986) 2051–2055.
[53] S.J. Jones, Improvement of growth in broiler chickens using trilostane, Poult. Sci. 69 (1990) 2143–2149.

[54] R. Nieto, R.M. Palmer, I. Fernández-Fígares, L. Pérez, C. Prieto, Effect of dietary protein quality, feed restriction and short-term fasting on protein synthesis and turnover in tissues of the growing chicken, Br. J. Nutr. 72 (1994) 499–507.
[55] M.E. Glickman, S.R. Rao, M.R. Schultz, False discovery rate control is a recommended alternative to Bonferroni-type adjustments in health studies, J. Clin. Epidemiol. 67 (2014) 850–857.
[56] P.J. Reeds, C.R. Fjeld, F. Jahoor, Do the differences between the amino acid compositions of acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? J. Nutr. 124 (1994) 906–910.
[57] T. Preston, C. Slater, D.C. McMillan, J.S. Falconer, A. Shenkin, K.C. Fearon, Fibrinogen synthesis is elevated in fasting cancer patients with an acute phase response, J. Nutr. 128 (1998) 1355–1360.
[58] M. Shankaran, C.L. King, T.E. Angel, W.E. Holmes, K.W. Li, M. Colangelo, J. C. Price, S.M. Turner, C. Bell, K.L. Hamilton, B.F. Miller, M.K. Hellerstein, Circulating protein synthesis rates reveal skeletal muscle proteome dynamics, J. Clin. Invest. 126 (2016) 288–302.
[59] E.L. O'Reilly, Acute Phase Proteins and Biomarkers for Health in Chickens, PhD, University of Glasgow, 2016.