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Space allowance and dietary fibre shape distinct adaptive proteomic responses in muscle and measurable effects on broiler meat quality

Seren Yigitturk [a,*], Ingrid C. de Jong [b], Sjef Boeren [c], Shai Barbut [d,e], Vincenzo Fogliano [a], Sara W. Erasmus [a,*]

[a] Food Quality and Design, Wageningen University & Research, P.O. Box 17, 6700, AA, Wageningen, the Netherlands
[b] Wageningen Livestock Research, Wageningen University & Research, 6700, AH, Wageningen, the Netherlands
[c] Laboratory of Biochemistry, Wageningen University & Research, Wageningen, the Netherlands
[d] Adaptation Physiology Group, Department of Animal Sciences, Wageningen University & Research, Wageningen, the Netherlands
[e] Department of Food Science, University of Guelph, Guelph N1G 2W1, Ontario, Canada

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ABSTRACT

The poultry industry faces increasing demand for sustainable production aligned with meat quality and animal welfare. This study examined effects of space allowance (39 vs. 21 kg/m^2) and dietary fibre (DF) supplementation (control vs. DF) on broiler pectoralis major muscle using mass spectrometry-based proteomics under higher-welfare conditions. While yield characteristics were unaffected, reduced space allowance significantly increased intramuscular fat and altered the proteome, with enrichment of proteins in oxidative phosphorylation, transcriptional stress and shifts in carbohydrate metabolism. Broilers receiving DF exhibited lower physical activity and feeding behaviours, with proteomic profiles linked to glycolysis, glycogen turnover and muscle architecture, as well as with reduced drip loss regardless of space allowance. Under higher space, DF induced broader proteomic adaptations, including cytoskeletal remodelling, lipid biosynthesis regulation and vascular homeostasis. These findings highlight how husbandry factors interact to shape meat phenotypes, offering a molecular framework to support quality-focused, welfare-aligned and environmentally conscious meat production.

1. Introduction

Meat has been a key component of human diets for millennia, with a demonstrated role in human evolution, and continues to serve human health and development today (Leroy et al., 2023). Among all meat sources, poultry production and consumption have grown rapidly and are projected to represent approximately 45% of the protein consumed globally by 2034 (OECD/FAO, 2025). This growth is driven by poultry's advantageous nutritional profile, offering high-quality, highly digestible protein with lower fat content, most of which is composed of unsaturated fatty acids and other nutrients (Connolly et al., 2022; Marangoni et al., 2015). These health attributes increasingly resonate with health-conscious consumers, for whom overall dietary health is a key consideration (OECD/FAO, 2025). When combined with economic efficiency, faster production cycles and a lower environmental footprint than other muscle foods, poultry stands out as a sustainable meat option that also appeals to environmentally conscious consumers (OECD/FAO, 2025).

At the same time, many consumers increasingly consider the ethical and welfare dimensions of intensive production, including husbandry, handling and slaughter practices (Barbut & Leishman, 2022; de Araújo, Patarata, & Fraqueza, 2022; European Commission, 2023; Fonseca & Sanchez-Sabate, 2022), as integral components of meat quality (Mir et al., 2017). These concerns contribute to a broader ethical orientation that shapes consumer purchasing decisions. In this context, consumers increasingly expect production systems to enable animals to experience a “good life” and to provide reliable, transparent information to maintain trust in the food system (Alonso et al., 2020; Peschel, Thomsen, Tsalis, & Grunert, 2025). A recent segmentation study across European countries further reported that approximately two-thirds of consumers prefer meat carrying farm animal welfare attributes, with space-related attributes playing a particularly important role (Peschel et al., 2025). However, while consumer perception is positive when such extrinsic parameters are considered, it becomes more negative when intrinsic meat quality, particularly sensory attributes, is compromised (de Araújo

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* Corresponding authors.
E-mail addresses: seren.yigitturk@wur.nl (S. Yigitturk), sara.erasmus@wur.nl (S.W. Erasmus).

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et al., 2022).

Given these evolving consumer expectations, it is important to consider how different production systems impact animals, as well as the environment. Intensive farming systems are often associated with higher productivity (Bilotto et al., 2024). Nonetheless, such systems have in some cases had deleterious environmental outcomes and have been identified as a major driver of biodiversity loss through land-use change, feed production and related ecosystem pressures (Bilotto et al., 2024; Pereira et al., 2025). Emerging strategies emphasise a transition from intensive to more extensive farming systems for broilers, which are among the most intensively reared livestock species for food (Marchewka et al., 2023). Such extensification factors can include reduced stocking density (increased space allowance) (Buijs et al., 2009; van der Eijk et al., 2022, 2023) and implementing dietary modifications (Ginindza, 2023; Yue 岳珂 et al., 2024). Stocking density has been shown to significantly influence broiler behaviour and stress physiology, as well as performance, yield and meat quality (Marchewka et al., 2023; Onbaşılar et al., 2008; van der Eijk et al., 2023). There is considerable variation in the classification thresholds used to define broiler stocking density across countries, which limits direct comparability between studies (Marchewka et al., 2023). Nevertheless, irrespective of these thresholds, lower densities allow more space for animals to express natural behaviours. This is often associated with reduced physiological stress and improved meat quality, although it may come with increased production costs (Marchewka et al., 2023; Simitzis et al., 2012; van der Eijk et al., 2022, 2023; Yue 岳珂 et al., 2024). To mitigate the challenges posed by high densities, dietary strategies involving functional feed ingredients are gaining interest (Obeidat et al., 2024). One such approach is the inclusion of lucerne or alfalfa roughage. This fibre-rich forage has prebiotic properties that positively modulate gut microbiota and digestive function and may improve a birds' stress resilience (Ginindza, 2023; Jha et al., 2019). Its antioxidant-rich secondary metabolites may also protect muscle tissues from oxidative stress, further supporting meat quality during growth and later meat processing (Ginindza, 2023; Yue 岳珂 et al., 2024).

To optimise the extensification factors, a deeper understanding of the underlying biochemical mechanisms governing broiler responses is crucial (Gobert et al., 2014). Mass spectrometry (MS)-based proteomics, supported by bioinformatics, provides a powerful approach to address this need. It enables quantitative profiling of structural, metabolic and stress-response proteins, including pathways related to oxidative balance, proteolysis and cellular signalling, within a single analysis (Gobert et al., 2014; Huang et al., 2020). As such, muscle proteomics offers a functional read-out of biological adaptation (Deshmukh et al., 2021) that integrates genetic background with on-farm environmental conditions (Yigitturk et al., 2025). This makes proteomics particularly well-suited to broiler research, as it provides a mechanistic bridge between husbandry factors, broilers' adaptability to their environment, welfare-related muscle physiology and subsequent intrinsic meat quality traits.

Despite extensive research on space allowance and growing interest in dietary fibre as independent strategies their combined effects and potential interactions within controlled experimental designs have not been systematically investigated. Moreover, studies integrating detailed individual animal data on on-farm conditions, performance, intrinsic meat quality and underlying muscle proteomics responses remain scarce. In our previous study within the *mEATquality* European Union project, we showed that genetic factors are essential to determine the response to on-farm enrichment factors (Yigitturk et al., 2025). Building on this work, the current study examined both the main effects and interactions of space allowance and dietary fibre provision in higher-welfare systems. Using a slower-growing genotype, Hubbard JA757 (EFSA AHAW Panel, 2023), we assessed broiler performance and intrinsic meat quality traits. To characterise the molecular mechanisms underlying these outcomes, we applied an optimised MS-based protein aggregation capture (PAC) method tailored for meat proteomics, which improves peptide recovery and the identification of unique peptides and proteins in muscle tissue (Batth et al., 2019).

2. Materials and methods

The workflow of the study is presented in Fig. 1.

2.1. Husbandry factors and experimental design

A randomised complete block 2 × 2 factorial design was used to test four treatment combinations: space allowance, expressed as stocking density (high density, **HD**: 39 kg/m^2, ~17.6 birds/m^2; low density, **LD**: 21 kg/m^2, ~9.5 birds/m^2) × diet (control, **CD** without added dietary fibre; dietary fibre-supplemented, **DF**). Each treatment had four replicate pens.

A total of 2928 day-old slower-growing JA757 broiler chicks (<50 g/day growth) (EFSA AHAW Panel, 2023), of mixed sex (50% male, 50% female), were sourced from a single commercial hatchery originating from a 39-week-old Hubbard parent stock. Broilers were placed at the experimental facility on day 0, reared without sex separation, and slaughtered at 49 days of age. The experiment was conducted at the Agrifirm facility (Laverdonk, NL) in a mechanically ventilated room containing 36 pens, of which 16 pens arranged in four blocks of four pens each were used. Pen-level broiler numbers are shown in Fig. 1.

Broilers were reared under controlled climatic and lighting conditions. Ambient temperature was set at 35 °C at placement and gradually reduced to 19–21 °C from Day 42 onwards. Relative humidity was maintained at ≥40% during the first week and at approximately 50–70% after Day 14. Artificial lighting was provided at a minimum intensity of 20 lx. Dark periods were scheduled as follows: on Day 0–4 from 21:00 to 00:00 h; on Day 5–10 from 20:00 to 00:00 h; on Day 10–13 from 20:00 to 00:00 h and from 04:00 to 06:00 h; and from Day 14 onwards, from 20:00 to 04:00 h. All pens were bedded with wood shavings, with one bale of litter provided per pen (1.48 kg/m^2). Feed and water systems varied by density treatment: HD pens were equipped with five pan feeders and 24 nipple drinkers with cups, whereas LD pens had three pan feeders and 12 nipple drinkers with cups. All birds were fed a commercially available four-phase broiler diet program (ABZ, Leusden, NL) formulated for slower-growing strains in accordance with the Dutch *Beter Leven* (Better Life) concept, a one-star welfare label. The feeding program consisted of a starter diet (crumble, day 0–14, raw protein content 200 g/kg), grower 1 (pellet, day 14–25, raw protein content 186 g/kg), grower 2 (pellet, day 25–35, raw protein content 175 g/kg), and a finisher diet (pellet, day 35–63, raw protein content 173 g/kg), with coccidiostats in all phases. In addition to the base diet, DF pens received lucerne (alfalfa) hay in two nets per pen. Broilers were reared to ~2.2 kg. Broilers that were clinically ill or were not vital were excluded from the study.

Broilers were transported to a commercial slaughter and processing plant in Diessen, the Netherlands (~35 min from the production facility). After a 1 h lairage period, broilers were electrically stunned (240 mA for 6 s) and immediately exsanguinated by trained slaughterhouse personnel, in accordance with standard commercial procedures and regulatory requirements. Carcasses were subsequently water scalded (60 °C for 90 s) and mechanically plucked.

The experiment has been approved by the institutional Animal Welfare Body (experiment number 201.D-0002.003; approval date 27-03-2023). All procedures were conducted in accordance with institutional animal welfare guidelines, EU Directive 2010/63/EU on the protection of animals used for scientific purposes and relevant Dutch legislation.

2.2. Broiler performance

Feed and water intake were recorded daily with automated systems, and body weights with automated scales. A sample of 50 broilers per pen was manually weighed at placement and during diet phase changes, and

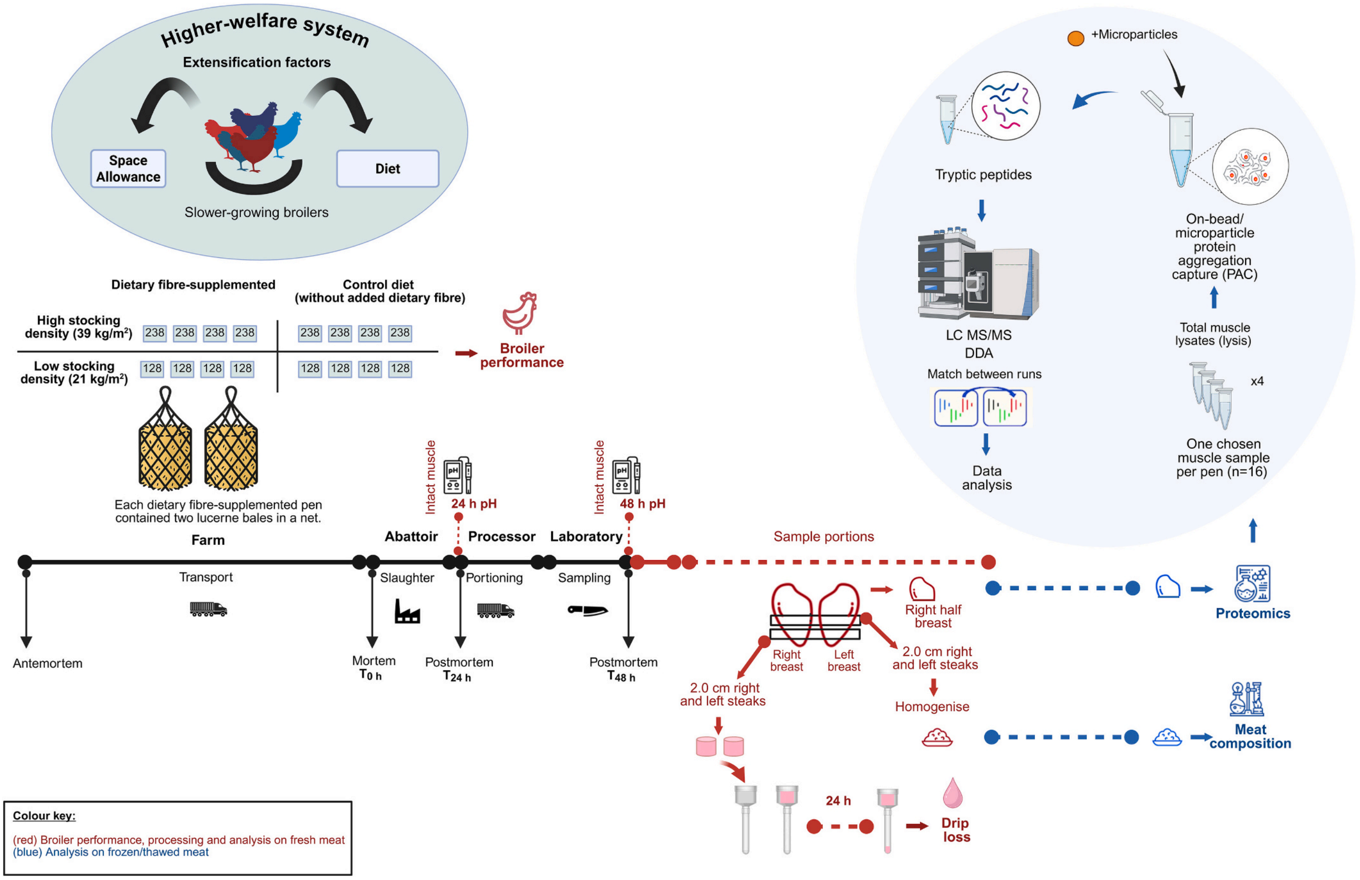

Fig. 1. Workflow of the study from farm to laboratory. The timeline illustrates the process from farm through transport, slaughter, portioning and sampling, with pH measured at 24 h ($T_{24\ h}$) and 48 h ($T_{48\ h}$). The study comprised four treatments: two space allowances (high vs. low stocking density) and two diets (dietary fibre-supplemented vs. control without added dietary fibre). Small boxes represent pens (n = 16, four replicates per treatment). In total, 2928 broilers slower-growing broilers (50% male, 50% female) were placed across pens: high stocking density (39 kg/m^2; eight pens, 238 chicks/pen) and low stocking density (21 kg/m^2; eight pens, 128 chicks/pen). Sample portions were designated for downstream analyses (broiler performance, meat composition, drip loss and proteomics). The upper-right panel outlines the mass spectrometry-based proteomics workflow applied to muscle samples. Further details are provided in Materials and methods (adapted from Yigitturk et al., 2025). Created in BioRender.

all broilers were weighed at marketing age. These were randomly sampled using a catching pen, with 25 males and 25 females (from the age at which they were visible) or 50 randomly sampled broilers per pen at other ages. These were thus not the same broilers across sampling points. Mortality-corrected pen data were used to calculate daily growth rate, feed conversion ratio and water-to-feed ratio.

2.3. Broiler sampling, muscle myopathy identification and meat quality analysis

At slaughter age (49 days; see Section 2.1), only male broilers (ten per pen; 40 per treatment, 160 total) were randomly selected for slaughter and subsequent postmortem analyses. All samples were subjected to the same standardised handling, slaughtering and processing procedures to ensure consistency across treatments, following the procedures previously described (Yigitturk et al., 2025). Hot carcass weights (with skin) were recorded post-processing, and carcasses were then air-chilled (at 1 °C). At 24 h postmortem, cold carcass weights (skin on) were recorded. Breast muscle pH was measured in triplicate using a portable meat pH meter equipped with an insertion glass electrode (HI99163, Hanna Instruments Nederland). Measurements were taken in the central region of the pectoralis major muscle following a small opening for electrode insertion. The electrode was calibrated prior to measurements using two standard buffer solutions at pH 4.01 and 7.01, and calibration was verified after approximately every 10 birds. Average pH values are reported. Carcasses were then vacuum-packed and transported at 4 °C to the laboratory (Wageningen University, NL), where pH was re-measured at 48 h postmortem to reflect the physiological condition of the muscle at the time of downstream analyses. The meat quality analyses in this study were sampled at this time point, aligning with industry practices to best represent consumer-facing products. The left and right pectoralis major (breast) muscles, as well as the corresponding drumsticks (skin on), were weighed. Samples from the upright portion of the pectoralis major were collected for proteomics. Proteomic samples were flash-frozen in liquid nitrogen and stored at −80 °C.

Muscle myopathies were evaluated by visually examining images (IRIS-Alphasoft electronic eye) of whole breasts, according to established criteria (Che et al., 2022; Kuttappan et al., 2016). Two muscle myopathies, i.e., white striping (**WS**) and spaghetti meat (**SM**), were evaluated. Mid-point 2 cm thick sections from the left and right pectoralis major were homogenised for proximate composition analyses. Moisture was determined according to AOAC Official Method 950.46 (AOAC, 2005), protein according to AOAC Official Method 992.15 (AOAC, 1992) and intramuscular fat (IMF) content according to AOAC Official Method 991.36, with analytical procedures as described previously (Yigitturk et al., 2025). All analyses were performed in duplicate. Moisture and protein were analysed on all available samples. For IMF analysis, three broilers per pen were selected across all treatments (n = 48) to represent within-pen natural variation in myopathy severity and its potential impact on meat composition traits.

Additional 2 cm-thick samples taken below the mid-point of the muscle were used for drip loss. Drip loss was assessed using the gravimetric EZ-drip loss method. Circular core samples (25 mm diameter, ~5 g) from the left and right breast muscles were weighed and stored at 4 °C for 24 h. Drip loss (%) was calculated as the proportion of exuded fluid relative to the sample weight. The procedure was performed as described in detail (Ludwiczak et al., 2025).

2.4. Statistical analysis for broiler performance, yield and meat quality

Performance data were analysed at the pen level using the GLIMMIX procedure in SAS (version 9.4). Phenotypic data were analysed using a two-way ANOVA in R (version 4.4.1) with the "doebioresearch" package (version 0.1.0). Assumptions of normality and homogeneity of variance were verified using the Shapiro-Wilk and Levene's tests. Post-hoc comparisons were performed using Tukey's test, with significance set at $p < 0.05$.

2.5. Proteomics analysis

2.5.1. Sample selection, protein extraction and PAC preparation

One broiler per pen was selected from the subset of ten used for meat quality analysis. In total, 62.3% of the initially screened samples were excluded from proteomics due to the presence of muscle myopathies (WS and/or SM). This exclusion was applied to ensure that the proteomic dataset reflected the effects of the experimental factors rather than myopathy-associated degeneration. Subsequently, samples showing larger residuals than their group averages for texture and colour (BMORS shear force; L*, a* and b* values; data collected but not included in the present analysis) were also excluded. The final selection of 16 pectoralis major muscles was based on the interquartile range, capturing the central 50% of the data points. Where multiple suitable samples remained, priority was given to those within the narrower percentile ranges (2.5–97.5%, followed by 5–95%) for texture, colour and body weights (live and carcass). This approach ensured a robust and representative dataset to evaluate the effects of space allowance and dietary fibre.

Liquid nitrogen-frozen muscle samples were cryo-milled and ~ 50 mg of sample was homogenised (in triplicate) in SDS-based lysis buffer, followed by acetone precipitation. Extracts were solubilised in Urea buffer and all extraction and solubilisation steps followed the protocol previously described (Yigitturk et al., 2025). After cell debris removal by centrifugation, protein concentration of the supernatants was determined (Pierce BCA, ThermoFisher Scientific). The average protein concentration was 6.5 μg/μL protein. For each sample, the replicate closest to its mean concentration was retained (total of 16 samples). Total muscle lysates (100 μg protein per sample) were processed using the PAC method as previously described (Yigitturk et al., 2025). Briefly, proteins were reduced with DTT, alkylated with acrylamide, captured on magnetic carboxylate-modified beads (1-μm-diameter, GE Healthcare), washed and digested. Proteolytic digestion was carried out overnight at room temperature by adding 100 μL bovine sequencing grade trypsin (0.5 μg/μL in 1 mM HCl) within 50 μL of 50 mM ammonium bicarbonate. Digestion was stopped by acidification to pH 3.0 using 10% TFA. Following a brief centrifugation, the supernatant (peptide-containing solution) was carefully transferred to new low-binding Eppendorf tubes for downstream analysis.

2.5.2. Liquid chromatography (LC) and mass spectrometry (MS)

For each sample, 0.5 μg of peptides was injected onto an analytical column (0.10 × 250 mm, prepared in-house) containing ReproSil Saphir 100 C18 1.5-μm beads (Dr. Maisch, Ammerbuch-Entringen, Germany). Peptides were loaded at constant pressure using 1% formic acid in water and separated on a Thermo Vanquish Neo nanoLC system (Thermo Fisher Scientific, Waltham, MA) with 4–29% acetonitrile gradient in 1% formic acid over 50 min at 0.5 μL/min. Electrospray ionisation was performed at 3.0 kV. MS analysis was performed on an Orbitrap Exploris 480 (ThermoFisher, Waltham, MA) operated in data-dependent acquisition mode. MS and MS/MS scans used automatic gain control targets. Ions were fragmented using HCD (28% NCE, 1.2 m/z isolation window). MS/MS spectra were collected at 15,000 resolution with a 1.1 s cycle time, targeting the most intense precursors (2+ to 5+) with a 12 s dynamic exclusion (±10 ppm).

The MS proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD069586 (Perez-Riverol et al., 2025).

2.5.3. Computational proteomics, bioinformatics and statistical analysis

Raw MS/MS data were processed in MaxQuant (version 2.0.3.0). The peptide spectra were searched by the Andromeda search engine (integrated into MaxQuant) against the UniProt *Gallus gallus* database (UP000000539 downloaded October 2024) and a database of frequently observed contaminants. The search included variable modifications of methionine oxidation, N-terminal acetylation, deamidation of asparagine (N) and glutamine (Q) and a fixed modification of cysteine to propionamide. Peptides were required to be at least seven amino acids long, with up to two missed enzymatic cleavages permitted. The false discovery rate (FDR) threshold of 1% was applied to both peptide and protein identifications. The MS runs were analysed with the "match between runs" option. Proteins identified by matches to reverse database and potential contaminants were excluded. Protein identification was based on detecting at least two peptides.

For comparative proteome analysis, normalised intensities were obtained using label-free quantification (LFQ) through the MaxLFQ algorithm (Cox et al., 2014). Proteins that were quantified at least three times in at least one group (Groups: HD-DF, HD-CD, LD-DF and LD-CD) were included in the analysis. This approach led to the identification of ~800 protein groups (8202 unique peptides; median sequence coverage of 29%; Supplementary Data 1). After filtering, data completeness and missingness patterns were assessed (Supplementary Fig. 1a and b).

Data were $\log_2$-transformed prior to statistical analysis. Two-factor ANOVA was performed in a full factorial design to determine: (i) the effect of space allowance (HD vs LD), (ii) the effect of dietary fibre (DF vs CD) and (iii) the interactions between space allowance and dietary groups (specifically comparing HD-DF vs HD-CD; LD-DF vs LD-CD).

Differentially expressed proteins (**DEPs**) were identified using two *R*-packages *limma* (v3.62.2) (Ritchie et al., 2015) and *prolfqua* (v1.3.6) (Wolski et al., 2023), each with two significance thresholds. Both methods apply empirical Bayes variance moderation (Kelter, 2021). Additionally, *prolfqua* is specifically optimised to handle missing data without requiring imputation (Wolski et al., 2023). Data for *limma* analysis were imputed by drawing from a Gaussian distribution with a width of 0.3 and a downshift of 1.8 on the $\log_2$-transformed data (provided as Supplementary Fig. 2). DEPs were selected based on (i) a significance score < 0.05 (Xiao et al., 2012) and (ii) an adjusted p-value (Benjamini-Hochberg correction) < 0.05 with an absolute fold change ≥1.5 (absolute $\log_2$ fold change ≥0.58). Venn diagrams (Figs. 2, 3 and 4) illustrate the intersection and divergence between methods. To maximise robustness, DEPs from both tools were considered. Volcano plots (Figs. 2 and 3) and Pearson correlation heatmaps (Fig. 5) present imputed values from *limma,* as no divergent DEP groups were identified by *prolfqua*.

Bar plots and protein-protein interaction networks in Figs. 2b, 3b and 4g present Gene Ontology Cellular Component (**GOCC**) of DEPs, generated using STRING (Szklarczyk et al., 2023) plugin in Cytoscape. To identify functional enriched biological processes, Cytoscape plugin ClueGO (Bindea et al., 2009) was employed, using annotations from Gene Ontology (GO) evidence levels and KEGG pathways. The enrichment analyses was performed using the hypergeometric test and multiple test correction (Benjamini-Hochberg; $p \leq 0.05$). GO term/pathway categories represented by a single term were excluded. Group labels within clusters were assigned based on the most significantly enriched term.

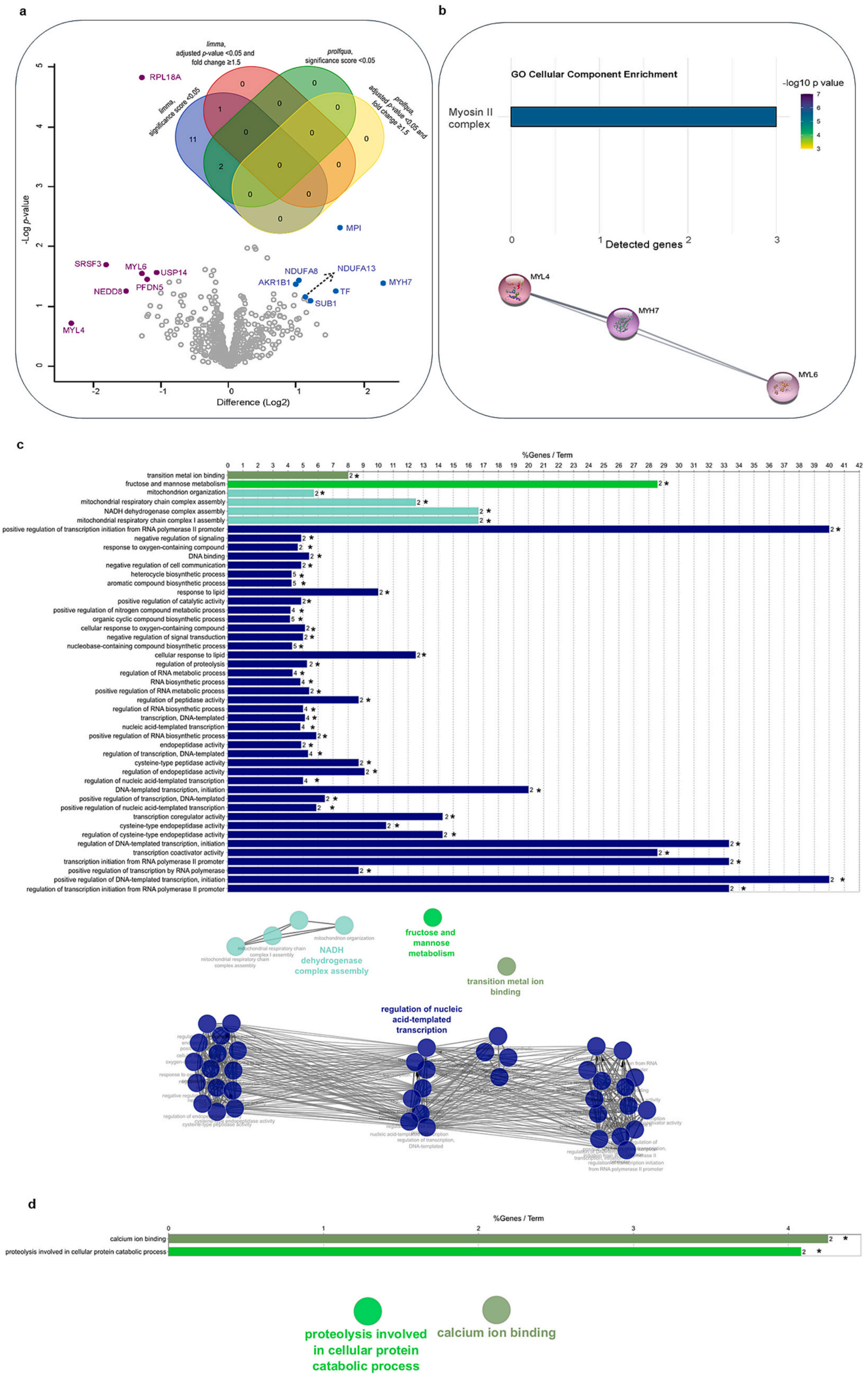

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Fig. 2. Quantitative postmortem muscle proteome differences between two levels of space allowance in broilers. a Venn diagram illustrating the overlap and divergence of DEPs identified using two *R*-packages (*limma* and *prolfqua*), each with two significance thresholds. Volcano plot displaying space-induced differences in protein abundance (imputed data), with significant DEPs highlighted (purple, higher in low stocking density; blue, higher in high stocking density). **b** Bar plot showing GOCC enrichment analysis (Benjamini-Hochberg correction; $p < 0.05$) of DEPs, with colour intensity representing statistical significance according to the scale, consistent across all figures for this analysis. Protein-protein interaction network from STRING, visualising the connectivity between the enriched myosin II complex proteins. **c** Bar plot representing functional group enrichment of DEPs in broilers raised at high stocking density, data analysed using ClueGo (Benjamini-Hochberg correction; $p \leq 0.05$). Bars indicate the number of genes from the analysed cluster found associated with the term, and the labels displayed on the bars are the percentage of found genes compared to all the genes associated with the term. Each term significance levels are indicated as ***: if the term $p < 0.001$, **: $0.001 \leq p < 0.01$ and *: $0.01 \leq p \leq 0.05$. Network analysis of the enriched functional terms/pathways, where the most significant term in each cluster is designated as the leading term and highlighted in the network. **d** Bar plot representing functional group enrichment of DEPs in broilers raised at low stocking density, analysed using ClueGO with the same statistical criteria as in panel (**c**). Network representation of the enriched terms/pathways, annotated similarly to panel (**c**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Performance, yield and meat quality characteristics in broilers

Broiler performance data were analysed at the pen level and are shown in Table 1. Broilers reared under LD showed significantly higher daily growth, higher feed intake and heavier body weight at marketing age (Table 1). These results indicate improved performance under increased space allowance, possibly due to reduced competition and disturbance during feeding. The feed conversion rate and mortality were unaffected by either factor or their interaction (Table 1).

Yield characteristics such as dressing percentage and the relative contributions of breast muscle and drumsticks are provided in Supplementary Table 1. Although population-level data indicated improved growth and body weight under LD (Table 1), these differences were not reflected in the subset of broilers analysed. Yield characteristics did not differ between treatments, likely reflecting the relatively small sample size and random selection of broilers for further analyses. Previous studies have reported inconsistent effects of density on yield characteristics (Marchewka et al., 2023). Some studies indicate higher yields at lower densities, supported by more recent evidence (Al-Baadani et al., 2023), while others report higher yields at intermediate or higher densities (Marchewka et al., 2023). Similarly, DF has shown variable results, with studies noting either beneficial or neutral impacts (Ginindza, 2023; Marchewka et al., 2023), potentially due to differences in fibre type, inclusion levels and the genetic backgrounds of broilers.

Meat quality traits, including postmortem pH (24 and 48 h), composition and drip loss are presented in Table 2. An interaction between density and DF was observed for meat pH at both 24 h and 48 h postmortem. Under HD conditions, DF increased meat pH ($p < 0.01$) at 24 h postmortem (5.86 ± 0.05) compared to the CD (5.78 ± 0.01). This effect was not observed under LD conditions. Previous studies investigated density have reported mixed results on meat pH. Some have suggested that increased density may accelerate postmortem glycolysis, thereby reducing meat pH and negatively affecting meat quality (Nasr et al., 2021; Tong et al., 2012; Wu et al., 2020; Yue 岳珂 et al., 2024). These variations may reflect considerable differences in how density is categorised across studies or the range of densities tested. Conversely, pasture access or DF has previously been associated with higher ultimate pH in broiler breast meat (ranging from 5.80 to 5.85) (Funaro et al., 2014; Ponte et al., 2008). Our findings support this observation, suggesting that DF was associated with a higher postmortem pH under HD conditions. It is worth noting that despite the differences, all pH values observed in this study remained within the normal physiological range of 5.7–6.1 reported for postmortem quality broiler breast (Beauclercq et al., 2022).

The meat composition results (shown in Table 2) indicate that HD significantly promoted IMF accumulation in the breast muscle. On a dry basis, IMF increased by 0.32 percentage points (20.4% relative) and by 0.09 percentage points (22.5% relative) on a wet basis. Similarly, previous studies have reported a significant positive correlation between reduced physical activity and increased fat deposition in broilers (Castellini et al., 2002; Simsek et al., 2009). Conversely, a higher IMF reported at LD (Simitzis et al., 2012; Wang et al., 2019) could be attributed to higher feed intake, whereas HD could be associated with elevated stress levels and increased competition for food resources (Dozier et al., 2005; Thema et al., 2022). Such discrepancies may also reflect that definitions of density vary across countries and studies (Marchewka et al., 2023).

The DF was associated with a significant reduction in both muscle moisture content and drip loss. Moisture content decreased by 0.36 percentage points (0.5% relative) while drip loss was reduced by 0.25 percentage points (25.5% relative; Table 2). Together, these findings suggest that DF may influence postmortem water retention in breast muscle, primarily by limiting fluid losses. Our findings align with previous studies conducted under different production contexts. In free-range broilers, which typically consume high amounts of DF and have more space allowance, decreased muscle moisture content (73.35% vs. 72.52%) and reduced drip loss (1.53% vs. 1.12%) have been reported (Funaro et al., 2014). In addition, broilers supplemented with alfalfa meal under indoor production systems also exhibited reduced drip loss (3.27% vs. 2.75%) (He et al., 2021). Notably, these studies reported no significant changes in muscle protein content, consistent with our findings (on both a dry and wet basis; Table 2). Genotype plays a key role, as slower-growing broilers generally exhibit improved water-holding capacity, although environmental factors can further enhance this trait (Akyüz & Onbaşılar, 2023; Marchewka et al., 2023). Drip loss is strongly influenced by postmortem pH (Marchewka et al., 2023; Tekin et al., 2025). The reduction in drip loss observed here, despite unchanged pH across dietary treatments, therefore suggests a pH-independent mechanism regulating muscle water retention.

Muscle myopathies were assessed to evaluate their implications on meat quality. The incidence of WS in modern broilers varies widely, ranging from 5% to 98%, and is influenced by multiple genetics and husbandry factors (Barbut et al., 2024; Petracci et al., 2019). However, how housing environment, management-related stress and dietary factors contribute to myopathies, and the resulting meat quality remains an active research question (Gündoğar et al., 2024; Onbaşılar et al., 2025; Varol Avcılar et al., 2019). Previously, we reported WS prevalence rates of 71.3% in faster-growing broilers and 53.2% in slow-growing broilers raised under higher welfare conditions (Yigitturk et al., 2025). In the current study, which focused on a slower-growing genotype raised under higher-welfare conditions (albeit with different densities and enrichment/roughage provision compared to our previous work), we observed a WS prevalence of 62.3%, with no severe cases (Supplementary Table 2). This relatively higher prevalence of the slower-growing genotype may reflect differences in their growth rate or environmental factors between studies. No significant differences were found among treatment groups, indicating that densities and DF did not affect WS prevalence or severity under the conditions tested. It might be interesting to mention that the lowest numerical WS incidence (57.5%) was observed in broilers raised at LD with DF. As noted above, all birds included in the downstream analyses in both studies were males, which may have contributed to the observed WS prevalence and severity, as male broilers are generally more prone to developing this myopathy than females (Barbut et al., 2024). It should also be mentioned that the

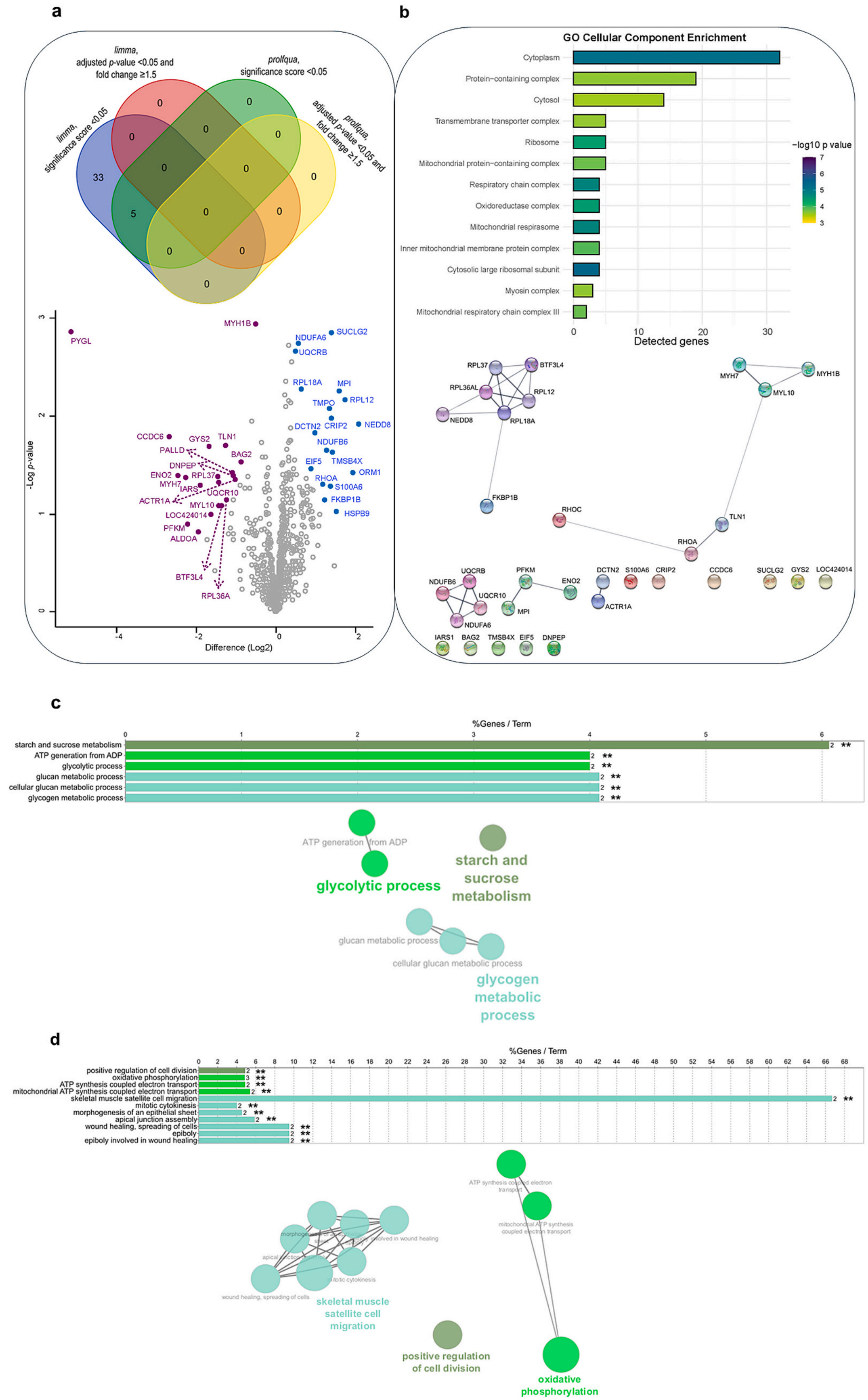

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Fig. 3. Quantitative postmortem muscle proteome differences in broilers in response to dietary fibre provision. a Venn diagram illustrating the overlap and divergence of DEPs identified using two *R*-packages (*limma* and *prolfqua*), each with two significance thresholds. Volcano plot displaying diet-induced differences in protein abundance (imputed data), with significant DEPs highlighted (purple, higher in broilers fed the fibre-supplemented diet; blue, higher in broilers fed the control diet without added dietary fibre. **b** Bar plot showing GOCC enrichment analysis (Benjamini-Hochberg correction; $p < 0.05$) of DEPs, with colour intensity representing statistical significance according to the scale, consistent across all figures for this analysis. Protein-protein interaction network from STRING, visualising the connectivity between the GOCC-enriched proteins. **c** Bar plot representing functional group enrichment of DEPs in broilers receiving the fibre-supplemented diet, data analysed using ClueGo (Benjamini-Hochberg correction; $p \leq 0.05$). Bars indicate the number of genes from the analysed cluster found associated with the term, and the labels displayed on the bars are the percentage of found genes compared to all the genes associated with the term. Each term significance levels are indicated as ***: if the term $p < 0.001$, **: $0.001 \leq p < 0.01$ and *: $0.01 \leq p \leq 0.05$. Network analysis of the enriched functional terms/pathways, where the most significant term in each cluster is designated as the leading term and highlighted in the network. **d** Bar plot representing functional group enrichment of DEPs in broilers fed the control diet, analysed using ClueGO with the same statistical criteria as in panel (**c**). Network representation of the enriched terms/pathways, annotated similarly to panel (**c**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

evaluation of SM revealed no cases in any treatment group (SM1 Sum = 0%, Supplementary Table 2).

The influence of muscle myopathy severity on meat composition and drip loss was further analysed (Supplementary Table 3). In the current study, myopathy severity showed no consistent impact on meat quality characteristics. While mild myopathy coincided with specific alterations in only dry protein content and drip loss under certain dietary and density contexts (HD-DF, LD-CD), the imbalanced distribution of myopathy severity across treatments limited the ability to draw definitive conclusions.

3.2. *Adaptive muscle proteomic responses to space allowance*

The study examined quantitative postmortem muscle proteome differences between the two levels of space allowance. To identify DEPs, we applied two empirical Bayes-based statistical approaches (*limma* and *prolfqua*) and thresholds as described in the Materials and methods section. This combined approach yielded 14 DEP groups as illustrated in Fig. 2a (Supplementary Data 2).

We first examined the cellular localisation of the DEPs based on GOCC enrichment analysis (Supplementary Data 3). The only significantly enriched term was the myosin II complex (GO:0016460), which included MYL4, MYH7 and MYL6 (Fig. 2b). Because myosin II is essential for muscle contraction, cytoskeletal organisation and myofibrillar integrity (Henderson et al., 2017), this enrichment suggests space-induced alterations in muscle architecture.

Functional enrichment analysis of the DEPs using GO annotations and KEGG pathways revealed space-induced proteomic adaptations affecting biological processes, metabolic functions and pathways (provided in Figs. 2c, d and Supplementary Data 4). Under HD conditions, most enriched terms clustered around the regulation of nucleic acid-templated transcription (GO:1903506). Within this cluster, processes such as regulation of proteolysis, cellular response to lipids, positive regulation of catalytic activity and regulation of peptidase activity all co-occurred with transcription-related terms (e.g., RNA biosynthetic process, transcription coactivator activity, transcription initiation from RNA polymerase II promoter) (Fig. 2c). These enrichments indicate that broilers at HD undergo a coordinated transcriptional reprogramming. The higher detection of SUB1 and LOC107055444, and their association with the transcriptional coactivator p15 (PC4), support an adaptive transcriptional regulatory mechanism with roles in genome stability (Garavís & Calvo, 2017). SUB1 is evolutionarily conserved and known to mediate oxidative stress responses (Wang et al., 2004; Yu et al., 2016). It is induced by oxidative stress, and purified Sub1 protein can protect DNA from oxidative damage (Yu et al., 2016) while supporting subsequent repair steps (Mortusewicz et al., 2008).

These transcriptional adaptations appear to be accompanied by shifts in energy metabolism. A cluster of enriched terms related to NADH dehydrogenase complex assembly (GO:0010257), including mitochondrial organisation (GO:0007005) and mitochondrial respiratory chain complex assembly (I) (GO:0033108, GO:0032981) (Fig. 2c), indicated altered mitochondrial function under HD conditions. The higher detection of NDUFA13 and NDUFA8 supports the higher reliance on mitochondrial oxidative phosphorylation (OXPHOS) for energy production (Wirth et al., 2016).

The simultaneous enrichment in fructose and mannose metabolism (KEGG:00051) (Fig. 2c), with higher detection of AKR1B1 and MPI, suggests activation of alternative carbohydrate pathways (Dashty, 2013). Such pathways have evolved as adaptive mechanisms, typically activated under metabolic stress. Recent findings from mammals indicate that environmental stressors, including heat stress linked to climate change, can stimulate fructose metabolism to support energy homeostasis (Johnson et al., 2020; Johnson et al., 2023). Broilers housed at HD may experience similar pressures due to increased thermal load from reduced airflow and bird proximity (Oluwagbenga & Fraley, 2023). They also exhibited reduced physical activity, as directly observed in the current study (de Jong et al., unpublished results). This suggests that, as in mammals, broilers under spatial constraints adapt carbohydrate metabolism pathways, potentially influencing changes in fat deposition and body composition when mobility is limited.

Additionally, the enrichment in transition metal ion binding (GO:0046914) (Fig. 2c) was observed, suggesting changes in cellular ion homeostasis. Metal ions are essential cofactors for mitochondrial function and redox balance. Therefore, changes in their binding may confirm adjustments in energy-related processes (Zhou et al., 2024). This category included MPI, a zinc-dependent enzyme (Bangera et al., 2019), and transferrin (TF), an iron-binding protein that maintains iron homeostasis under oxidative stress (Horrocks et al., 2011). Notably, increased TF abundance has also been reported in broilers with woody breast myopathy, which is similarly associated with increased oxidative stress (Zhang et al., 2020).

Collectively, the proteomic alterations observed at HD may suggest increased metabolic, oxidative and thermal stress, reflected by transcriptional reprogramming and shifts toward mitochondrial energy production and alternative carbohydrate metabolism. However, these interpretations remain hypotheses based on protein-level data and further targeted experiments are necessary to validate these adaptive responses.

In contrast, under LD, we detected the enrichment of proteolysis involved in cellular protein catabolic processes (GO:0051603; Fig. 2d), driven by NEDD8 and USP14. This points to a more regulated protein turnover mechanism, consistent with their roles in ubiquitin-like modification and deubiquitination (Chen & Dou, 2010; Glickman & Ciechanover, 2002). Although proteolysis also appeared under HD, it clustered with processes related to the regulation of nucleic acid-templated transcription (Fig. 2c) and involved different associated genes (NDUFA13 and TF). In our previous work, proteasome-driven protein degradation accompanied muscle loss in faster-growing broilers when protein synthesis was insufficient (Yigitturk et al., 2025). Here, however, in a slower-growing genotype raised with more space allowance, such regulated protein turnover may contribute to muscle remodelling. In contrast, under HD, proteolysis was associated with transcriptional stress pathways, consistent with more metabolically stressed state. This interpretation is supported by the significantly higher detection of 60S ribosomal protein L18A (RPL18A; ~2.5 fold increase; *limma*, significance score < 0.05 and *limma* adjusted *p*-value

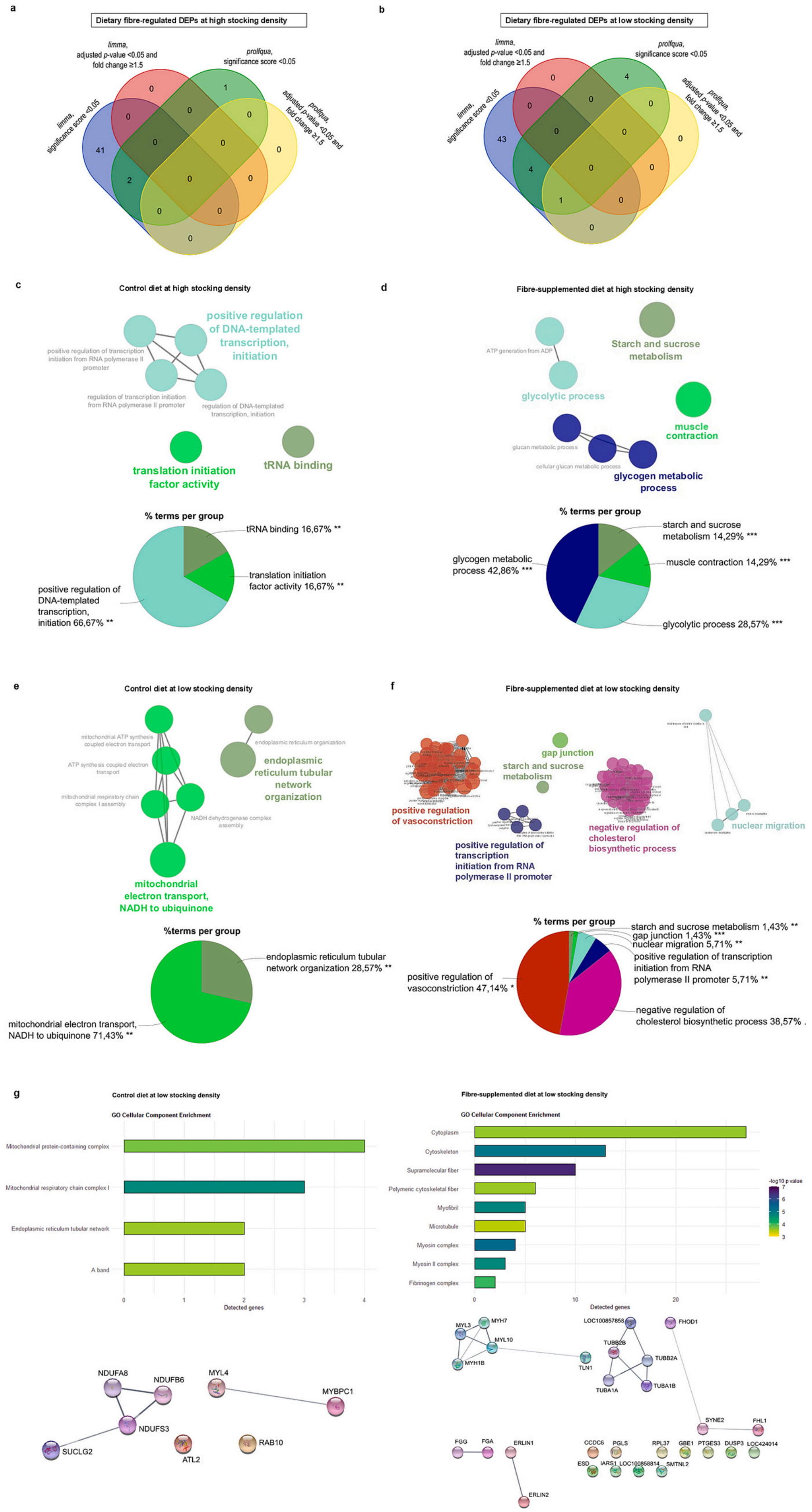

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Fig. 4. Quantitative postmortem muscle proteome differences in broilers in response to dietary fibre provision under different space allowances. a Venn diagram illustrating the overlap and divergence in dietary fibre-regulated DEPs in broilers at high stocking density. DEPs were identified using two *R*-packages (*limma* and *prolfqua*), each applied with two different significance thresholds. **b** Venn diagram for broilers raised at low stocking density, identified using the same approaches described in panel a. **c** Network analysis of the enriched functional terms/pathways in broilers raised at high stocking density without added fibre, where the most significant term in each cluster is designated as the leading term and highlighted in the network, data analysed using ClueGo (Benjamini-Hochberg correction; $p \leq 0.05$). The pie chart shows functional groups for the user genes. The label displayed on the chart shows the percentage of genes found compared to all the genes associated with the group. Group significance levels are indicated as ***: if the term $p < 0.001$, **: $0.001 \leq p < 0.01$ and *: $0.01 \leq p \leq 0.05$. **d** ClueGO functional group annotation network for broilers raised at high stocking density with fibre provision, with annotations as described in panel (**c**). **e** ClueGO functional group annotation network for broilers raised at low stocking density without added fibre, with annotations as described for panel (**c**). **f** ClueGO functional group annotation network for broilers raised at low stocking density with fibre provision, with annotations as described in panel (**c**). **g** Bar plot showing GOCC enrichment analysis (Benjamini-Hochberg correction; $p < 0.05$) of DEPs in broilers raised at low stocking density without and with fibre provision, with colour intensity representing statistical significance according to the scale, consistent across all figures for this analysis. Protein-protein interaction network from STRING, visualising the connectivity between the GOCC-enriched proteins.

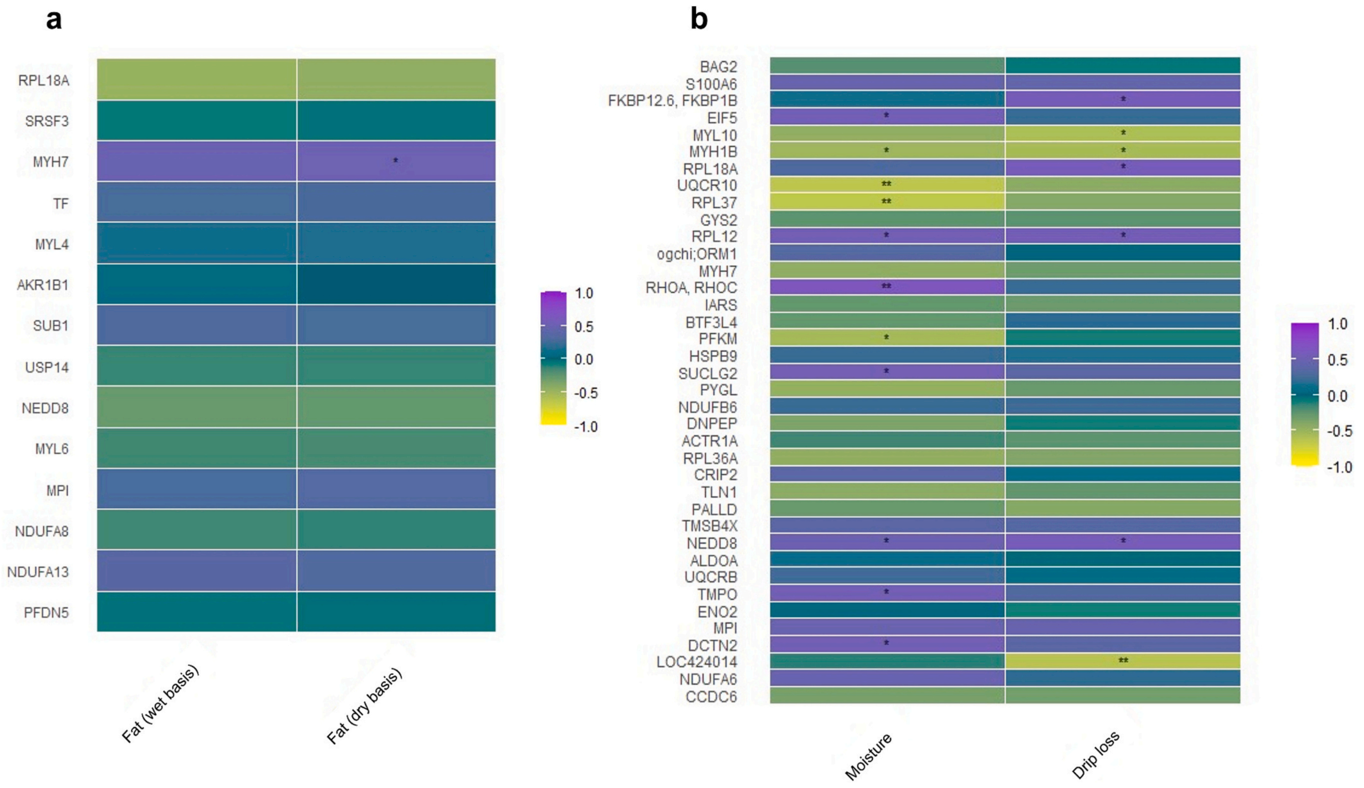

Fig. 5. Linking proteomics DEPs to intrinsic meat quality traits. a Pearson correlation heatmap between protein abundance of space-induced DEPs and intramuscular fat content, measured on a wet and dry basis. **b** Pearson correlation heatmap between protein abundance of diet-induced DEPs and moisture and drip loss traits. Colours represent Pearson correlation coefficients (r, scale: −1 to +1), with yellow indicating a strong negative correlation and purple indicating a strong positive correlation. Stars indicate significance levels: $***p < 0.001$, $**p < 0.01$ and $*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Performance of broilers under different space allowance and diet treatments.

Parameter	LS means treatments				SE	*p*-value		
	HD-CD	HD-DF	LD-CD	LD-DF		Space allowance	Diet	Space allowance x Diet
Growth (g/day)	45.46	45.54	46.48	46.58	0.30	**0.01**	0.80	0.98
Feed intake (g/day)	85.3^b	86.0^b	87.5^a	87.7^a	0.50	**< 0.01**	0.30	0.55
Feed conversion rate	1.88	1.89	1.88	1.88	0.01	0.97	0.63	0.65
Mortality (%)	0.31	0.62	0.39	0.58	0.19	0.93	0.26	0.79
Body weight at marketing (g)	2270	2274	2320	2324	14.9	**0.01**	0.80	0.98

Values are least squares means (LSmeans) and standard error (SE). HD = high density (39 kg/m^2); LD = low density (21 kg/m^2); CD = control diet; DF = dietary fibre-supplemented diet. Superscript letters (a, b) within a row indicate significant differences, where relevant, between treatment means ($p < 0.05$). p values are shown for the main effects and their interaction; significant values are indicated in bold.

<0.05), which indicates higher protein synthesis (Konikkat & Woolford, 2017).

Additionally, LD showed enrichment of calcium ion binding (GO:0005509) (Fig. 2d), driven by MYL4 and MYL6. These genes encode myosin light chains involved in muscle contractile activity and calcium handling (Berchtold et al., 2000), suggesting better muscle functional

Table 2
Meat quality of broilers under different space allowance and diet treatments.

Treatments	pH postmortem 24 h	pH postmortem 48 h	Moisture (%)	Protein (dry basis, %)	Protein (wet basis, %)	Fat (dry basis, %)	Fat (wet basis, %)	Drip loss (%)
Space allowance								
HD	5.82 ± 0.05	5.86 ± 0.05	74.07 ± 0.29	91.79 ± 1.07	23.88 ± 0.25	$1.89^{a} \pm 0.26$	$0.49^{a} \pm 0.08$	0.78 ± 0.13
LD	5.82 ± 0.02	5.87 ± 0.03	74.05 ± 0.30	91.87 ± 1.42	23.84 ± 0.31	$1.57^{b} \pm 0.28$	$0.40^{b} \pm 0.07$	0.94 ± 0.33
p value	0.71	0.67	0.91	0.88	0.73	**< 0.05**	**< 0.05**	0.17
Diet								
CD	5.81 ± 0.03	5.86 ± 0.04	$74.24^{a} \pm 0.25$	92.29 ± 1.26	23.85 ± 0.33	1.78 ± 0.31	0.46 ± 0.09	$0.98^{a} \pm 0.30$
DF	5.83 ± 0.04	5.88 ± 0.04	$73.88^{b} \pm 0.18$	91.37 ± 1.04	23.87 ± 0.22	1.69 ± 0.32	0.44 ± 0.08	$0.73^{b} \pm 0.12$
p value	0.12	0.27	**< 0.05**	0.12	0.92	0.49	0.56	**< 0.05**
Interaction								
HD-CD	$5.78^{a} \pm 0.01$	$5.84^{a} \pm 0.04$	74.21 ± 0.33	92.13 ± 1.26	23.84 ± 0.33	2.00 ± 0.28	0.52 ± 0.08	0.81 ± 0.13
HD-DF	$5.86^{b} \pm 0.05$	$5.89^{a} \pm 0.04$	73.92 ± 0.17	91.74 ± 1.04	23.92 ± 0.18	1.79 ± 0.23	0.46 ± 0.06	0.75 ± 0.14
LD-CD	$5.83^{ab} \pm 0.02$	$5.88^{a} \pm 0.03$	74.28 ± 0.18	92.74 ± 1.25	23.86 ± 0.38	1.57 ± 0.18	0.39 ± 0.03	1.16 ± 0.34
LD-DF	$5.80^{ab} \pm 0.02$	$5.86^{a} \pm 0.04$	73.83 ± 0.20	91.00 ± 1.05	23.81 ± 0.28	1.58 ± 0.39	0.42 ± 0.10	0.72 ± 0.12
p value	**< 0.01**	**< 0.05**	0.51	0.17	0.65	0.43	0.24	0.11

Values are means ± standard deviation. HD = high density (39 kg/m^2); LD = low density (21 kg/m^2); CD = control diet; DF = dietary fibre-supplemented diet. Sample sizes: For main effects (space allowance and diet), $n = 80$ for pH, moisture, protein and drip loss, and $n = 24$ for fat contents. For interaction groups, $n = 40$ for pH, moisture, protein, and drip loss, and $n = 12$ for fat contents. Data were analysed by ANOVA (randomised complete block design) followed by Tukey's post hoc test. Significant values are indicated in bold. Significant differences, where relevant, are indicated by superscript letters (a, b) within a column. For pH at 48 h postmortem, no post hoc differences were detected despite a significant interaction in ANOVA.

capacity (Heissler & Sellers, 2014) under LD relative to HD. These results are consistent with the higher physical activity observed in the LD group (de Jong et al., unpublished results) and highlight the influence of stocking density on muscle metabolism, structure and stress resilience.

3.3. *Adaptive muscle proteomic responses to dietary fibre provision*

To assess the proteomics adaptations in broilers in response to DF, a total of 38 DEP groups were identified using the same statistical criteria described earlier, as illustrated in Fig. 3a (Supplementary Data 5).

The cellular distribution of these diet-induced DEPs (Fig. 3b and Supplementary Data 6) revealed significant enrichment of proteins located in cytoplasmic and cytosolic compartments. Additionally, proteins associated with the cytosolic large ribosomal subunit and ribosome were enriched, highlighting changes in translational activity (Feher, 2012). The simultaneous enrichment of mitochondrial and respiratory chain complex terms indicates changes in OXPHOS and energy metabolism (Feher, 2012). The presence of transmembrane transporter complex proteins revealed alterations in electron transport and ion homeostasis, particularly calcium regulation driven by FKBP1B / FKBP12.6 (Marx et al., 2001). Furthermore, the myosin complex (GO:0016459), comprising MYH7, MYL10, and MYH1B, was also significantly enriched, pointing to structural or contractile adaptations in muscle (Henderson et al., 2017). Collectively, these findings indicate that DF modulates metabolic pathways, protein translation machinery and cytoskeletal organisation.

Functional enrichment analysis of DEPs that were more abundant in DF broilers revealed significant enrichment in carbohydrate metabolism-related pathways (Fig. 3c and Supplementary Data 7). Specifically, pathways involved in starch and sucrose metabolism (KEGG:00500), ATP generation from ADP (GO:0006757), glycolytic processes (GO:0006096) and glucan/glycogen metabolic processes (GO:0044042, GO:0006073, GO:0005977) were significantly enriched. These results suggest enhanced muscle energy metabolism, particularly through glycolysis (ENO2 and PFKM) (Fuller & Kim, 2021). Additionally, enhanced detection of glycogen-related enzymes (GYS2 and PYGL) highlights an adaptive metabolic shift toward improved glycogen storage and utilisation. It potentially enhances immediate ATP availability and metabolic flexibility in response to DF (Kanungo et al., 2018).

In contrast, DEPs that were more abundant in CD broilers (presented in Fig. 3d and Supplementary Data 7) revealed significant enrichment in OXPHOS-related pathways and mitochondrial ATP synthesis coupled electron transport (GO:0006119, GO:0042773, GO:0042775). Key proteins driving these enrichments included NDUFB6, UQCRB and RHOA. Additionally, processes related to skeletal muscle satellite cell migration (GO:1902766) and positive regulation of cell division (GO:0051781) were enriched, primarily through RHOA, RHOC and S100A6. Physiologically, RHOA and RHOC regulate actin cytoskeleton dynamics (Mosaddeghzadeh & Ahmadian, 2021; Vaezi et al., 2002). S100A6 is a Ca^{2+}-binding protein involved in cell proliferation and cytoskeletal remodelling (Donato et al., 2017). Together, these CD-enriched pathways point to higher mitochondrial energy production alongside enhanced muscle fibre turnover or remodelling. Additionally, farm-based observations from the current study (data not shown) indicate that CD broilers exhibited higher physical activity and more frequent feeding behaviours (de Jong et al., unpublished results). These behaviours likely increased mechanical load on the muscle.

Collectively, our proteomics data indicate that DF promotes dynamic glycogen turnover in pectoralis major. Broilers fed DF showed higher abundance of key glycolytic enzymes (PFKM, ENO2) as well as enzymes involved in both synthetic (GYS2) and degradative (PYGL) arms of the glycogen cycle (Kanungo et al., 2018). This profile, therefore, suggests a "store-and-spend" glycogen turnover rather than a simple increase or decrease in net stores. Conversely, the CD pushes muscle toward higher reliance on oxidative metabolism and a more active cytoskeletal/muscle remodelling and/or proliferative state. These divergent metabolic programmes likely arise from DF-induced changes in nutrient flow and gut microbial metabolites (Jha & Mishra, 2021). Additionally, DF can influence feed intake and satiety, thereby affecting energy expenditure and digestive physiology, further contributing to these responses (Ginindza, 2023). Behavioural differences observed in the current study may also contribute to variation in glycogen utilisation and ongoing muscle remodelling. Because pre-slaughter glycogen content and early-postmortem pH were not measured, the impact of these proteomic shifts on rigor-mortis kinetics remains to be tested.

3.4. *Adaptive muscle proteomic responses to dietary fibre provision under different space allowances*

Following analysis of the main effects, we further examined the influence of DF on proteomic responses within each space allowance. We identified 44 DEP groups under HD and 52 DEP groups under LD conditions, as illustrated in Figs. 4a and b (Supplementary Data 8 and 9).

Functional enrichment analysis under HD revealed that DEPs detected at higher levels in CD broilers were primarily linked to transcription/translation regulation (e.g., AIMP1, EIF2S3, EIF4G2, SUB1, LOC107055444) (Fig. 4c and Supplementary Data 10). These broilers appear to activate pathways related to protein synthesis and transcriptional responses. This response may reflect adaptive responses to environmental stress (Garavís & Calvo, 2017; Mortusewicz et al., 2008; Wang et al., 2004; Yu et al., 2016) induced by HD. Alternatively, it may represent metabolic adaptations driven by an accelerated nutrient-utilisation mechanism (Jha & Mishra, 2021) under the CD.

When DF was supplied under HD, DEPs clustered around carbohydrate metabolism and muscle-contractile functions (Fig. 4d and Supplementary Data 10). This pattern suggests a shift in muscle metabolic machinery toward maintaining a rapid energy supply (Fuller & Kim, 2021; Kanungo et al., 2018) and supporting contractile activity (Henderson et al., 2017). However, GOCC enrichment analysis did not identify significant enrichment in protein cellular localisation under these conditions.

Under LD, DEPs identified at higher levels in CD broilers were predominantly associated with endoplasmic reticulum organisation (e.g., ATL2, RAB10) and mitochondrial electron transport/Complex I assembly (e.g., NDUFA8, NDUFB6) (Fig. 4e and Supplementary Data 11). This suggests that muscle cells prioritised organelle remodelling (Chang & Blackstone, 2013), consistent with the space allowance effect. At the same time, they invested in ATP-generating machinery (OXPHOS) (Wirth et al., 2016), aligning with responses previously linked to dietary effects.

Under DF at LD, DEPs also included proteins involved in carbohydrate-related metabolism (e.g., GBE1, PYGL) and in the negative regulation of cholesterol/steroid/lipid/fatty acid biosynthesis (e.g., ERLIN proteins and SREBP signalling pathway) (Huber et al., 2013). Proteins linked to maintaining blood/vascular haemostasis were also enriched with DF, which may indicate improved muscle perfusion (e.g., FGA, FGG, SMTNL2) (Murali & MacDonald, 2018; Wolberg, 2023). Further, DF promoted cell-cell communication via gap junction and cytoskeletal components (tubulin family). It also supported cytoskeletal reorganisation that facilitates organelle and nuclear positioning (e.g., FHOD1, SYNE2, TLN1) (Bone & Starr, 2016; Conti et al., 2009) (Fig. 4f).

These findings were supported by GOCC enrichment analysis (Fig. 4g and Supplementary Data 12). In CD broilers, enrichment was observed in mitochondria-related components, along with the endoplasmic reticulum tubular network and muscle structural proteins related to the A-band. Cytoskeletal and muscle-structural terms, including supramolecular fibres, myosin complexes and cytoskeleton-related proteins were strongly enriched with DF. These diet-induced shifts highlight that DF distinctly modifies cellular structural organisation and contractile adaptations in muscle tissue (Henderson et al., 2017) under LD conditions.

In conclusion, while both density groups exhibited beneficial proteomic adaptations in response to DF, the LD broilers showed a more diverse shift in muscle metabolism and structure compared to HD broilers (Figs. 4c-f). This suggests that DF, when coupled with higher space allowance, unlocks broader physiological benefits, potentially enhancing both muscle function and welfare outcomes. These findings strengthen the evidence from our previous study (Yigitturk et al., 2025), in which positive effects of on-farm environmental enrichment were observed only in slower-growing, not in faster-growing broilers. Taken together, the data suggest that four key factors related to the extensiveness of production: (1) genetics, (2) diet, (3) quantity of space (space allowance) and (4) quality of space (on-farm enrichment materials) (Ludwiczak et al., 2023; Marchewka et al., 2023) exert their full potential only when applied in the right combination and may be less effective or even counterproductive when implemented under suboptimal conditions. This context-dependent efficacy appears to be a core theme in designing extensification strategies.

Supporting these findings, on-farm behavioural observations (data not shown) revealed that broilers under LD with DF exhibited reduced ingestion behaviours, increased inactivity and frequently rested near the roughage bales (de Jong et al., unpublished results). Although higher activity is generally encouraged as a positive welfare trait, the lower activity levels observed in this group may not necessarily be negative. Instead, they may reflect a more relaxed state or engagement in comfort behaviours. This may be due to higher satiety from DF or the opportunity to rest undisturbed near the bales (Forslind et al., 2021, 2022; Tickle et al., 2018). Supporting this interpretation, these behavioural changes did not compromise performance or yield (Table 1, Supplementary Table 1). Moreover, the more restful behavioural profile may reflect a shift in energy allocation away from physical activity to support anabolic investment (Forslind et al., 2021, 2022; Tickle et al., 2018) Such a shift could explain how broilers offset the energetic cost of muscle remodelling and could align with proteomic indicators of improved structural organisation, enhanced cell-cell communication and metabolic efficiency (Figs. 4f and g).

3.5. *Proteomic insights into intrinsic meat quality traits in broilers*

Proteomic profiles of broilers raised under HD revealed coordinated structural and metabolic adaptations aligned with increased IMF content (Table 2). Correlation analysis identified MYH7, typically linked to slow-twitch oxidative fibres (Li et al., 2024), as significantly associated with IMF (Fig. 5a). Although the pectoralis major is predominantly composed of fast-twitch (type II) fibres (Lilburn et al., 2019), the higher abundance of MYH7 suggests adaptive remodelling of contractile or cytoskeletal proteins. Human studies show that a higher proportion of oxidative fibres (type I) is linked to greater intramyocellular lipid content, reflecting enhanced lipid storage capacity (van Loon et al., 2003). A full fibre-type transition is unlikely in broiler breast muscle. However, higher MYH7 expression may represent selective molecular adaptations within glycolytic muscle fibres that favour lipid accumulation under spatial restriction (de Meeûs d'Argenteuil et al., 2021). Supporting this interpretation, higher MYH7 expression in broiler breast has previously been linked to the regulation of muscle growth and development (Kanakachari et al., 2022).

Correlation analysis revealed that several proteins enriched under HD conditions (Fig. 2c and Supplementary Data 4) were positively associated with IMF (Fig. 5a). These proteins involved in nucleic acid-templated transcriptional regulation (MPI, NDUFA13, SUB1, TF), transition metal ion binding (MPI, TF) and fructose and mannose metabolism (AKR1B1, MPI). The DEPs identified in this study were mapped onto key energy metabolism pathways and are shown in Fig. 6. AKR1B1 converts glucose to sorbitol/fructose while generating cytosolic NADPH (Pastel et al., 2012) and MPI recycles mannose-6-P to fructose-6-P (Sharma et al., 2014). Together, AKR1B1 and MPI reroute carbohydrate flux and supply the metabolic precursors and reducing power required for lipid biosynthesis (Pastel et al., 2012; Sharma et al., 2014). This is consistent with findings in mammalian systems where fructose metabolism potently stimulates de-novo lipogenesis (Johnson et al., 2020; Johnson et al., 2023).

Notably, broilers raised under HD also exhibited reduced physical activity (de Jong et al., unpublished results), which may have contributed to IMF by reducing energy expenditure (Simsek et al., 2009). In contrast, RPL18A was significantly higher in the LD group and negatively correlated with IMF, suggesting a metabolism toward higher ribosomal capacity, translational activity (Konikkat & Woolford, 2017) and leaner tissue accretion. This proteomic profile is consistent with the improved growth performance observed under LD (Table 1), while total protein and moisture contents remained unaffected by density (Table 2).

Together, these findings indicate that the fattening phenotype observed under HD arises from the interplay of cytoskeletal remodelling, transcriptional activation, mitochondrial activity, redox-regulatory adaptations and behavioural energy conservation. While single protein-fat content associations were modest (Fig. 5a), the cumulative effects suggest an environment-induced lipogenic adaptation. This highlights the importance of interpreting proteomic data in a systems-level biological context, in which multiple DEPs can collectively contribute to phenotypic outcomes.

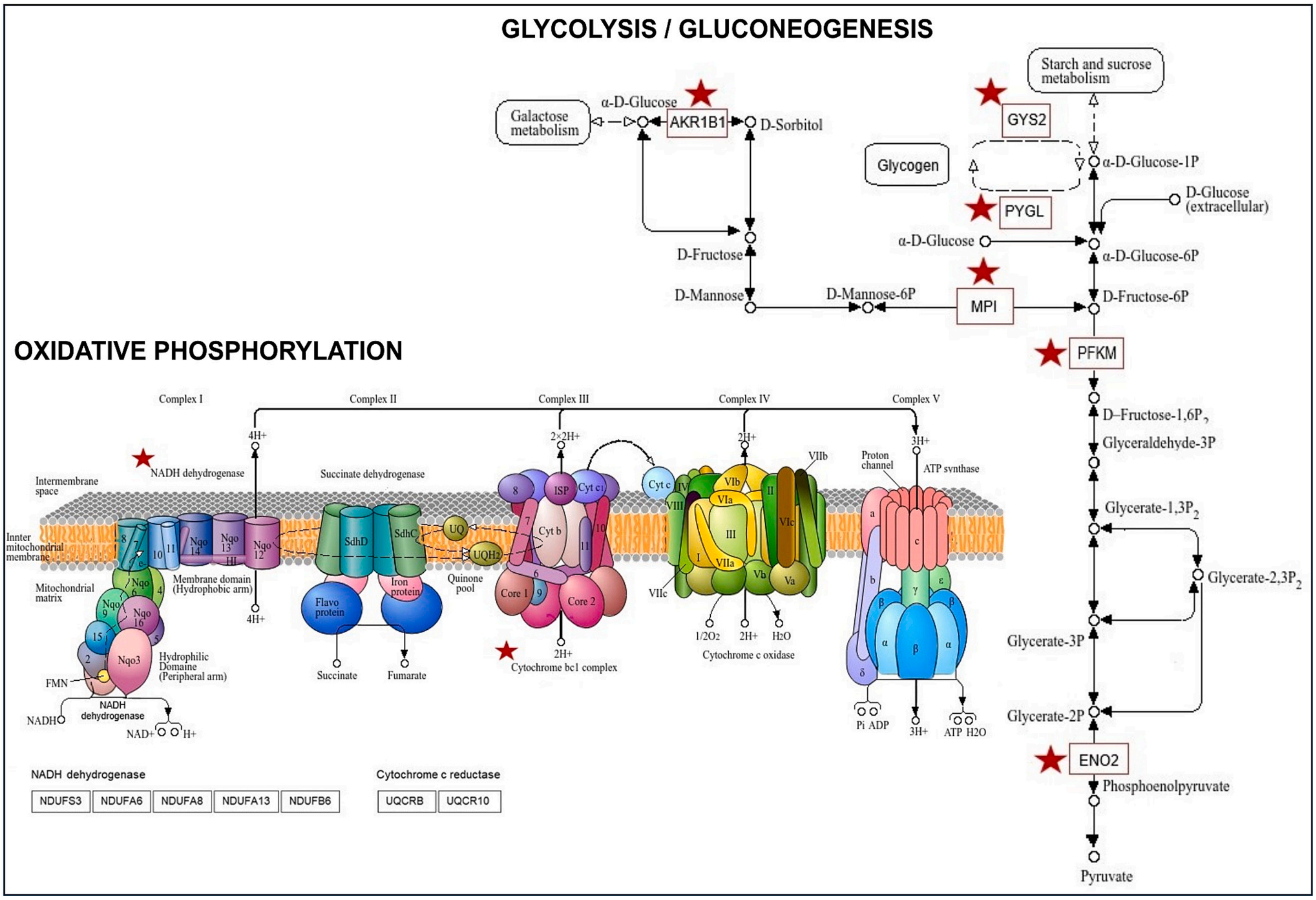

Fig. 6. DEPs involved in key energy metabolism pathways. The illustration combines some proteins identified in this study from glycolysis/gluconeogenesis (KEGG: 00010), fructose and mannose metabolism (KEGG: 00051) and oxidative phosphorylation (KEGG:00190), with annotations based on data provided by Kanehisa Laboratories. Proteins were adapted from KEGG pathway maps and the figure was modified for clarity by omitting unrelated branches. DEPs identified in this study are indicated with red stars. Source: KEGG PATHWAY Database (kegg.jp/kegg/pathway.html). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Broilers receiving the DF displayed enrichment in enzymes involved in glycolysis (ENO2, PFKM) and glycogen metabolism (GYS2, PFKM, PYGL; Fig. 6). These pathways may contribute to improved postmortem water retention. The importance of glycogen-cycle enzymes in modulating water dynamics was also highlighted previously, with low water-holding capacity (WHC) linked to postmortem denaturation of glycogen phosphorylase in broiler breast muscle (Bowker & Zhuang, 2015). Additionally, myosin complex proteins (MYL10 and MYH1B) and LOC424014 (methyltransferase type 11 domain-containing protein) were significantly associated with reduced drip loss, pointing to possible contributions of structural (Henderson et al., 2017) and post-translational modifications (So et al., 2021) to water retention. These results align with previous findings showing that increased WHC in broiler breast muscle is associated with upregulation of glycolysis/gluconeogenesis metabolism (PGM1, LDHA, CKM, FBP2, GAPDH, PGK) and some cytoskeletal proteins (MYH3, MYH1, MYH2, ACTN2) (Zheng et al., 2014). Additionally, under HD conditions, DF led to a modest but significantly higher muscle pH at 24 h postmortem (0.08 units; Table 2). Although increased glycolytic activity is typically associated with lactic acid accumulation and pH decline (Bochereau et al., 2024; Kiyimba et al., 2023), this was not observed here. Instead, the combined proteomic and phenotypic evidence suggests that DF supported more controlled glycogen turnover, thereby preventing rapid postmortem acidification.

In contrast, CD broilers exhibited enrichment in OXPHOS related proteins (NDUFB6, UQCRB, RHOA) and cytoskeletal regulators (RHOA, RHOC, S100A6) (Fig. 3d and Supplementary Data 7). While these processes may benefit muscle remodelling, potentially reflecting increased physical activity and feeding behaviours (de Jong et al., unpublished results), they may also compromise water retention. Notably, FKBP1B/FKBP12.6, a regulator of electron transport, Ca^{2+}-mediated ion homeostasis (Marx et al., 2001) and cellular stress responses (Kim et al., 2018), was more abundant in CD broilers and positively correlated with drip loss (Fig. 5b).

Positive correlations between drip loss and some ribosomal proteins (RPL18A, RPL12), as well as the ubiquitin-like modifier NEDD8, point to increased translational activity and protein turnover (Glickman & Ciechanover, 2002; Konikkat & Woolford, 2017). Conversely, the opposing trends in RPL37 and RPL36A suggest an inconsistency among ribosomal protein responses. This may reflect functionally distinct ribosomal subpopulations that differentially regulate postmortem water dynamics, a hypothesis warranting further investigation.

Together, these data suggest that higher drip loss in CD broilers may result from a multifactorial mechanism through a more OXPHOS reliance (Fig. 6), Ca^{2+}-mediated stress signalling, cytoskeletal plasticity and higher translational muscular dynamics. These results are consistent with mechanisms summarised in a review which links WHC variation across species to metabolic, structural and stress response proteins (Huang et al., 2020).

Beyond its demonstrated effects on meat quality and welfare, DF may also enhance flavour development. This is likely driven by its prebiotic properties, which shape gut microbiota and influence the generation of flavour-active metabolites (Yue 岳珂 et al., 2024). Furthermore, alterations in cholesterol/steroid/lipid/fatty acid biosynthesis observed in fibre-supplemented broilers under LD offer promising avenues to explore sensory traits in relation to proteomic profiles. Additionally, the higher IMF detected under HD may contribute to improved flavour perception (Yue 岳珂 et al., 2024), emphasising a nuanced view of the trade-offs between growth environment, welfare and sensory quality.

4. Conclusion

The results of this study demonstrate that both space allowance and dietary fibre trigger distinct proteomic responses in the broiler pectoralis major muscle, with measurable effects on meat quality. Broilers raised at HD showed proteomic profiles associated with oxidative metabolism, stress and transcriptional adaptation, accompanied by increased IMF and potential shifts in cytoskeletal architecture. Genotypes that maintain stable IMF and show a weaker stress-like proteomic shift may represent preferable selection targets for breeding programs aimed at welfare-aligned broiler production. These proteomic traits may therefore serve as indicators of environmental adaptability. The inclusion of DF, particularly under space-limited conditions, promoted glycolytic and glycogen metabolic processes, enhanced structural protein networks and reduced indicators of transcriptional and oxidative stress. However, when space allowance was high, DF unlocked broader biological benefits for muscle integrity and quality. These included modulation of cholesterol/steroid/lipid/fatty acid biosynthesis, cell-cell communication and muscle structural reorganisation, possibly facilitated by behavioural relaxation and greater anabolic investment. From a breeding perspective, the regulated metabolic and structural proteins identified in this study provide candidate molecular indicators that can be validated across genotypes to support selection for robust water-retention. From a production perspective, improvements in water-retention were consistent across space allowances, indicating a generalisable benefit of DF. Notably, breed-specific behavioural responses observed in Hubbard JA757, a slower-growing genotype, suggest that genetic background plays a role in how broilers respond to these environmental and dietary interventions.

Importantly, these findings have implications not only for meat quality, including consumer- relevant attributes such as flavour development and animal welfare, but also for environmental sustainability and resource efficiency. Interventions that improve meat quality, reduce drip loss and promote more stable postmortem muscle characteristics may collectively minimise resource waste and environmental impact per unit of edible meat. By aligning production strategies with both quality and welfare goals, this study provides a systems-level perspective for developing ethical, efficient and high-quality broiler farming strategies. Future research should focus on validating these molecular associations and further exploring how extensification factors influence other aspects of meat quality.

CRediT authorship contribution statement

Seren Yigitturk: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ingrid C. de Jong:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Sjef Boeren:** Writing – review & editing, Supervision, Resources, Data curation. **Shai Barbut:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Vincenzo Fogliano:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Sara W. Erasmus:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2026.148964.

Data availability

I have shared the data at the Attach File step.

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