71st ICoMST
GIRONA - CATALONIA - SPAIN
3rd-8th August - 2025

SWATH-MS SHOTGUN PROTEOMICS TO CHARACTERIZE THE WOODEN BREAST MYOPATHY AND EFFECTS OF EXERCISE ON MUSCLE PROTEOME IN BROILER CHICKENS

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I. INTRODUCTION

Wooden breast (WB) is a prevalent muscle disorder in broiler chickens and is strongly related with rapid growth and high breast yield from intensive breeding [1, 2]. It affects primarily the *Pectoralis major* muscle, exhibiting hard and rigid texture with reduced water-holding capacity, which lowers consumer appeal and causes immense economic losses [2]. Although its exact etiology remains unclear, hypoxia-induced oxidative damage is increasingly recognized as a key factor in its pathogenesis [2]. Increasing broiler activity has been proposed as a cost-effective strategy to mitigate WB, potentially by enhancing blood flow and oxidative capacity [3]. This study applied a shotgun proteomics approach using sequential window acquisition of all theoretical mass spectra (SWATH-MS) and compared the early *post-mortem* muscle proteome of normal and WB-affected broilers with and without exposure to physical exercise.

II. MATERIALS AND METHODS

Ross 308 broiler chicks were assigned to 16 pens (15 broliers per pen, 2 m^2 each) located in a single hall. Pens were divided into 2 treatments: with or without physical hurdles. From day 5 onward, the hurdle height and incline were 5 cm at 30°; from day 10 onward, 20 cm at 55°; and from day 20 onward, 25 cm at 55°. At 42 days of age, 24 broilers from each treatment were randomly selected, euthanized and slaughtered. WB myopathy was assessed by palpation following a previously established method [4]; birds classified as WB hard were considered affected. Samples from the cranial (superficial part) of the *Pectoralis major* were collected early post-mortem, snap-frozen in liquid nitrogen, and stored at −80 °C for proteomics. Total muscle proteins from randomly selected unaffected (i.e., control) and WB-affected birds (n=4 per treatment) were extracted from frozen samples, quantified using the Bradford assay, and protein bands were prepared using SDS-PAGE as described [5], before their excision and analysis by SWATH-MS. Statistical analyses were performed using the MetaboAnalyst 6.0 platform. First, the data were normalized using $\log_{10}$ transformation and Pareto scaling. Then, pairwise comparisons between control and WB-affected muscle proteomes within each treatment were performed using volcano plots, applying a 1.2-fold change and a significance level of $P < 0.05$. Subsequently, bioinformatics analyses were applied on the differentially abundant proteins (DAPs) using the open-source tool Metascape® to assess the enriched Gene Ontology (GO) terms. Additionally, pathway and process enrichments were visualized using heatmap clustering analyses.

III. RESULTS AND DISCUSSION

A total of 1419 proteins were identified and quantified at an FDR of 1%. With hurdle treatment, 69 proteins were differentially abundant between control and WB-affected muscles (Figure 1A), with 41 down-regulated in WB. Without hurdle treatment, 115 proteins were differential abundant (Figure 1B), with 94 down-regulated in WB. The overlap analysis identified only FABP4 (fatty acid-binding protein) as common protein. The results of the pathway enrichment analysis are given in Figure 1C & D, highlighting to the top enriched GO terms within each comparison. Heatmap clustering of the up- and down-regulated proteins (Figure 1E) identified significantly enriched GO terms specific to each condition. Seven GO terms were specific to WB-affected muscles with hurdle treatment: “generation of precursor metabolites and energy”, “supramolecular fiber organization”, “striated muscle tissue development”, “ribose phosphate metabolic process”, “neuromuscular process”, “regulation of cation channel activity”, and “postsynaptic actin cytoskeleton organization”. Five GO terms were specific for control with hurdle: “cellular response to tumor necrosis factor”, “supramolecular fiber organization”, “neuromuscular process”, “muscle system process”, “positive regulation of organelle organization”. Four GO terms were specific for WB-affected muscles without hurdle: “generation of precursor metabolites and energy”, “cellular response to nutrient levels”, “ribose phosphate metabolic process”, “glucose metabolic process”. Thirteen GO terms were specific for control without hurdle: “generation of precursor metabolites and energy”, “supramolecular fiber organization”, “reticulophagy”, “carbohydrate catabolic process”, “ribose phosphate metabolic process”, “spindle assembly”, “renal sodium excretion”, “muscle system process”, “telomerase holoenzyme complex assembly”, “regulation of plasma membrane bounded cell projection

organization", "cardiac muscle hypertrophy", "positive regulation of organelle organization", "organelle localization". The results revealed disparity and distinct biological processes behind control and WB-affected broiler muscles under hurdle and non-hurdle conditions. The recurrent enrichment of "generation of precursor metabolites and energy" and "ribose phosphate metabolic process" pathways in WB-affected muscles reflects disrupted energy metabolism, likely due to mitochondrial dysfunction [6]. Enrichment of "striated muscle tissue development" and "regulation of cation channel activity" in WB-affected muscles with hurdle suggests activation of pathways related to muscle remodeling and calcium signaling. The control group with hurdle showed enrichment of "muscle system process" and "positive regulation of organelle organization," suggesting enhanced muscle resilience and maintenance. Interestingly, both WB and control groups with hurdle shared pathways related to the "supramolecular fiber organization" and "neuromuscular process," suggesting structural and functional adaptations induced by physical activity. In contrast, WB-affected birds without hurdle exhibited unique enrichment in "glucose metabolic process" and "cellular response to nutrient levels," indicating a stress-induced metabolic response. The control group without hurdle displayed the most diverse GO profile, with unique enrichment in pathways like "reticulophagy," "carbohydrate catabolic process," and "telomerase holoenzyme complex assembly," indicating efficient energy use.

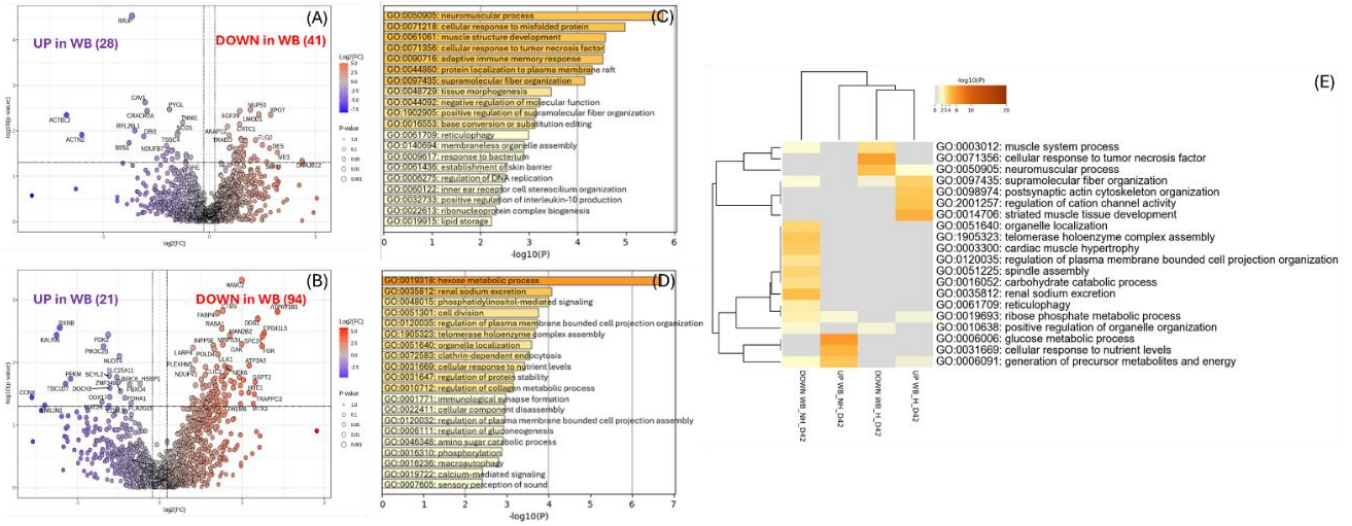


Figure 1. (A, B) Volcano plots showing the DAPs for each treatment comparison. (C, D) Top 20 enriched Gene Ontology (GO) terms. (E) Heatmap showing significantly enriched GO terms for up- and down-regulated proteins under hurdle (H) and non-hurdle (NH) treatments.

IV. CONCLUSION

This study is the first to demonstrate using SWATH-MS proteomics that WB myopathy in broiler chickens is associated with significant alterations in muscle proteome, particularly in pathways related to energy metabolism, muscle structure, and cellular stress responses. Physical activity altered these pathways, suggesting a potential influence on the progression of WB myopathy. Notably, WB-affected birds without exercise showed distinct molecular signatures, while exercise exhibited enrichment of GO terms linked to muscle development.

ACKNOWLEDGEMENTS

This work was financed by the WB2go project (Broiler Wooden Breast myopathy: Underlying mechanisms, and maternal nutrition for alleviation) supported by the Research Council of Finland (funding number 348385).

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