Influence of feeding leucine-supplemented milk replacer on the muscle proteome and metabolic pathways in neonatal dairy calves

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This study evaluated the influence of leucine supplementation on the muscle proteome in neonatal dairy calves fed milk replacer. Our findings demonstrated that leucine supplementation altered the muscle proteome with changes in expression of proteins that promote protein synthesis and metabolic efficiency, which potentially improved growth and long-term productivity. These results highlight the potential of targeted neonatal nutritional interventions — such as leucine supplementation — to optimize muscle development and efficiency in dairy calves, contributing to more sustainable and productive livestock production systems.

Summary

Early postnatal nutrition is critical for muscle growth and metabolic programming in livestock. Leucine, a branched-chain amino acid, activates the mTOR signaling pathway to stimulate protein synthesis, yet its effects on the muscle proteome of neonatal dairy calves are not well understood. This study evaluated how leucine supplementation in milk replacer (MR) affects muscle proteomic profiles after 28 days of treatment. Thirty-five newborn Holstein heifer calves were randomly assigned to one of three groups: the control group (no added amino acids), Leucine-supplemented (5% in MR) or Alanine-supplemented (isonitrogenous to leucine). All calves were fed equal volumes of MR for 56 days. Muscle biopsies collected on day 28 were analyzed using data-independent acquisition (DIA) proteomics via Orbitrap Exploris 480, with protein quantification in Spectronaut. Protein-protein interaction (PPI) networks and Gene Ontology (GO) enrichment were analyzed using STRING v12.5. Leucine supplementation upregulated 25 proteins compared to the control group and 15 compared to the Alanine-supplemented group, with enrichment in pathways related to amino acid and nucleotide metabolism, protein synthesis and cell proliferation. Hormone-related proteins (dehydroepiandrosterone, estradiol, progesterone) and caseins were enriched in both comparisons, suggesting hormonal responses to supplemental amino acids. Proteins associated with catabolic processes were downregulated, indicating a shift toward an anabolic muscle environment. These findings demonstrate that leucine supplementation in milk replacer might promote favorable proteomic and metabolic changes in neonatal calves, which could enhance muscle development and efficiency of growth — key goals for improving productivity and sustainability in modern dairy production.

Introduction

Achieving optimal growth and productivity in dairy cattle is a central goal of the industry, with early-life nutrition playing a critical role in determining lifetime performance factors such as future milk production and overall herd health. Proper muscle development and efficient nutrient utilization during the neonatal stage are essential to establishing this foundation. However, many preweaning feeding strategies may not fully support these outcomes. In the first weeks of life, calves depend entirely on milk or milk replacer for nutrition because their rumen is not yet developed. As a result, digestion takes place mainly in the abomasum and intestines (Abe et al., 1979), allowing nutrients to be absorbed without substantial fermentation. Evidence suggests that providing a higher nutritional plane during the preweaning period can enhance milk yield later in life (Soberon et al., 2012), highlighting the importance of optimizing early-life

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feeding practices to support long-term productivity.

Leucine, an essential branched-chain amino acid, is known for its unique ability to stimulate skeletal muscle protein synthesis and regulate metabolism through activation of the mTORC1 signaling pathway (Drummond and Rasmussen, 2008). Although leucine is abundant in muscle tissue and recognized for its metabolic functions, its specific role in supporting muscle development in neonatal dairy calves remains relatively underexplored. Because skeletal muscle grows rapidly during the preweaning period, ensuring an adequate and balanced supply of amino acids during this stage could improve muscle growth, feed efficiency and, ultimately, productivity. This study aimed to address this knowledge gap by determining the effect of leucine supplementation in milk replacer on the muscle proteome of neonatal dairy calves at 28 days of age, providing insight into the mechanisms of early-life nutritional programming.

Experimental Procedures

All animal procedures were approved by the North Dakota State University Animal Care and Use Committee. The study was conducted at the NDSU Dairy Unit using 35 neonatal Holstein heifer calves. After a three-day colostrum-feeding period following birth, calves were assigned to individual calf hutches and began their respective dietary treatments. Calves were randomly assigned to one of three treatments: the control group (no added amino acids), Leucine (Leu) supplemented at 5% in the milk replacer or Alanine (Ala) supplemented isonitrogenously to match the nitrogen content of the Leu treatment. Milk replacer was provided twice daily, with 3 quarts per feeding, for a total of 56 days. At the end of the treatment period, calves were weaned and moved to group housing.

On day 28 of amino acid treatment, muscle biopsies were collected from the longissimus dorsi, snap-frozen in liquid nitrogen and stored at minus 80 degrees Celsius until analysis. Samples were submitted to the IDeA National Resource for Quantitative Proteomics for analysis. Proteomic profiling was performed using Data-Independent Acquisition (DIA) on an Orbitrap Exploris 480 mass spectrometer, and protein quantification was conducted using Spectronaut software. Protein-protein interaction (PPI) networks and Gene Ontology (GO) enrichment analyses were subsequently performed using STRING v12.5 to identify functional pathways and interactions associated with treatment effects.

Results and Discussion

Leucine supplementation resulted in distinct proteomic changes in the muscle tissue of neonatal dairy calves at 28 days of age. Compared to the control group, 59 proteins were differentially expressed in the leucine group (25 upregulated, 34 downregulated), while 24 proteins differed between the leucine and alanine groups (15 upregulated, nine downregulated; Table 1). In contrast, the alanine group showed a larger number of differentially expressed proteins relative to the control group (84 total; 19 upregulated, 65 downregulated) (Table 1), suggesting that leucine induces more targeted molecular effects than alanine.

Protein-protein interaction network analyses further highlighted the coordinated nature of these molecular changes. The upregulated proteins in leucine-supplemented calves (vs. control and alanine) formed highly connected networks (PPI enrichment *P*-values ≤ 4e-06 and 7.2e-07, respectively; Figure 1), indicating organized activation of anabolic and metabolic pathways. In contrast, downregulated proteins in leucine vs. alanine lacked significant network connectivity (PPI *P*-value = 1), reflecting a less coordinated suppression of pathways. Notably, the proteomic response to leucine supplementation was more focused than the broader, less targeted changes observed with alanine supplementation, which may be indicative of the role that leucine has as a regulator of muscle development at the molecular level. The CSN (casein) proteins were commonly found among the upregulated proteins in leucine-supplemented groups (Leu vs. Con and Leu vs. Ala), highlighting a potential link between leucine and casein-associated pathways in muscle growth and nutrient utilization. This suggests that leucine may not only enhance muscle protein synthesis through classical anabolic signaling but also modulate structural and regulatory proteins such as caseins that contribute to muscle fiber integrity and metabolic activity.

Interestingly, as shown in Figure 2A, the pathways associated with the upregulated proteins in leucine

Table 1: Differentially expressed proteins in muscle tissue of neonatal calves at 28 days of treatment across dietary groups ($P \leq 0.05$).

Sl no.	Treatment groups	Total genes	Upregulated	Downregulated
1	Alanine vs. Control	84	19	65
2	Leucine vs. Control	59	25	34
3	Leucine vs. Alanine	24	15	9

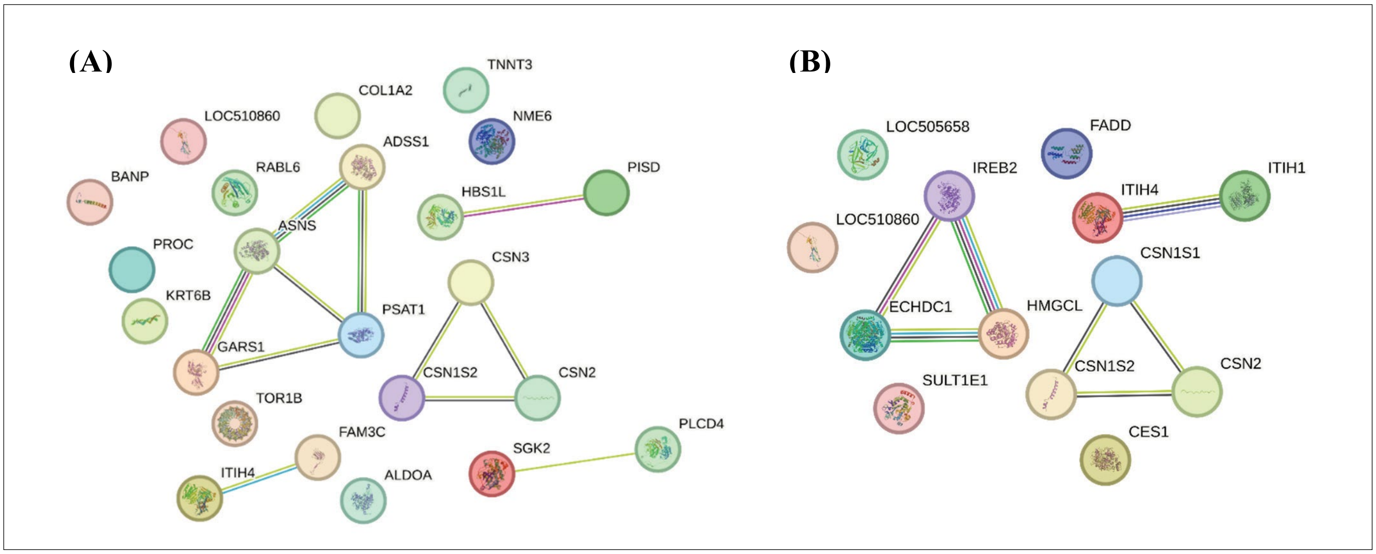

Figure 1: Protein-protein interaction networks of significantly upregulated proteins in neonatal calf muscle following leucine supplementation. (A) Network of proteins significantly upregulated (FDR ≤ 0.05) in the Leucine vs. Control comparison. (B) Network of proteins significantly upregulated (FDR ≤ 0.05) in the Leucine vs. Alanine comparison.

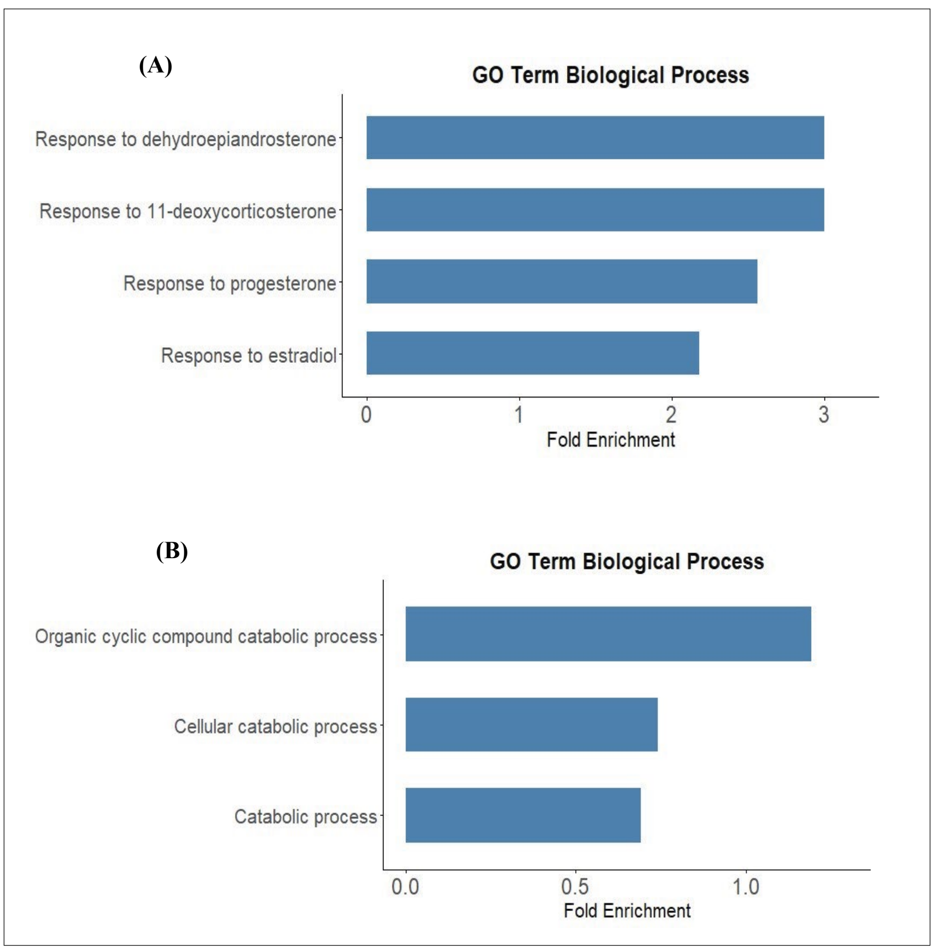

Figure 2: Gene Ontology (GO) biological processes enriched among differentially expressed proteins in neonatal calf muscle following leucine supplementation. (A) GO biological processes associated with proteins significantly upregulated (FDR ≤ 0.05) in Leucine vs. Control and Leucine vs. Alanine comparisons. (B) GO biological processes associated with proteins significantly downregulated (FDR ≤ 0.05) in Leucine vs. Control comparison.

supplementation groups were involved with steroid hormone responses, including pathways involving dehydroepiandrosterone, estradiol and progesterone. These findings suggest that leucine may influence hormonal pathways that contribute to muscle growth and nutrient metabolism during the neonatal stage. The downregulated proteins in leucine-supplemented calves were largely associated with catabolic processes, further supporting a metabolic shift toward an anabolic state (Figure 2B).

These findings demonstrate that targeted leucine supplementation to calves fed milk replacer influences the expression of proteins that enhance anabolic processes and reduce catabolic pathways, with potential implications for improving feed efficiency and long-term productivity in dairy cattle. Optimizing early-life amino acid nutrition through milk replacer formulations represents a promising strategy for developmental programming in modern dairy production systems.

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