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Effect of Omega-3 fatty acids and N-acetyl L-tryptophan supplementation on skeletal muscle proteolysis, amino acid metabolism, and inflammatory biomarkers in transition dairy cows

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Abstract

The postpartum period in dairy cows is characterized by profound metabolic challenges, including negative energy balance (EB) and extensive protein mobilization. Excessive muscle proteolysis may compromise health and performance. This study evaluated the effects of omega-3 (O3) fatty acids and N-acetyl L-tryptophan (NAT) supplementation on skeletal muscle protein metabolism, plasma biomarkers, and amino acid profiles in high-yielding multiparous Holstein cows. Forty-eight cows producing ≥30 kg milk/day were assigned randomly to four groups: control, O3, NAT, and O3+NAT from −21 to +42 days relative to calving. Muscle biopsies were collected on days +21 and +42 postpartum for analysis of ubiquitin–proteasome system (*UPS*) genes (*UBE2G1*, *UBE2G2*, *UBA1*, *MuRF1*, *Atrogin-1*) and mTOR signaling genes (*mTOR*, *EIF3K*, *4EBP1*). Blood samples were meausured at -21, 0 (calving), +21 and +42 days relative to calving for creatinine, 3-methylhistidine (3-MH), and amino acids. Supplementation with O3 and NAT reduced plasma TNF-α, Haptoglobin, creatinine and 3-MH versus control ($P<0.05$), down-regulated proteolysis genes, and up-regulated anabolic signaling, with the greatest responses to O3+NAT ($P<0.01$). Several essential amino acids (His, Lys, Met) were higher with supplementation, while branched-chain amino acids were lower ($P<0.05$). In conclusion, O3 and NAT, particularly the combined O3+NAT group, were associated with attenuated muscle proteolysis and supported anabolic signaling during early lactation. These results reflect that combined O3 and NAT supplementation may provide an enhanced response in regulating skeletal muscle protein metabolism and improving amino acid metabolism during early lactation with practical implications for improving lactation efficiency, metabolic health, and protein utilization in high-producing dairy cows.

Key Words: Transition cow, skeletal muscle proteolysis, omega-3 fatty acids, N-acetyl-L-tryptophan, ubiquitin–proteasome system, amino acid metabolism.

Introduction

Successful adaptation to early lactation depends on the cow's ability to coordinate profound metabolic, endocrine, and nutritional adjustments immediately after calving. Because nutrient demand for milk synthesis increases more rapidly than voluntary feed intake, most dairy cows experience a period of negative energy balance (NEB) during the transition period[1,2]. To maintain energy homeostasis, body reserves are mobilized, with adipose tissue supplying fatty acids and skeletal muscle contributing glucogenic amino acids required to support hepatic glucose production[3]. Although mobilization of skeletal muscle protein is an essential adaptive response that supports glucose homeostasis during early lactation, excessive proteolysis has been linked to reduced reproductive performance, impaired immune competence, metabolic disturbances, and reduced milk yield, all of which can negatively affect overall metabolic efficiency[4,3].

Skeletal muscle protein turnover is tightly regulated by a balance between protein synthesis and degradation. The ubiquitin–proteasome system (UPS) is the primary pathway for proteolysis, involving ubiquitin-activating enzyme (UBA1), ubiquitin-conjugating enzymes (UBE2G1, UBE2G2), and E3 ligases (MuRF1, Atrogin-1), which mediate targeted degradation of myofibrillar proteins[5,6]. In contrast, the mechanistic target of rapamycin (mTOR) pathway regulates protein synthesis, with downstream effectors such as EIF3K and 4EBP1 facilitating translational initiation. Dysregulation of these pathways during early lactation accelerates muscle protein loss and can negatively affect metabolic efficiency. Therefore, nutritional strategies capable of suppressing excessive proteolysis while promoting anabolic signaling may improve metabolic adaptation and postpartum recovery.

Nutritional strategies to minimize proteolysis and enhance protein accretion in transition cows are gaining increasing attention. Among these, omega-3 (O3) fatty acid supplementation has been associated with multiple metabolic benefits in dairy cows, including reduced plasma concentrations of non-esterified fatty acids (NEFA), anti-inflammatory effects, modulation of the endocannabinoid system (ECS), and improved insulin sensitivity[3]. In addition, O3 supplementation has been reported to enhance reproductive performance[3,7], and may suppress ubiquitin–proteasome system (UPS)-mediated skeletal muscle proteolysis, thereby contributing to improved protein preservation during early lactation[8,9].

N-acetyl L-tryptophan (NAT), an acetylated rumen-protected source of tryptophan (Trp), has dual roles as a substrate for protein synthesis and as a precursor for bioactive molecules including serotonin, melatonin, and kynurenine[10]. These metabolites participate in the regulation of oxidative balance, immune responses, and whole-body energy metabolism[11]. Dietary Trp supplementation has been reported to improve antioxidant capacity[12], modulate immune responses, and stimulate feed intake in dairy cows[13,14]. Furthermore, NAT availability may alter mTOR activity and thereby influence protein synthesis[15].

Although the individual metabolic effects of O3 and NAT have been investigated, their combined influence on skeletal muscle protein metabolism during the postpartum period remains unknown. Because these nutritional interventions target distinct yet potentially complementary metabolic pathways, their concurrent supplementation may produce synergistic effects on muscle protein turnover. The present study represents a complementary component of a broader research project investigating the physiological effects of dietary O3 and NAT supplementation in transition dairy cows. Previous findings from this experiment[16], primarily described adipose tissue adaptations and systemic metabolic responses, whereas the current study specifically focuses on skeletal muscle biology by evaluating the expression of genes involved in protein turnover together with changes in plasma amino acid profiles. Together, these complementary studies provide a more

comprehensive understanding of tissue-specific metabolic adaptations to nutritional intervention during the transition period.

Accordingly, this study evaluated the effects of dietary O3, NAT, and their combined supplementation on skeletal muscle gene expression, biomarkers of protein metabolism, and plasma amino acid profiles in multiparous Holstein cows during early lactation. We hypothesized that combined supplementation would attenuate proteolysis, enhance anabolic signaling, and improve circulating amino acid availability compared with either supplement alone or the control diet. Furthermore, NAT supplementation may influence melatonin-related pathways, given the role of melatonin in antioxidant defense.

Materials and Methods

Animal ethics and welfare

All animal experiments were authorized by the Animal Care and Use Committee of Sari Agricultural Sciences and Natural Resources University, Sari, Iran (project no. 1403-12-17). Procedures conformed to national ethical guidance issued by the Iranian Ministry of Science, Research and Technology. Written informed consent and permission for the use of client-owned animals were obtained from the farm owner (Mahdasht Dairy Farm) prior to the start of the study. No animals were euthanized, sacrificed, or killed during or after the experiment; all cows remained in the commercial herd after completion of sampling. Muscle biopsies were performed under local anesthesia on live animals. This study was conducted in accordance with ARRIVE guidelines.

Animals and dietary treatments

Forty-eight multiparous Holstein dairy cows (parity 2.8 ± 0.6, range 2–4, average body weight (BW) 600 ± 50 kg (SD) at calving) were enrolled in a randomized 2 × 2 factorial design study conducted at a commercial dairy farm (Mahdasht Dairy Farm, Sari, northern Iran; 36°34′ N, 53°05′ E; 16 m above sea level). Cows were housed in freestall barns with ad libitum access to water and a total mixed ration (TMR) formulated to meet or exceed NRC (2001) requirements for lactating cows (Table 1). The trial began at -20.2 ± 2.1 days prepartum (mean ± SD) and continued through +42 days postpartum. No acclimatization period was required as cows were already adapted to a similar TMR. The TMR, consistent across all groups, included corn silage, alfalfa hay, soybean meal, corn grain, and a vitamin-mineral premix. Cows were assigned randomly to one of four treatment groups (n = 12 per group): (1) control (0 g/cow O3, 0 g/cow NAT), (2) O3 (100 g/cow/day O3, 0 g/cow NAT), (3) NAT (0 g/cow O3, 100 g/cow/day NAT), or (4) combined O3 and NAT (100 g/cow/day O3, 100 g/cow/day NAT). O3 was provided as rumen-protected fish oil (EPA: DHA ratio 2:1, 60% active ingredient; Megalac Omega, Volac International), and NAT was administered as rumen-protected N-acetyl-tryptophan (98% purity, AminoMax, Balchem Corp.). Supplements were top-dressed on the TMR twice daily at 0700 and 1500 h, with complete intake visually confirmed by farm staff. Individual feed intake was calculated as TMR offered minus TMR refused, and milk yield was recorded automatically for every cow at the three daily milkings (8-h intervals).

Blood Sampling and Plasma Analysis

Blood samples were collected from the coccygeal vein at -20.2, 0 (calving), +21 and +42 days relative to calving at 0600 h, prior to feeding, into 10-mL vacuum tubes containing EDTA. Samples were kept on ice immediately after collection and centrifuged at 3,000 ×g for 15 min at 4 °C to obtain plasma. Aliquots of plasma were stored at −20 °C until analysis.

Measurements of TNF-α, Haptoglobin, NEFA and BHBA

Plasma concentrations of TNF-α were determined by the ELISA method using Bovine commercial kit (Eastbiopharm Inc, China) according to manufacturer's instructions. The samples were analyzed for Hp (Tridelta PHASE Haptoglobin Assay cat. No. TP-801, with Haptoglobin control (cat. No. TP-801-CON) on a Atellica CH from Siemens (Atellica® CH 930 Analyzer, Erlangen, Germany). Selected previously published variables (BW, BCS, NEFA, BHBA) are presented here only as contextual data to aid interpretation of the muscle-related findings of Ghorbanalinia et al., 2026, https://doi.org/10.1038/s41598-026-38923-8)[16]. The present manuscript addresses distinct research objectives and focuses on inflammatory biomarkers, plasma amino acid profiles, muscle protein turnover markers, and skeletal muscle gene expression. The previously published variables are identified and cited to ensure transparency regarding the use of the same experimental population.

3-methylhistidine (HPLC–MS/MS)

Plasma 3-methylhistidine (3-MH) was quantified according to Houweling et al. [17]. Briefly, plasma proteins were precipitated with perchloric acid and centrifuged, and the supernatant was analyzed by HPLC–MS/MS using a C18 reversed-phase column (150 × 2.1 mm, 3 µm particle size). Separation was achieved with a water–acetonitrile gradient containing 0.1% formic acid. Detection was performed in positive electrospray ionization mode using multiple reaction monitoring (MRM) of the protonated molecular ion at m/z 170 and its characteristic fragment ion at m/z 124.

Creatinine (HPLC-UV)

Plasma creatinine was determined using the rapid RP-HPLC method described by Zhao et al. [18]. Proteins were precipitated with acetonitrile, centrifuged, and 20 µL of supernatant was injected for analysis. Separation was carried out on a C18 reversed-phase column (250 × 4.6 mm, 5 µm particle size) under isocratic conditions with a sodium acetate buffer (pH =5.0) containing 1% methanol at a flow rate of 1.0 mL min^{-1}. Detection was performed by UV absorbance at 235 nm, with a total runtime of 4 min.

Free amino acids (LC–MS/MS)

Concentrations of leucine, isoleucine, valine, alanine, phenylalanine, tyrosine, histidine, lysine, and methionine were measured using LC–MS/MS as described by Liu et al. [19]. Plasma proteins were precipitated with cold methanol containing isotope-labeled internal standards. The supernatant was injected onto a C18 reversed-phase column (2.1 × 100 mm, 1.7 µm particle size) and separated using a water–acetonitrile gradient containing 0.1% formic acid. Detection was performed in positive electrospray ionization mode with MRM.

Muscle Tissue Biopsies

Semitendinosus muscle biopsies were obtained after the morning milking at +21 and +42 d postpartum using the technique of Thanh et al. [20]. The second biopsy was collected from the same semitendinosus muscle at a site selected to avoid the previous biopsy site. Briefly, a 20 × 20 cm area over the sampling site was shaved, scrubbed with chlorhexidine (Dermachlor 4%) to reduce microbial contamination, and then disinfected with an iodine-based antiseptic. Local anesthesia was achieved by subcutaneous and intramuscular infiltration of 2% lidocaine hydrochloride (approximately 10–15 mL) around the biopsy site. After allowing 5–10 min for desensitization, a small skin incision (~1 cm) was made using a sterile scalpel blade. A muscle biopsy (~100–150 mg) was obtained using a Bergström needle (6 mm diameter) with suction assistance. The incision was closed using a single absorbable suture, and the site was again disinfected with povidone-iodine. Samples were snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

Quantitative Real-Time PCR (qPCR) Analysis of Gene Expression

Total RNA was isolated from 20 mg of muscle using the Parstous Total RNA Extraction Kit (A101231) per the manufacturer's protocol. RNA integrity was assessed on an Agilent 2100 Bioanalyzer, and only samples with RIN > 8.0 were used. Genomic DNA was digested using DNase I before reverse transcription. First-strand cDNA was synthesized from 1 µg RNA with the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher, 18091050) and stored at −20 °C until analysis. Quantitative real-time PCR was performed on a LightCycler® 96 (Roche) in accordance with MIQE guidelines [21] using SYBR Green master mix (YTA, YT2551) and gene-specific primers (Table 2) targeting mRNA levels of UPS-related genes (*UBE2G1*, *UBE2G2, UBA1, MuRF1, Atrogin-1*) and mTOR pathway genes (*mTOR, EIF3K, 4EBP1*). Cycling conditions were 95$^{\circ C}$ for 5 min, followed by 40 cycles of 95$^{\circ C}$ for 10 s, 60$^{\circ C}$ for 40 s, and 72$^{\circ C}$ for 20 s. Melt-curve analysis confirmed amplicon specificity. Relative transcript abundance was calculated by the $2^{\wedge -\Delta\Delta CT}$ method, normalized to *LRP10*, *GAPDH*, and *EMD* (Emerin) [22]. Primer efficiencies were between 1.9 and 2.1, with $R^2 > 0.98$ for all primers (data not shown).

Statistical Analysis

Data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) using a mixed-effects model for the 2 × 2 factorial design with repeated measures. The model included fixed effects of O3 (0 vs. 100 g/cow/day), NAT (0 vs. 100 g/cow/day), their interaction (O3 × NAT), and time (+21 vs. +42 days for gene expression; -20.2, 0, +21, +42 days for metabolites), with cow included as a random effect to account for repeated measurements within cow. The main effects of O3 and NAT and their interaction were evaluated. When significant effects were detected, least-squares means were compared using Tukey's adjustment for multiple comparisons. Normality was assessed using the Shapiro-Wilk test in PROC UNIVARIATE, and non-normally distributed variables (Haptoglobin, TNF, and 3-MH) were log-transformed before analysis. Parity and BW at enrollment were initially tested as covariates but did not significantly affect any of the response variables ($P > 0.05$) and were therefore removed from the final models. Pearson's and partial correlations were analyzed using PROC CORR, and network visualization was performed using Cytoscape. Significance was declared at $P<0.05$, and $0.05 \leq P < 0.10$ was considered a tendency. Data are presented as least squares means ± standard error of the mean (SEM). Power analysis (PROC POWER) confirmed that n=12 per group provided 80% power to detect a 15% difference in metabolite concentrations and gene expression ($\alpha=0.05$).

Results

Cow performance, feed intake, and energy balance

The enrolled cows were high-producing multiparous Holsteins, with an overall average milk yield of approximately 40 kg/d during early lactation. Dry matter intake (DMI) and milk yield were not affected by dietary supplementation ($P > 0.05$), although milk yield changed significantly over time ($P = 0.001$). Cows receiving combined O3+NAT supplementation maintained higher BW and BCS than control cows ($P < 0.05$), as reported previously[16].

Plasma TNF-α, Haptoglobin, NEFA and BHBA

Plasma concentrations of TNF-α and haptoglobin were influenced significantly by dietary treatment (Table 3). Cows supplemented with O3+NAT exhibited the lowest concentrations of TNF-α and haptoglobin compared with Control. TNF-α declined from 2.2 ng/mL in controls to 1.01 ng/mL in the O3+NAT group ($P < 0.05$), and Haptoglobin similarly decreased from 91.8 mg/dL in control cows to 70.4 mg/dL in cows receiving O3+NAT ($P = 0.02$). Both variables also changed significantly over time ($P < 0.01$), with no significant treatment × time interaction (Table

3). As reported previously for the same cows[16], plasma NEFA and BHBA concentrations were lower in supplemented cows, particularly in the O3+NAT group, whereas TNF-α and haptoglobin are reported here for the first time.

Plasma biomarkers of muscle proteolysis

Dietary treatment also affected plasma concentrations of the muscle protein breakdown biomarkers 3-MH and creatinine (P = 0.02, Table 3). Mean 3-MH concentrations were 5.81, 3.10, 3.60, and 1.30 µmol/L in Control, O3, NAT, and O3+NAT cows, respectively, with the lowest concentration observed in the combined treatment group. Similarly, plasma creatinine concentrations averaged 155, 133, 128, and 102 µmol/L in Control, O3, NAT, and O3+NAT cows, respectively. In contrast to the inflammatory and energy-related biomarkers, neither 3-MH nor creatinine concentrations were affected by sampling time (P = 0.52 and 0.42, respectively), and no treatment × time interactions were detected (P ≥ 0.23).

Plasma amino-acid profile

Dietary treatment significantly affected the concentrations of several circulating amino acids (Table 3). Plasma histidine, lysine, and methionine concentrations were higher in supplemented cows than in control cows, with the greatest concentrations observed in the O3+NAT group. Histidine increased from 40.25 nmol/mL in Control cows to 54.65 nmol/mL in O3+NAT cows, while lysine and methionine increased from 63.30 to 81.50 nmol/mL and from 17.11 to 22.80 nmol/mL, respectively (TRT P ≤ 0.03). In contrast, concentrations of the branched-chain amino acids (BCAA; leucine, isoleucine, and valine) were lower in supplemented cows than in Control cows, with the lowest concentrations observed in the O3+NAT group. Leucine, isoleucine, and valine concentrations decreased from 149.8, 178.9, and 270.9 nmol/mL in Control cows to 126.1, 140.91, and 230.9 nmol/mL in O3+NAT cows, respectively (TRT P ≤ 0.03). Sampling time and treatment × time interactions were not significant for the measured amino acids (P > 0.05, Table 3).

Skeletal-muscle gene expression

qPCR analyses of semitendinosus muscle biopsies collected at +21 and +42 d postpartum showed greatest reductions in the combined O3+NAT group in the expression of genes associated with protein turnover among dietary treatments (Figures 1 and 2). Expression of UPS related-genes (*MuRF1*, *Atrogin-1*, *UBA1*, *UBE2G1/2*) was lower in supplemented cows than in control cows, with the lowest expression generally observed in the combined O3+NAT group. Conversely, expression of *mTOR*, *EIF3K* was higher in supplemented groups, with the highest expression generally detected in cows receiving O3+NAT. Mixed-model analyses included fixed effects of O3, NAT, Day and their interactions, Cow as random, and Day as repeated measure, letters in Figures denote Tukey-adjusted differences at P < 0.05 (within day).

Discussion

Cow performance and feed intake

The limited effects of treatment on DMI and milk yield, together the greater BW and BCS maintained by supplemented cows, suggest that O3 and NAT supplementation may support body reserve preservation without markedly altering overall productive performance during early lactation. These findings indicate that the differences observed in inflammatory, metabolic, and skeletal muscle–related biomarkers were not accompanied by differences in feed intake or milk yield.

Systemic inflammation

Reductions in plasma TNF-α and haptoglobin concentrations observed in cows supplemented with O3 and NAT indicate a modulation of systemic inflammatory status during early lactation, a period characterized by substantial metabolic and inflammatory challenges. The anti-inflammatory response associated with O3 supplementation is consistent with the reported anti-inflammatory effects of long-chain n-3 fatty acids, which may involve to inhibit nuclear factor kappa B (NF-κB) signaling and promoting the production of specialized pro-resolving lipid mediators, including resolvins and protectins, which facilitate the resolution of inflammation rather than simply suppress it[23,24]. These mechanisms may contribute to the lower inflammatory biomarker concentrations observed in the present study.

In addition to inflammatory markers, O3 and NAT supplementation was associated with lower circulating concentrations of TNF-α and Haptoglobin ($P<0.05$), also NEFA and BHBA (previously reported), were also lower. Reduced NEFA concentrations suggest attenuated adipose tissue mobilization, while lower BHBA concentrations could be explained by reduced ketogenesis during early lactation. Similar metabolic responses to O3 fatty acids[25,26,27], and Trp[28,29] supplementation have been reported previously in dairy cows, highlighting their capacity to modulate inflammatory and metabolic responses without adversely affecting milk yield or body condition. Notably, both O3 and NAT each lowered these variables relative to Control, with the greatest reductions observed in the combined O3+NAT group, may provide an enhanced response of these nutrients on systemic inflammatory and metabolic regulation during the postpartum period.

Gene Expression and Muscle Proteolysis

The down-regulation of *MuRF1* and *Atrogin-1* observed in supplemented groups indicates that dietary supplementation was accompanied by altered expression of key regulators of skeletal muscle proteolysis in postpartum dairy cows. These genes are central components of the UPS, which is widely recognized as a primary pathway governing muscle protein degradation in cattle and other mammalian species[24,25]. Although the precise mechanisms underlying these responses cannot be fully elucidated without direct assessment of upstream signaling pathways, the observed transcriptional changes suggest that O3 and NAT may influence proteolytic gene expression. The effects of O3 may be mediated, at least in part, through reducing NF-κB activation and inflammatory signaling[26,8]. This aligns with studies in both cattle and non-ruminant species showing that O3 reduces pro-inflammatory cytokines production and improves insulin sensitivity, thereby limiting activation of proteolytic gene expression[22,23]. Trp supplementation appears to provide a distinct yet complementary mechanism. As a precursor of serotonin and melatonin, Trp plays a regulatory role in stress and endocrine responses[28,13]. By improving serotonin availability, Trp may attenuate the catabolic impact of cortisol, while concurrently supporting IGF-1 secretion and mTOR signaling pathway[14,11,30]. Both pathways are critical for promoting protein synthesis and counteracting muscle degradation. In the present study, control cows exhibited greater expression of *UPS* genes, indicating enhanced muscle proteolysis during early lactation. The greatest suppression of *UPS* genes observed in the O3+NAT group, suggesting that the effects of O3 and NAT may be additive, O3 may act mainly through reduced inflammatory signaling, whereas NAT may favor anabolic signaling. Our results showed that supplementation with O3+NAT has the greatest benefit by down-regulation of UPS-related genes and supporting genes involved in anabolic and translation-related pathways such as *mTOR* and *EIF3K*.

Amino Acid Profiles

McCabe et al quantified adipose and skeletal muscle mobilization, showing that skeletal muscle protein contributes substantially to hepatic gluconeogenesis and milk protein synthesis when

dietary intake is insufficient[3]. Consistent with this, protein mobilization in early lactation, while supporting glucose availability and milk production, has been shown to impair muscle recovery and long-term productivity[31]. Martens et al highlighted that hormonal changes in early lactation promotes muscle breakdown to fill the energy gap, emphasizing that while skeletal muscle supports key metabolic functions, excessive reliance on it can negatively impact health and performance[2].

In our study, lower concentrations of histidine, lysine, and methionine in control cows reflect their mobilization from skeletal muscle for gluconeogenesis or milk protein synthesis during NEB. The restoration of these amino acids in supplemented cows, particularly under O3+NAT treatment, may indicate altered amino acid availability or utilization for anabolic purposes[11,32,33]. In contrast, circulating concentrations of the BCAAs (leucine, isoleucine, and valine) were higher in control cows than in supplemented groups. Elevated circulating BCAAs are typically seen in catabolic states such as starvation and low-insulin conditions, indicating increased muscle proteolysis and altered BCAA catabolism. The higher BCAA levels in early lactation reflect such catabolic pressure, which is supported by proteolysis markers like 3-MH[34,35]. Previous studies have shown that O3 supplementation modulates systemic metabolic markers in ruminants, further supporting the biological plausibility of our findings[36,37]. Thus, the lower BCAA concentrations observed in supplemented cows may reflect altered amino acid utilization and reduced reliance on skeletal muscle protein during early lactation.

An additional and potentially interesting finding was the reduction in circulating tyrosine concentrations in supplemented cows, with the lowest concentration observed in the O3+NAT group. Tyrosine serves as a precursor for catecholamine biosynthesis, including dopamine, norepinephrine, and epinephrine[38]. Tyrosine residues also play a central role in thyroid hormone synthesis through their iodination within thyroglobulin and subsequent formation of T3 and T4[39]. Although thyroid hormones, catecholamines, and oxidative status were not directly assessed in the present study, the treatment-associated reduction in circulating tyrosine raises the possibility that O3 and NAT supplementation may alter amino acid utilization and metabolic or endocrine regulation during early lactation. Further studies incorporating thyroid hormones, catecholamines, and direct markers of oxidative status are needed to clarify the biological significance of this response.

These results are consistent with previous reports highlighting NEB as a major driver of skeletal muscle mobilization to support gluconeogenesis and milk production. However, the reductions in proteolytic markers and the down-regulation of *UPS* genes in cows supplemented with O3, NAT, or both illustrate the potential of targeted nutrition to mitigate catabolism. The most pronounced effects were observed in the combined O3+NAT group, indicating a synergistic action. Additionally, the restoration of essential amino acids in supplemented cows implies improved nitrogen utilization and anabolic efficiency. Rumen-protected tryptophan (RPT) feeding improved nutrient utilization efficiency and lactation performance of dairy cows, which enhanced the quality of milk[11].

Plasma Biomarkers of Muscle Breakdown

Creatinine and 3-MH are established biomarkers of muscle proteolysis, reflecting creatinine turnover and myofibrillar protein degradation, respectively[40]. The higher concentrations observed in control cows are consistent with greater muscle protein turnover during early lactation. The reduction of both metabolites in supplemented cows supporting the gene expression data and suggesting that supplementation attenuates muscle protein breakdown. These findings extend previous reports showing that O3 fatty acids reduce NEFA concentrations and that Trp improves

redox balance and metabolic resilience[8,41,42,28,43]. The greatest reduction in these metabolites in the combined O3+NAT group suggests that O3 and NAT may exert additive effects on muscle protein breakdown, consistent with the previously documented roles of O3 in reducing inflammation and improving insulin sensitivity[8,44], and of Trp in modulating endocrine and antioxidant pathways[28,13]. To our knowledge, this is the first study to demonstrate the combined benefits of O3 and NAT supplementation in postpartum dairy cows.

Although we did not directly measure endocrine mediators or inflammatory pathways (e.g., NF-κB), prior research has shown that long-chain n-3 PUFA supplementation modulates metabolic and inflammatory markers in adipose tissue, liver, and immune cells[45,46]. RPT also influences endocrine parameters like melatonin, prolactin, and IGF-1 [11], and affects circulating serotonin and calcium metabolism in lactating cows[47]. These findings provide biologically plausible mechanisms for the effects of O3 and NAT supplementation, and future studies should directly assess these pathways.

Limitations

This study has several limitations. Gene expression was assessed only at the transcript level, protein abundance and activity of the UPS and mTOR pathways were not measured. Muscle proteolysis was inferred from indirect markers (plasma 3-MH and creatinine) rather than measured directly. Endocrine mediators (e.g., insulin, IGF-1, cortisol, melatonin) and direct markers of oxidative stress were not quantified, limiting mechanistic insight. Biopsies were collected only at +21 and +42 days postpartum from the semitendinosus, so responses at baseline or in other muscles are unknown. Only a single dose of each supplement was tested, and control cows received no placebo carrier. The study was conducted on a single commercial herd under Iranian management and climatic conditions, which may affect generalizability. Finally, longer-term effects on production, reproduction, and health beyond 42 days postpartum were not evaluated.

Conclusion

O3 and NAT supplementation, particularly in combination, was associated with lower circulating markers of muscle proteolysis and higher expression of anabolic signaling genes during early lactation. These responses occurred without changes in feed intake or milk yield. The findings suggest that combined O3 and NAT supplementation may help support skeletal muscle protein ,balance during the postpartum period. Further studies that include protein-level measurements endocrine profiling, multiple muscle sites, and longer follow-up are required to confirm mechanisms, establish optimal doses, and determine effects on production, health, and .reproductive performance

Data availability

The data that support the findings of this study are available, on reasonable request, from the corresponding author.

Author contributions

Mansoureh Ghorbanalinia: Writing & Performing project Resources. **Essa Dirandeh:** Review & editing, Supervision, Project administration, Methodology, Data curation, Conceptualization. **Zarbakht Ansari-Pirsaraei:** Formal analysis. **William W. Thatcher** and **Hassan Sadri:** review & editing.

Additional Information

All information presented in text.

Declaration of generative AI and AI-assisted technologies in the writing

During the preparation of this work, the authors did not use any artificial intelligence (AI) or AI-assisted technologies.

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Declaration of 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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Table 1. Ingredients and nutrient composition (g/kg DM unless otherwise noted) of pre and postpartum diets.

Item	**Prepartum**	**Postpartum**
Ingredient (g/kg DM[1])		
Alfalfa hay mid	244	228
Wheat straw	96	--
Corn silage	288	220
Beet pulp, dehydrated	--	41
Corn grain, ground	141	164
Barley grain, rolled	85	145
Soybean meal, 48%	70	145
Soybean whole, roast	--	8
Cottonseed whole	10	--
Wheat	21	--
Sodium bicarbonate	--	9
Salt	--	4
DCP	--	3
Magnesium oxide	--	3
Glucose	20	13
Choline chloride	8	4
Palm oil[2]	5	3
Mineral and vitamin premix[3]	12	10
Composition (g/kg DM)		
NEL (Mj/kg DM)	6.7	7.3
Fat	32.7	40.8
Crude protein	133.5	171.0
Neutral detergent fiber	368.0	320.0
Acid detergent fiber	231.0	223.0
Non fibrous carbohydrate	365.3	403.9

[1]Gram per kilogram of dry matter.
[2]Energizer-RP10; Iffco, Johor Bahru Johor, Malaysia.
[3]Contained (per kg): 500,000 IU of Vitamin A, 100,000 IU of Vitamin D, 1,000 mg of Vitamin E, 9,000 mg of P, 195,000 mg of Ca, 2,000 mg of Mn, 55,000 mg of Na, 2,000 mg of Zn, 2,000 mg of Fe, 280 mg of Cu, 100 mg of Co, 100 mg of Br, 1 mg of Se, and 3,000 mg of Anti-oxidant.

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Table 2. Primer sequences used for qPCR analysis of genes associated with muscle turnover in Holstein dairy cows.

Gene	Primer sequences 5′→3′	Accession number or reference	Length (bp)
mTOR	F: CGAAACCCTGGATGTCCCAA R: AGGACACCAGCCAATGTAGC	XM_002694043.2	94
UBA1	F: GGGGAACCGGCATTGATGT R: AGGGCACTTCGGACAATACG	NM_001102477.1	118
UBE2G1	F: TATGCTGGCAGACCCCAATG R: TCTTACACAGCGGGCAACTT	NM_001082458.1	109
UBE2G2	F: TCACCCCAACATCTACCCAGA R: AGGAGGATCTTCTCCACGCT	NM_001076328.1	129
EIF3K	F: CCAGGCCCACCAAGAAGAA R: TTATACCTTCCAGGAGGTCCATGT	NM_001034489	125
4EBP1	F: CCCTGGAGGTACCAGGATCA R: CATCGCCTGTAGGGCTAGTG	NM_001077893.2	125
MuRF1	F: CCTGATCCAGGATGGAAACCC R: CAGCCTGCTGGAAGATGTCGT	NM_001046295.1	149
Atrogin-1	F: GAAACGCTTCCTGGACGAGA R: TCTTCTTGGCTGCAACGTCA	NM_001046155.1	125
LRP10	F: CCAGAGGATGAGGACGATGT R: ATAGGGTTGCTGTCCCTGTG	BC149232	139
GAPDH	F: AATGGAAAGGCCATCACCATC R: GTGGTTCACGCCCATCACA	U85042	204
EMD	F: GCCCTCAGCTTCACTCTCAGA R: GAGGCGTTCCCGATCCTT	NM_203361	100

Forward (F) and reverse (R) primer sequences are shown along with the corresponding accession number or reference and the expected amplicon length (bp). Genes include: *mTOR* = mammalian target of rapamycin; *UBA1*= ubiquitin-like modifier activating enzyme 1; *UBE2G1*=ubiquitin conjugating enzyme E2 G1; *UBE2G2*=ubiquitin conjugating enzyme E2 G2; *EIF3K* = eukaryotic translation initiation factor 3 subunit K; *4EBP1* = eukaryotic translation initiation factor 4E binding protein 1; *MuRF1* = muscle ring-finger protein-1; *Atrogin-1*= muscle atrophy F-box; *LRP10*= lipoprotein receptor-related protein 10; *GAPDH* =Glyceraldehyde 3-phosphate dehydrogenase, *EMD*= emerin.

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Table 3. Effect of experimental treatments (Control, O3, NAT, and O3+NAT) on haptoglobin, TNF-α, NEFA, BHBA connotations and plasma biomarkers of muscle breakdown (3-MH and creatinine) and amino acid profiles in Holstein dairy cows.

Variable	Treatments[1]				P-value		
	Control	O3	NAT	O3+NAT	TRT	Time	TRT*Time
Haptoglobin (mg/dL)	91.80±1.80[a]	78.50±1.51[b]	79.80±1.21[b]	70.40±1.30[c]	0.02	<0.01	0.42
TNF-α (ng/mL)	2.20±0.20[a]	1.40±0.21[b]	1.60±0.21[b]	1.01±0.10[c]	0.02	<0.01	0.103
NEFA* (mmol/L)	0.39±0.01[a]	0.31±0.01[b]	0.30±0.01[b]	0.24±0.01[c]	0.03	<0.01	< 0.05
BHBA* (mg/dL)	9.06 ± 0.10[a]	8.33 ± 0.10[b]	8.36 ± 0.10[b]	7.70 ± 0.10[c]	0.03	<0.01	< 0.01
3-MH (µmol/L)	5.81 ± 0.30[a]	3.10 ± 0.31[b]	3.60 ± 0.44 [b]	1.30 ± 0.50[c]	0.02	0.52	0.23
Creatinine (µmol/L)	155 ± 4.5[a]	133 ± 5.5[b]	128 ± 6.3[b]	102 ± 7.2[c]	0.02	0.42	0.56
Amino acid profile (nmol/mL)							
Leucine	149.80±3.10 [a]	136.70±3.00 [b]	135.60±3.20 [b]	126.10±3.33[c]	0.01	0.35	0.65
Isoleucine	178.90±7.20 [a]	159.11±7.51 [b]	157.85±7.62 [b]	140.91±7.50 [c]	0.03	0.23	0.54
Valine	270.90±8.20 [a]	252.87±8.31 [b]	249.91±8.45 [b]	230.9±7.21 [c]	0.01	0.31	0.48
Alanine	210.91±7.80 [a]	190.51±7.49 [b]	187.80±7.90 [b]	170.43±7.30[c]	0.01	0.64	0.61
Phenylalanine	51.90±1.10 [a]	48.30±1.11[b]	47.86±1.28 [b]	46.15±1.01[c]	0.04	0.75	0.37
Tyrosine	45.85±1.20 [a]	42.81±1.20 [b]	42.60±1.31 [b]	40.10±1.05 [c]	0.04	0.44	0.81
Histidine	40.25±2.11 [c]	48.90±2.01[b]	46.54±2.08 [b]	54.65±2.21[a]	0.03	0.19	0.59
Lysine	63.30±2.71 [c]	73.50±2.11 [b]	75.60±2.24 [b]	81.50±2.41 [a]	0.01	0.29	0.31
Methionine	17.11±1.04 [c]	19.61±1.34 [b]	19.90±1.45 [b]	22.80±1.04 [a]	0.01	0.38	0.43

Data are presented as least squares mean ± SEM (n = 12 cows/group). Values with different superscript letters (a, b, c, d) within a row differ significantly ($P < 0.05$). TRT = treatment effect; Time = sampling day; TRT × Time = interaction effect.

*These data were previously published in Ghorbanalinia et al. (2026) and are included here only to support interpretation of muscle-related outcomes.

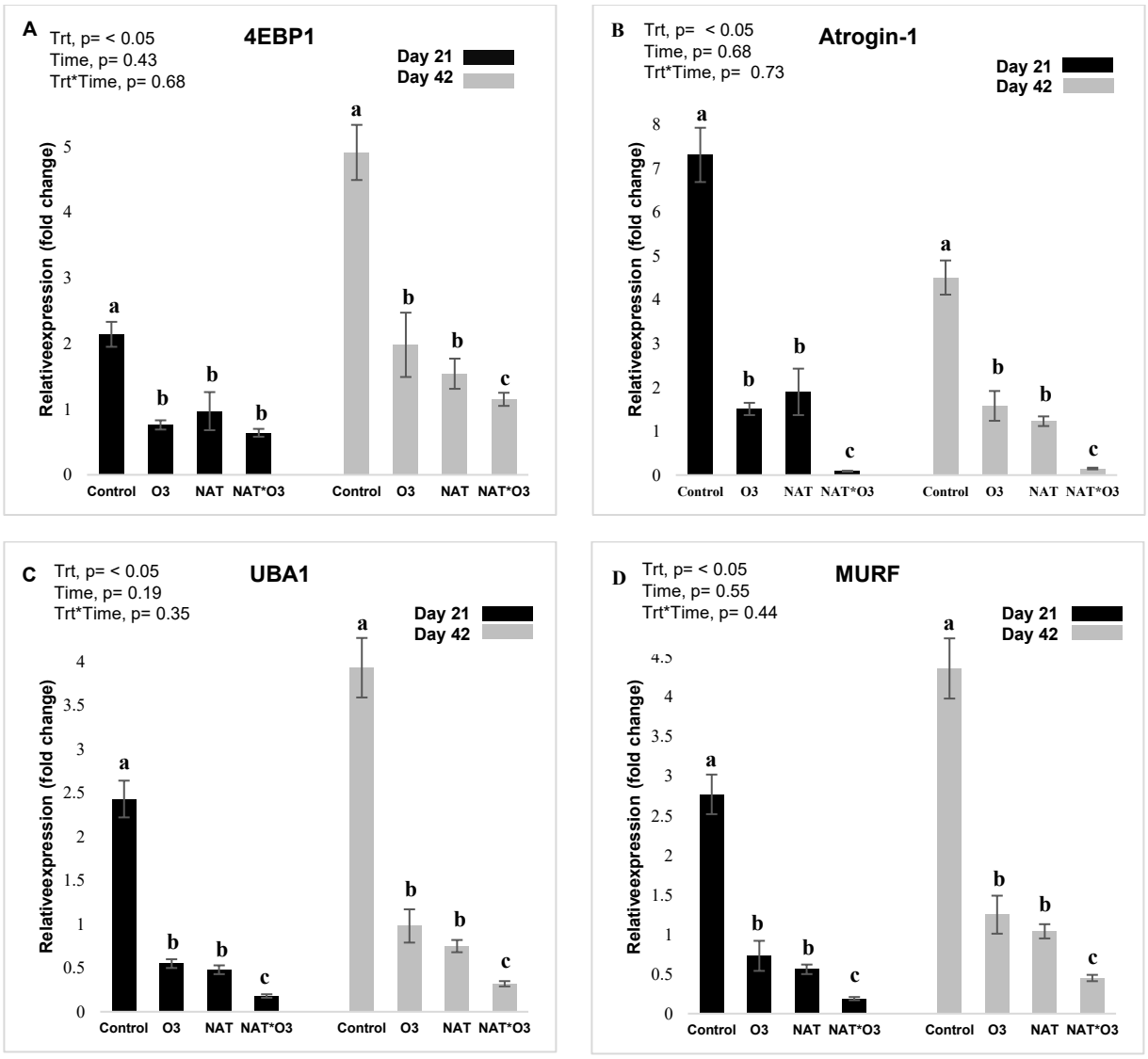


Fig 1. Panels A–D show qPCR expression for *4EBP1*, *Atrogin-1*, *UBA1* and *MuRF1* in semitendinosus biopsies at +21 and +42 d postpartum. Treatments: Control, O3, NAT, O3+NAT (n = 12 per group). Expression was normalized to *LRP10*, *GAPDH*, and *EMD* and analyzed on the ΔCT scale. Genes associated with protein degradation (MuRF1, UBA1, and Atrogin-1) and translation initiation (4EBP1) showed distinct responses to dietary treatments. Within each sampling day, bars with different letters differ significantly ($P < 0.05$). Statistics: linear mixed model (PROC MIXED) with fixed effects O3, NAT, Day (+21, +42) and their interactions; Cow as random subject; Day as repeated factor (covariance chosen by AIC; Kenward–Roger denominator df).

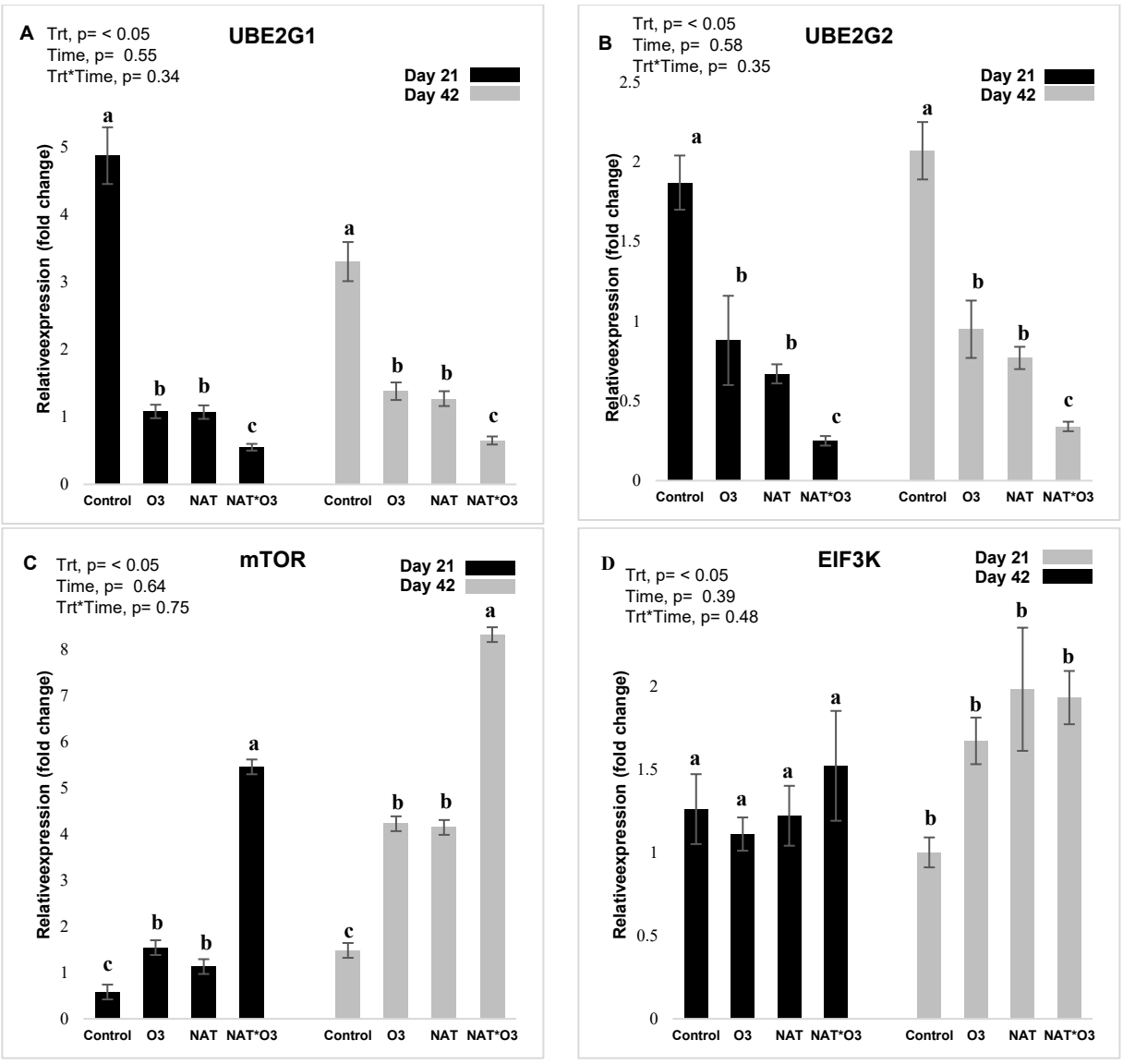


Fig 2. Panels E–H display qPCR expression for the *UBE2G1*, *UBE2G2*, *mTOR*, *EIF3K*, measured at +21 and +42 d postpartum. Treatments: Control, O3, NAT, O3+NAT (n = 12 per group). Expression was normalized to *LRP10*, *GAPDH*, and *EMD* and analyzed on the ΔCT scale. Gene associated with protein synthesis pathways (*mTOR*) and protein degradation pathways (*UBE2G1*, *UBE2G2*) showed distinct regulation in response to dietary treatments. Within each sampling day, bars with different letters differ significantly ($P < 0.05$). Statistics: linear mixed model (PROC MIXED) with fixed effects O3, NAT, Day (+21, +42) and their interactions; Cow as random subject; Day as repeated factor (covariance chosen by AIC; Kenward–Roger denominator df).