Effects of an exogenous ketone ester using multi-omics in skeletal muscle of aging C57BL/6J male mice

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Exogenous ketone ester supplementation provides a means to increase circulating ketone concentrations without the dietary challenges imposed by ketogenic diets. Our group has shown that oral R,S-1,3, butanediol diacetoacetate (BD-AcAc2) consumption results in body weight loss or maintenance with moderate increases in circulating ketones. We have previously shown a diet consisting of 25% BD-AcAc2 can maintain lean body mass (LBM) and induce fat mass (FM) loss in young, healthy male mice, but the underlying mechanisms are still unknown. Therefore, the purpose of this study was to determine if a diet consisting of 25% BD-AcAc2 (ketone ester, KE) would alter body composition, transcriptional regulation, the proteome, and the lipidome of skeletal muscle in aged mice. We hypothesized that the KE group would remain weight stable with improvements in body composition compared to controls, resulting in a healthy aging phenotype. Male C57BL/6J mice (n = 16) were purchased from Jackson Laboratories at 72 weeks of age. After 1 week of acclimation, mice were weighed and randomly assigned to one of two groups (n = 8 per group): control (CON) or KE. A significant group by time interaction was observed for body weight (P < 0.001), with KE fed mice weighing significantly less than CON. FM increased over time in the control group but was unchanged in the KE group. Furthermore, LBM was not different between CON and KE mice despite KE mice weighing less than CON mice. Transcriptional analysis of skeletal muscle identified 6 genes that were significantly higher and 21 genes that were significantly lower in the KE group compared to CON. Lipidomic analysis of skeletal muscle identified no differences between groups for any lipid species, except for fatty acyl chains in triacylglycerol which was 46% lower in the KE group. Proteomics analysis identified 44 proteins that were different between groups, of which 11 were lower and 33 were higher in the KE group compared to CON.
In conclusion, 72-week-old male mice consuming the exogenous KE, BD-AcAc₂, had lower age-related gains in body weight and FM compared to CON mice. Furthermore, transcriptional and proteomics data suggest a signature in skeletal muscle of KE-treated mice consistent with markers of improved skeletal muscle regeneration, improved electron transport chain utilization, and increased insulin sensitivity.

**KEYWORDS**
skeletal muscle, ketone ester, nutrition, sarcopenia, proteomics, lipidomics

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**Introduction**

Ketogenic diets (KDs) improve or maintain metabolic function and attenuate age-related increases in adiposity and body weight, presumably by increasing circulating ketone concentrations in both animal models and humans (1, 2). Clinical studies have demonstrated the beneficial effect of KDs as an efficient nutritional strategy in the treatment of obesity (3–5). This effect occurs not only in modifying body weight and body composition but also in modulating epigenetic markers, as well as circulating levels of cytokines and circulating levels of cytokines and markers of oxidative stress and this effect appears to be induced by the action of ketone bodies synergistically with the weight loss induced by the VLCKD treatment (6–8). In mice, KDs have been shown to extend longevity and healthspan by decreasing oxidative and endoplasmic reticulum (ER) stress resulting in lower protein turnover in skeletal muscle, which may allow greater maintenance of muscle mass and function with both age and disease (9, 10). These findings have practical implications in humans, but by nature of the requirement for extensive carbohydrate restriction and high fat content (often >75%), KDs present adherence challenges and may not be a long-term strategy for all humans.

Exogenous ketone ester (KE) supplementation provides a means to increase circulating ketone concentrations without the dietary challenges imposed by KDs. Ketone esters have been used in many forms such as R,S-1,3-butanediol diacetoacetate (BD-AcAc₂, ketone diester), D-β-hydroxybutyrate-(R)-1,3 butanediol (ketone monoester), and more recently Bis-hexanoyl (R)-1,3-butanediol (BH-BD) (11–13). Studies in animal models show that oral BD-AcAc₂ consumption results in body weight loss or maintenance with moderate increases in circulating ketones (0.5–1.0 mM) (14–16). Recently, we examined concentration-dependent effects of BD-AcAc₂ on body weight, adiposity, energy intake, and energy expenditure in lean mice showing that on an ad libitum basis, mice consuming a 25% (by kcals) KE diet consumed the same amount of food as an ad libitum fed control group, but had a significant difference in body weight (BW) and fat mass (FM), and maintained lean body mass (LBM) (17). Furthermore, after adjustment for LBM and FM, there was no difference in resting energy expenditure (REE) compared to control (17). Our group has also shown that BD-AcAc₂ induces loss of BW and FM in diet-induced obesity without changes in LBM or changes in skeletal muscle thermogenic activity (18). Furthermore, when housed in thermoneutral conditions, mice consuming BD-AcAc₂ decreased body weight resulting in lower adiposity. The decrease in body weight observed in KE-fed mice transpired without an increase in REE or TEE (19). These findings suggest that a diet consisting of 25% BD-AcAc₂ can maintain LBM and induce FM loss in young, healthy male mice, but the underlying mechanisms are still unknown.

Despite extensive evidence of reductions or maintenance of body weight and adiposity, little is known regarding the effects of BD-AcAc₂ on skeletal muscle. Previously, we found that a KE diet does not produce transcriptional changes consistent with mitochondrial biogenesis or respiration (18). Therefore, the purpose of this study was to determine if a diet consisting of 25% BD-AcAc₂ would alter body composition, transcriptional regulation, the proteome, and the lipidome of skeletal muscle in aged B6 mice. We hypothesized that the KE group would remain weight stable with improvements in body composition (higher LBM and lower FM) compared to controls resulting in a healthy aging phenotype.
Materials and methods

Study design and diets

Male C57BL/6 mice (n = 16) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) at 72 weeks of age and acclimated for 7 days. At 73 weeks of age, mice were weighed and randomly assigned control (CON, n = 8) or KE (KE, n = 8) diet. Mice were fed ad libitum for 9 weeks. The KE diet (Dyets Inc., Bethlehem, PA, #104419) with fat and protein content equal between the diets. Each diet was 3.7 kcal/g and details of the individual diets (CON and KE) are described in Deemer et al. (17). The UAB Institutional Animal Care and Use Committee (IACUC) approved the investigation.

Husbandry

Mice were single-housed and maintained on a standard 12:12 light-dark cycle. All animals had access to ad libitum water and food throughout the study. Food intake and body weight were recorded every day, and fresh food was given each day at the same time. Two body composition (fat and lean body mass) measurements were completed in the University of Alabama at Birmingham (UAB) Small Animal Phenotyping Core on day 33 and day 66 of the study by quantitative magnetic resonance (QMR; EchoMedical MRI, Houston, TX, USA). Due to COVID-19 shutdowns body composition was not obtained at baseline.

RNA isolation and nanostring analysis

Tissue samples were flash frozen in liquid nitrogen at the time of dissection and stored at −80°C. RNA from vastus lateralis was isolated according to manufacturer instructions (RNAEasy Mini Kit; Qiagen; Germany). All procedures occurred at room temperature. Briefly, an approximately 30 mg sample was homogenized in Buffer RLT with metal beads using a bead mill (Fisherbrand™ Bead Mill Homogenizer) for 2 min at 5 m/s. Lysate was centrifuged for 3 min and the supernatant was transferred to a new microcentrifuge tube. A 1:1 by volume of 70% ethanol was added to the supernatant and mixed by trituration. Approximately 700 µL of sample was transferred to the Qiagen spin column and isolation of mRNA was completed per manufacturer instructions. The A260/A280 ratio was measured to quantify mRNA purity (Thermo Scientific™ NanoDrop™ Lite Spectrophotometer) and isolated mRNA was concentrated to 15 ng/µL prior to NanoString analysis. Concentrated mRNA was analyzed using a 770-plex Mouse PanCancer Pathways kit (nCounter; NanoString, Seattle, WA, United States) and raw image counts (RCC files) were obtained using a SPRINT Profiler (NanoString) following a 16-h hybridization assay at 65°C.

Data was analyzed by ROSALIND©, with a HyperScale architecture developed by ROSALIND, Inc., (San Diego, CA, USA). Read Distribution percentages, violin plots, identity heatmaps, and sample MDS plots were generated as part of the QC step. Normalization, fold changes, and p-values were calculated using criteria provided by Nanostring. Housekeeping probes to be used for normalization are selected based on the geNorm algorithm as implemented in the NormqPCR R library (20). Fold changes and p-values are calculated using the fast method as described in the nCounter® Advanced Analysis 2.0 User Manual. P-value adjustment is performed using the Benjamini-Hochberg method of estimating false discovery rates (FDR).

Proteomics

Proteomics analysis was carried out as previously referenced with minor differences (21). Proteomics samples were compared in the control (n = 7) and KE groups (n = 6). All protein extracts were obtained using T-PERTM Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Cat# 78510) containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific, Cat# 78429) and Dounce-homogenized in lysis buffer, 20–30 strokes per sample, and centrifuged (~12 Kg) for 10 min at 4°C to remove debris. Protein extracts were then quantified using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Cat# PI23225). Forty (40) µg of protein was diluted in 35 µL of NuPAGE LDS sample buffer (1x final conc., Invitrogen, Cat# NP0007) for each sample. Proteins were then reduced with dithiothreitol (DTT) and denatured at 70°C for 10 min prior to loading onto Novex NuPAGE 10% Bis-Tris protein gels (Invitrogen, Cat# NP0315BOX) and separated (35 min at 200 V). Gels were stained overnight with Novex Colloidal Blue Staining kit (Invitrogen, Cat# LC6025). Following de-staining, each lane was cut into 6-molecular weight (MW) fractions and equilibrated in 100 mM ammonium bicarbonate (AmBc). Each gel plug was then digested overnight with Trypsin Gold, Mass Spectrometry Grade (Promega, Cat# VS280) following the manufacturer’s instructions. Peptide extracts were reconstituted in 0.1% formic acid/ddH2O at 0.1 µg/µL. Peptide digests (8 µL each) were injected onto a 1,260 Infinity nHPLC stack (Agilent Technologies), and separated using a 75 micron i.D. x 15 cm pulled tip C-18 column (Jupiter C-18 300 Å, 5 micron, Phenomenex).

The XCalibur RAW files were collected in profile mode, centroided and converted to MzXML using ReAdW v. 3.5.1. Mgf files were then created using MzXML2Search (included in TPP v. 3.5) for all scans. The data was searched using SEQUEST (Thermo Fisher Scientific), which was set for three maximum missed cleavages, a precursor mass window of 20 ppm, trypsin
digestion, variable modification C at 57.0293, and M @ 15.9949 as a base setting. Searches were performed with redundant sequences removed on the *mus musculus* specific subset of the UniProt100 database. The list of peptide IDs generated based on SEQUEST search results were filtered using Scaffold (Protein Sciences, Portland, OR, USA). The cut-off values included a minimum peptide length of > 5 AA’s, with no MH + 1 charge states, with peptide probabilities of > 80% C.I., and with the number of peptides per protein ≥ 2. The protein probabilities were set to a > 99.0% C.I. and an FDR < 1.0. Scaffold incorporates the two most common methods for statistical validation of large proteome datasets, the false discovery rate (FDR) and protein probability (22–24). Relative quantification across experiments was then performed via spectral counting (25, 26). Spectral count abundances were then normalized between samples (27).

For protein abundance ratios determined, we set a 1.5-fold change as the threshold for significance, determined empirically by analyzing the inner-quartile data from the control experiments using In-In plots, where the Pierson’s correlation coefficient (R) is 0.98, and > 95–99% of the normalized intensities fell between the set fold changes. In each case, both tests (t-test and fold change) were required to pass in order to be considered significant. All multivariate analyses, including 2D HCA HeatMaps and PCA plots were carried out using Qlucore Omics Explorer (Qlucore, Lund Sweden). Gene ontology assignments and pathway analysis were carried out using MetaCore (GeneGO Inc., St. Joseph, MI, USA). Interactions identified within MetaCore are manually correlated using full text articles. Detailed algorithms have been described previously (28, 29).

**Lipidomics**

Lipid species were analyzed using multidimensional mass spectrometry-based shotgun lipidomic analysis (30). In brief, muscle homogenates containing 0.6 mg of protein (quantified by Pierce BCA assay) was transferred to a disposable glass test tube. A premixture of lipid internal standards (IS) was added prior to conducting lipid extraction for quantification of the targeted lipid species. Lipid extraction was performed using a modified Bligh and Dyer procedure (31), and each lipid extract was reconstituted in chloroform:methanol (1:1, v:v) at a volume of 400 µL/mg protein.

For shotgun lipidomics, lipid extract was further diluted to a final concentration of ~500 fmol total lipids per µL. Mass spectrometric analysis was performed on a triple quadrupole mass spectrometer (TSQ Altis, Thermo Fisher Scientific, San Jose, CA, USA) and a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA, USA), both of which were equipped with an automated nanospray device (TriVersa NanoMate, Advion Bioscience Ltd., Ithaca, NY, USA) as described (32). Identification and quantification of lipid species were performed using an automated software program (33, 34). Data processing (e.g., ion peak selection, baseline correction, data transfer, peak intensity comparison and quantitation) was performed as described (34). The result was normalized to protein content (nmol lipid/mg protein or pmol lipid/mg protein).

**Statistical analysis of body weight, food intake, and body composition**

Graphical and formal statistical tests performed using PROC UNIVARIATE revealed that all variables were normally distributed. Differences within and between groups were analyzed using a GROUP x TIME repeated measures analysis of variance (ANOVA) in SAS (version 9.4m7, Cary, NC, USA). For body weight and food intake, weekly mean values of mice within a group were compared over the 9 weeks. Body composition variables were compared between day 33 and day 66 between and within groups. Due to COVID-19 restrictions, a baseline body composition measurement was not performed. Tissue weights between groups were analyzed by one-way ANOVA. Tukey-Kramer post hoc testing was used to explore significant differences determined by RMANOVA. Significance was set a priori at P < 0.05. Data are expressed as means ± standard error of the mean (SEM).

**Results**

**Body weight, muscle weight, body composition, and food intake**

Groups were not significantly different in body weight at baseline. There was a significant group by time interaction in the overall analysis for body weight (P < 0.001), with KE fed mice weighing significantly less than CON at endpoint. However, these differences were not significant after post hoc adjustments for multiple testing. Interestingly, there was a within-group increase in body weight in the CON group in week 2 compared to week 8 (P < 0.01), week 9 (P < 0.001), and week 10 ([P < 0.001] Figure 1A). There were no between or within group changes in energy intake (kcal/day) (Figure 1B). For body composition, there was an overall group by time interaction (P < 0.01), which was driven by a within-group increase in FM in the CON group (P < 0.01) while there was no significant within-group change in the KE group (Figures 1C,D). There were also no significant differences in LBM, muscle wet weight of vastus lateralis, gastrocnemius, soleus, and tibialis anterior between groups (data not shown).

**Gene expression**

A broad approach was employed to compare gene expression in vastus lateralis between groups using the ROSALIND gene array. This analysis was completed at the
A Weekly mean body weight of ketone ester (KE) mice was significantly less than control (CON) mice only during the last 3 weeks of the dietary intervention. (B) Weekly energy intake was similar between CON and KE groups. (C) Fat mass measured by quantitative magnetic resonance (QMR) was greater in CON compared to KE mice, and increased from day 33 to day 66 in CON mice only. (D) Lean mass measured by QMR was not different between CON and KE mice despite KE mice weighing less than CON mice.

endpoint (week 83) of the study. Of the 773 genes analyzed, 6 were significantly higher in the KE group compared to CON (Table 1). 23 genes were also significantly lower in the KE group compared to CON (Table 2).

Lipidomics

Lipidomics was performed on vastus lateralis in CON (n = 7) and KE groups (n = 6). There were no differences between groups for any lipid species, except for fatty acyl chains in triacylglycerol (nmol/mg protein) which was 46% lower in the KE group compared to CON (P = 0.05, Table 3).

Proteomics

There were 1,020 protein IDs with > 99% C.I. and < 1% FDR from the initial analysis. Proteomics was performed on the gastrocnemius in CON (n = 7) and KE groups (n = 6). Many proteins identified were in low enough abundance and presented with a fair amount of heterogeneity “zero” values across each group and were therefore eliminated from the analysis. Of those proteins that were more homogeneous, 545 high confidence hits were observed in at least 3 or more specimens per group with a signal over zero. A non-parametric statistical analysis was then applied to these remaining proteins along with a fold change of > 1.5 or < −1.5, which led to 44 proteins that were statistically changed in abundance in the KE compared to CON. Of those, 11 were significantly higher in KE compared to CON (Table 4) and 33 were significantly lower (Table 5) in KE compared to CON.

Discussion

The purpose of this study was to determine the effects of a 25% KE diet on body weight, body composition, and the skeletal muscle transcriptional, proteomic, and lipidomic responses in
TABLE 1 | Genes that were significantly higher in ketone ester (KE) compared to control (CON).

| Gene       | Gene name                             | Cellular process                      | Fold change | p-value |
|------------|---------------------------------------|---------------------------------------|-------------|---------|
| Gadd45a    | Growth arrest and DNA damage inducible alpha 45 | regulation of cell cycle              | 1.86        | 0.006   |
| Pla2g4e    | Phospholipase A2 group IVE             | cytosol phospholipase                 | 1.23        | 0.038   |
| Runx1      | RUNX family transcription factor 1     | transcription regulator                | 1.69        | 0.040   |
| Tnc        | Tenascin C                            | extracellular matrix protein          | 1.44        | 0.032   |
| Cal51a     | Cell division cycle 1A                 | protein phosphatase                   | 1.26        | 0.027   |
| Bax        | BCL2 associated X, apoptosis regulator  | mitochondrial membrane protein        | 1.13        | 0.021   |

TABLE 2 | Genes that were significantly lower in ketone ester (KE) compared to control (CON).

| Gene       | Gene name                             | Cellular process                      | Fold change | p-value |
|------------|---------------------------------------|---------------------------------------|-------------|---------|
| Pim1       | Pim-1 proto-oncogene, serine/threonine kinase | transmembrane glycoprotein              | −1.56279    | 0.049   |
| Fgfr4      | Fibroblast growth factor receptor 4    | growth factor                          | −1.37815    | 0.049   |
| Prkg       | Protein kinase C gamma                | transferase activity                   | −1.29662    | 0.032   |
| Smad3      | SMAD family member 3                  | transcription regulator                | −1.24761    | 0.023   |
| Ikr3       | Interleukin 1 receptor associated kinase 2 | signal transduction                   | −1.22257    | 0.041   |
| Ch1        | Ch1 proto-oncogene                    | ubiquitin protein ligase               | −1.18758    | 0.048   |
| Igf3p3     | Insulin like growth factor binding protein 3 | protease inhibitor                    | −1.15034    | 0.049   |
| Tsc2       | TSC complex subunit 2                 | GTPase activator                      | −1.1314     | 0.015   |
| Axin1      | Axin 1                                | angiogenesis                           | −1.10821    | 0.029   |
| Mycn       | MYCN proto-oncogene, BHLH transcription factor | transcription regulator                | −1.82609    | 0.021   |
| Itga3      | Integrin subunit alpha 3              | membrane protein                       | −1.46154    | 0.002   |
| Rac3       | Rac family small GTPase 3             | transcription regulator                | −1.37600    | 0.004   |
| Mocom      | MDS1 And EVI1 complex locus protein EV1 | transcription regulator                | −1.26208    | 0.007   |
| Rpska5     | Ribosomal protein S6 kinase A5        | protein signaling                      | −1.22216    | 0.015   |
| Tgfr3      | Transforming growth factor beta 3      | growth factor                          | −1.17609    | 0.006   |
| Tsc1       | TSC complex subunit 1                 | protein signaling                      | −1.13642    | 0.001   |
| Camka2b    | Calcium/calmodulin dependent protein kinase II beta | calcium signaling                  | −1.12545    | 0.001   |
| Traf7      | TNF receptor associated factor 7       | ubiquitin protein ligase               | −1.10585    | 0.003   |
| Prom1      | Prominin 1                            | cholesterol binding                    | −1.37095    | 0.009   |
| Hdac6      | Histone deacetylase 6                 | transcription regulator                | −1.22481    | 0.036   |
| Dvl2       | Disheveled segment polarity protein 2  | cytoplasmic protein                    | −1.21628    | 0.012   |
| Pfb6       | PHD finger protein 6                   | zinc finger protein                    | −1.16103    | 0.038   |
| Ptp11      | Protein tyrosine phosphatase non-receptor type 11 | protein phosphatase                  | −1.11103    | 0.016   |

aging 72-week-old C57BL/6J mice for nine weeks. We found mice consuming a KE diet remained weight stable, with no changes in FM or LBM, yet the control group increased body weight and fat mass over the course of the study. There were no differences in energy intake between the groups, which agrees with our previous findings in juvenile mice (19). Interestingly, the KE group displayed a unique skeletal muscle transcriptional and proteomic profile compared to the control group yet had a very similar lipidomic profile.

Transcriptional analysis indicated six genes were higher in the KE group compared to CON. Growth Arrest And DNA Damage Inducible Alpha (Gadd45a) expression was 1.85-fold higher in KE mice compared to CON. Previous longitudinal data indicates that Gadd45a is elevated in aged mouse skeletal muscle during the transition to sarcopenia, which involves progressive muscle atrophy (35). Others have reported that Gadd45a expression represents a protective negative feedback response to denervation, which delays the rate of atrophy and myofiber type transition, potentially preserving myofiber type during chronic denervation (36). We found that tenascin-C (Tnc) was up-regulated 1.44-fold in the KE group, and previous findings have implicated Tnc in the formation, maturation, and stabilization of the neuromuscular junction (37). In line with these findings, RUNX Family Transcription Factor 1 (Runx1) was up-regulated 1.69-fold in the KE group. Runx1 is required to sustain skeletal muscle by preventing denervated myofibers from undergoing myofibrillar disorganization and autophagy (38). Runx1 ablation results in excessive autophagy during denervation which leads to severe atrophy, suggesting that these findings are protective with regards to age-related
autophagy and atrophy of skeletal muscle (39). Other studies have found similar results in aged mice, showing Gadd45α and Runx1 are elevated with a KD, which has been shown to mitigate sarcopenia (10). Another transcript, calponin homology-associated smooth muscle protein (Chasm), which is required for tropomyosin binding, was 1.79-fold lower in KE compared to CON (40). Some have suggested the protein form of CHASM is a discrete marker of Type IIa muscle fibers, which supports the idea that the KE group may be undergoing a transition from Type II to Type I fibers to attenuate muscle denervation induced with aging (41, 42). Decreased calcineurin A (CnA) in our proteomic analysis (−2.82-fold compared to CON) also suggests muscle protection since CnA has been shown to indirectly protect muscle fibers

| Lipid                          | CON    | KE     | p-value |
|-------------------------------|--------|--------|---------|
| Phosphatidylcholine (nmol/mg protein) | 49.5 ± 9.2 | 47.0 ± 3.7 | 0.534 |
| Lyso-Phosphatidylcholine (nmol/mg protein) | 1611.5 ± 304.4 | 1920.8 ± 393.0 | 0.138 |
| Phosphatidylethanolamine (nmol/mg protein) | 43.2 ± 5.2 | 48.0 ± 8.9 | 0.253 |
| Lyso phosphatidylethanolamine (nmol/mg protein) | 803.7 ± 160.1 | 845.1 ± 159.2 | 0.650 |
| Carnitine (pmol/mg protein)     | 272.5 ± 41.3 | 272.2 ± 52.2 | 0.991 |
| Cardiolipin (nmol/mg protein)   | 4.0 ± 0.9  | 3.7 ± 0.5  | 0.501 |
| Lyso cardiolipin (nmol/mg protein) | 17.1 ± 7.3  | 25.8 ± 8.4  | 0.070 |
| Phosphatidylserine (pmol/mg protein) | 5.0 ± 0.8  | 4.8 ± 0.7  | 0.523 |
| Phosphatidylserine (pmol/mg protein) | 2.5 ± 0.5  | 2.4 ± 0.2  | 0.620 |
| Phosphatidylglycerol/Bis (monoacylglycerol) phosphate | 2.0 ± 0.2  | 1.9 ± 0.3  | 0.317 |
| Phosphatidic acid (pmol/mg protein) | 31.4 ± 6.4  | 30.8 ± 2.1  | 0.846 |
| Triacylglycerol (nmol/mg protein) | 813.1 ± 303.4 | 592.4 ± 256.3 | 0.188 |
| Fatty acyl chains in triacylglycerol (nmol/mg protein) | 2452.2 ± 914.2 | 1787.3 ± 711.2 | 0.050 |

*De novo fatty acids were examined cumulatively as 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 22:0, 22:1. Values are means ± SD. Significant was set a priori at P < 0.05. Data are represented as Mean ± SD. The bold value represents the statistical significance.

| UniProtKB names                        | Gene name               | Prot Acc# | GeneID | Avg (C)  | Avg (KE)  | P      | SAM   | Fold (KE/C) |
|----------------------------------------|-------------------------|-----------|--------|----------|-----------|--------|-------|-------------|
| COP9 signalosome complex subunit 4     | COP9 signalosome subunit 4 (Cops4) | O88544    | 26891  | 1.0      | 2.7       | 0.002  | 3.26  | 2.63        |
| Serine/threonine-protein kinase mTOR   | mechanistic target of rapamycin (Mtor) | Q9LN9     | 56717  | 1.5      | 2.9       | 0.016  | 1.33  | 1.96        |
| Quinone oxidoreductase                 | crystallin, zeta (Cryz) | P47199    | 12972  | 2.7      | 4.9       | 0.007  | 0.93  | 1.84        |
| Electron transfer flavoprotein, dehydrogenase (Rfidh) | electron transferring flavoprotein, dehydrogenase (Rfidh) | Q921G7    | 66841  | 3.8      | 6.8       | 0.004  | 0.90  | 1.80        |
| Thioderodoxin-like protein 1           | thioderodoxin-like 1 (Tncl1) | Q8CDN6    | 53382  | 1.3      | 2.3       | 0.012  | 0.97  | 1.76        |
| Probable ubiquitin carboxyl-terminal hydrolase FAF-X | ubiquitin specific peptidase 9, X chromosome (Usp9x) | P70398    | 22284  | 8.8      | 15.2      | 0.010  | 0.75  | 1.72        |
| Protein Col6a3                         | collagen, type VI, alpha 3 (Col6a3) | J3QQ16    | 12835  | 4.8      | 8.2       | 0.026  | 0.73  | 1.69        |
| Voltage-dependent anion-selective channel protein 1 | voltage-dependent anion channel 1 (Vdac1) | Q60932    | 22333  | 2.8      | 4.7       | 0.048  | 0.56  | 1.67        |
| Mutant fibrillin-1                     | fibrillin 1 (Fbn1)     | O88840    | 14118  | 4.0      | 6.7       | 0.049  | 0.62  | 1.66        |
| Protein Tnxb                          | tenascin Xb (Tnxb)     | O35452    | 81877  | 14.4     | 23.8      | 0.012  | 0.79  | 1.66        |
| ENH isoform 3a                        | PDZ and LIM domain 5 (Pdlim5) | D9J303    | 56376  | 8.5      | 12.8      | 0.021  | 0.64  | 1.50        |
TABLE 5 Proteomic differences in mouse muscle tissues: Ketone ester (KE) vs. control diets. 33 proteins were significantly lower in KE compared to CON.

| UniProtKB names                      | Gene name                                                                 | Prot Acc#  | GeneID   | Avg (C) | Avg (KE) | P        | SAM | Fold (KE/C) |
|--------------------------------------|---------------------------------------------------------------------------|------------|----------|---------|----------|---------|------|-------------|
| Serine protease inhibitor A3K        | serine (or cysteine) peptidase inhibitor, clade A, member 3K (Serpina3k) | P07759     | 20714    | 8.1     | 2.5      | 0.022   | 0.81 | −3.27       |
| Serine/threonine-protein phosphatase 2B | protein phosphatase 3, catalytic subunit, alpha isoform (Ppp3ca)         | P63328     | 19055    | 3.8     | 1.3      | 0.017   | 0.88 | −2.82       |
| 10 kDa heat shock protein            | heat shock protein 1 (chaperonin 10) (Hsp10)                               | Q64433     | 15528    | 4.6     | 1.7      | 0.020   | 1.00 | −2.66       |
| Annexin A1                           | annexin A1 (Anxa1)                                                        | P10107     | 16952    | 5.1     | 2.0      | 0.029   | 0.84 | −2.58       |
| Alpha-2-macroglobulin                | pregnancy zone protein (Pep)                                              | Q61838     | 11287    | 23.9    | 9.9      | 0.003   | 0.98 | −2.40       |
| Acyl-coenzyme A thioesterase 13      | acyl-CoA thioesterase 13 (Acot13)                                         | Q9CQ84     | 66834    | 8.6     | 3.8      | 0.041   | 0.53 | −2.24       |
| ES1 protein homolog, mitochondrial   | DNA segment, Chr 10, Johns Hopkins University 81 expressed (D10mitu81e)  | Q9D172     | 28295    | 13.5    | 6.2      | 0.001   | 1.14 | −2.15       |
| Murinoglobulin-1                     | murinoglobin 1 (Mog1)                                                     | P28665     | 17836    | 20.6    | 9.7      | 0.002   | 1.13 | −2.13       |
| Transgelin                           | transgel (Tagln)                                                          | P37804     | 21345    | 5.1     | 2.4      | 0.043   | 0.58 | −2.12       |
| Gelsolin, isoform CRA_c              | gelsolin (Gsn)                                                            | Q6PAC1     | 227753   | 3.9     | 1.9      | 0.016   | 0.74 | −2.08       |
| Dual specificity protein phosphatase 3 | dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related) (Dups3) | Q9D7×3   | 72439    | 8.1     | 3.9      | 0.004   | 1.01 | −2.07       |
| 5-formylglutathione hydrolase         | esterase D/formylglutathione hydrolase (Eisd)                              | Q9R0P3     | 13885    | 2.7     | 1.3      | 0.010   | 1.05 | −2.02       |
| Vesicle-associated membrane protein  | vesicle-associated membrane protein, associated protein A (Vapa)          | Q9WV55     | 30960    | 6.5     | 3.3      | 0.030   | 0.67 | −1.98       |
| Galectin-1                           | lectin, galactose binding, soluble 1 (Lgals1)                             | P16045     | 16852    | 10.6    | 5.4      | 0.002   | 1.15 | −1.98       |
| Endoplasmalin                         | heat shock protein 90, beta (Gsp90), member 1 (Hsp90b1)                     | P08113     | 22027    | 5.7     | 3.0      | 0.007   | 0.89 | −1.90       |
| Glutathione S-transferase kapp1       | glutathione S-transferase kapp1 (Gstk1)                                   | Q9DCM2     | 76263    | 3.4     | 1.8      | 0.021   | 0.88 | −1.84       |
| 6-phosphogluconolactonase            | 6-phosphogluconolactonase (Pglu)                                         | Q9CC6O     | 66171    | 4.2     | 2.3      | 0.015   | 0.84 | −1.83       |
| Thioredoxin reductase 1              | thioredoxin reductase 1 (Tndr1)                                           | Q9JM6H     | 50493    | 3.8     | 2.1      | 0.041   | 0.64 | −1.82       |
| EH domain-containing protein 2       | EH-domain containing 2 (EHd2)                                             | Q8BH64     | 259300   | 5.8     | 3.2      | 0.038   | 0.62 | −1.81       |
| Smoothelin-like protein 1            | smoothelin-like 1 (Smtm11)                                                | Q99LM3     | 68678    | 2.2     | 1.2      | 0.048   | 0.64 | −1.79       |
| Carboxylesterase 1C                  | carboxylesterase 1C (Ces1c)                                              | P23953     | 13884    | 9.5     | 5.5      | 0.030   | 0.72 | −1.72       |
| Hemopexin                            | hemopexin (Hpx)                                                           | Q91×72     | 15458    | 6.5     | 3.8      | 0.042   | 0.53 | −1.70       |
| Alpha-1-antitrypsin 1-2              | serine (or cysteine) peptidase inhibitor, clade A, member 1B (Serpina1b) | P22599     | 20701    | 12.6    | 7.5      | 0.019   | 0.82 | −1.69       |
| Annexin A2                           | annexin A2 (Anxa2)                                                        | P071356    | 12306    | 14.4    | 8.8      | 0.023   | 0.62 | −1.64       |
| Phosphorylase b kinase                | phosphorylase kinase alpha 1 (Phka1)                                      | P18826     | 18679    | 10.3    | 6.4      | 0.030   | 0.60 | −1.62       |
| Fatty acid-binding protein, adipocyte | fatty acid binding protein 4, adipocyte (Fabp4)                           | P04117     | 11770    | 36.9    | 23.0     | 0.029   | 0.59 | −1.60       |
| Alpha-1-antitrypsin 1-3              | serine (or cysteine) peptidase inhibitor, clade A, member 1C (Serpina1c) | Q00896     | 20702    | 11.1    | 7.0      | 0.005   | 0.86 | −1.59       |

*(Continued)*
from atrophy by raising the proportion of slow fibers in muscles (43). This occurs, in part, by maintaining peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) through activation of the myocyte enhancer factor-2 (MEF2) and Nuclear factor of activated T-cells (NFAT) transcription factors (43). Atrogin-1, a muscle-specific E3 ubiquitin ligase that is upregulated during atrophy, may contribute to this process because it initiates CnA degradation in cardiomyocytes (44, 45). Therefore, a reduction in CnA could influence the fiber switching that occurs in aging. Taken together, these findings suggest that skeletal muscle may be remodeling toward a slow fiber phenotype in the KE group, potentially protecting against denervation induced muscle fiber loss during sarcopenia. The implications of these findings are not entirely clear and suggest that skeletal muscle satellite cells, and our proteomic data show muscle weakening with age (55). Our proteomic data partially support these findings since Annexin A1 was lower in the KE group compared to CON. PDZ and LIM domain 5 (PDLIM5) is required for proper myogenesis (46). Furthermore, pharmacological inhibition of CAMK2B activity suppresses denervation-induced muscle atrophy (47). SMAD Family Member 3 (Smad3) was also 1.25-fold lower in KE compared to control, which could translate to less atrophy since activation of Smad proteins inhibit the function of Akt and the expression of Atrogin-1 and Muscle-specific RING finger protein 1 (MurF1) by Forkhead box O transcription factors (48). The COP9 singlaseome (Cops4), which is similar in structure and function to that of the 19S regulatory particle of 26S proteasome, was 2.63-fold higher in KE and has been shown to interact with SCF-type E3 ubiquitin ligases and act as a positive regulator of E3 ubiquitin ligases. Taken together, these findings suggest that the KE may protect against muscle atrophy in aged mice even though skeletal muscle weight was not different between groups, nor were Atrogin-1 and MAFA mRNA expression. Previous reports indicate that at least one form of KE can attenuate muscle wasting with some diseases in humans (49).

Several transcripts in the KE group were involved in myogenesis, muscle repair, and muscle stem cells. For example, Pim-1 oncogene protein (Pim1) was 1.56-fold lower in the current study and Pim1 is required for proper myogenesis (50). Furthermore, Prominin 1 (Prom1) expression was 1.37-fold higher in KE, which is required for stem cell maintenance and activation (51) along with a reduction in the transcripts for MDS1 And EVI1 Complex Locus Protein EV1 (Mecom) and Rac Family Small GTPase 3 (Rac3), both of which have been implicated in stem cell control. Our proteomic data support these findings, with Galectin-1 protein content lower in KE mice than CON. Galectin-1 regulates myotube growth in regenerating skeletal muscle (52) and treatment with galectin-1 improves myogenic potential in some mouse models (53). This pathway acts through the Annexin family, and we found that Annexin A1 and Annexin 2 mRNA expression were downregulated, in KE mice, which could partially explain our proteomic findings with galectin-1 (52). Annexin A1 affects myoblast fusion causing a slowdown in regeneration of injured muscle but does not cause muscle damage or decrease the ability of injured myofibers to repair (54), while a lack of Annexin 2 results in poor myofiber repair and progressive muscle weakening with age (55). Our proteomic data partially support these findings since Annexin A1 was lower in the KE group compared to CON. PDZ and LIM domain 5 (PDLIM5) plays a positive role in the proliferation and differentiation of skeletal muscle satellite cells, and our proteomic data show it was 1.5-fold lower in KE compared to control (56). The silencing of PDLIM5 increases the nuclear accumulation of

### TABLE 5 (Continued)

| UniProtKB names | Gene name | Prot Acc# | GeneID | Avg (C) | Avg (KE) | P      | SAM | Fold (KE/C) |
|-----------------|-----------|-----------|--------|---------|----------|--------|-----|-------------|
| Ubiquitin-conjugating enzyme E2 variant 2 | ubiquitin-conjugating enzyme E2 variant 2 (Ube2v2) | Q9D2M8 | 70620 | 4.2 | 2.7 | 0.044 | 0.72 | −1.55 |
| Citrate lyase subunit beta-like protein | citrate lyase beta like (Clybl) | Q8R4N0 | 69634 | 2.8 | 1.8 | 0.020 | 0.68 | −1.55 |
| Ras-related protein Rab-18 | Rab18, member RAS oncogene family (Rab18) | P35293 | 19330 | 2.1 | 1.3 | 0.043 | 0.62 | −1.53 |
| Methylcrotonoyl-CoA carboxylase | methylcrotonoyl-Coenzyme A carboxylase 1 (alpha) (Mcc1) | Q99MR8 | 72039 | 2.3 | 1.5 | 0.023 | 0.81 | −1.51 |
| Proflin | proflin 1 (Pfn1) | Q8CEH8 | 18643 | 12.1 | 8.0 | 0.026 | 0.67 | −1.51 |
| O-acetyl-ADP-ribose deacetylase | MACRO domain containing 1 (MacroD1) | Q922B1 | 107227 | 5.2 | 3.4 | 0.030 | 0.64 | −1.50 |

Our results also highlighted the activation of atrophy pathways. For example, we found a small yet significant decrease in Calcium/Calmodulin Dependent Protein Kinase II Beta (Camk2b) mRNA expression in KE, which has been identified as a downstream target of p38 mitogen-activated protein kinase (p38α MAPK) and positive regulator of muscle atrophy (46). Furthermore, pharmacological inhibition of CAMK2B activity suppresses denervation-induced muscle atrophy (47). SMAD Family Member 3 (Smad3) was also 1.25-fold lower in KE compared to control, which could translate to less atrophy since activation of Smad proteins inhibit the function of Akt and the expression of Atrogin-1 and Muscle-specific RING finger protein 1 (MurF1) by Forkhead box O transcription factors (48). The COP9 singlaseome (Cops4), which is similar in structure and function to that of the 19S regulatory particle of 26S proteasome, was 2.63-fold higher in KE and has been shown to interact with SCF-type E3 ubiquitin ligases and act as a positive regulator of E3 ubiquitin ligases. Taken together,
difference in insulin sensitivity (65). Our proteomics data show Acyl-CoA dehydrogenase inhibited ketosis (BD-AcAc) and that HDAC6 deficiency promotes autophagy and decreases mitochondrial homeostasis (80). Given findings that a basal amount of HDAC6 mRNA in response to high-fat diet (57) and inflammatory markers (65) were significantly lower (46%) in the KE group compared to CON. Dvl2 functions as part of the upstream Wnt signal transduction pathway of β-catenin and GSK-3β, and can positively regulate the Wnt signal pathway (69). TRAF7 regulates skeletal muscle through its activity as a ubiquitin ligase and depletion of TRAF7 accelerates myogenesis, in part through downregulation of nuclear factor-κB (NF-κB) activity (70, 71). The downregulation of TRAF7, along with decreased Dvl2 in the KE group suggests that BD-AcAc2 may reduce cellular stress. Furthermore, profilin 1 protein was lower in the KE group. Increased expression of profilin-1 in vascular smooth muscle cells induces stress fiber formation and causes cardiac hypertrophy and fibrosis by modulating actin polymerization (72, 73). Histone deacetylase 6 (Hdac6) mRNA expression was also lower, and it controls fusion of autophagosomes to lysosomes (74). Evidence suggests that HDAC6 also plays a role in the eventual clearance of aggresomes, implying a functional connection between HDAC6 and autophagy (74–76). While we can only speculate about the possible role of BD-AcAc2 on cellular stress, others have found that a KD attenuates the increase in cellular stress due to aging (10).

Other proteins that were higher in the KE group compared to CON involved elements of the electron transport chain (ETC). For example, crystallin zeta (CryZ), a quinone oxidoreductase and threodoxi-like 1, which controls disulfide oxidoreductase activity was higher in KE compared to CON. VDAC1 deficiency causes multiple defects in the cytosol, by regulating the influx and efflux of metabolites, cations, and nucleotides (77). Human VDAC1 deficiency compromises pyruvate oxidation and ATP production (78). Others have shown VDAC1 deficiency causes multiple defects in the mitochondria and ETC complexes in both oxidative and glycolytic striated muscle biopsies. This includes loss of ion homeostasis resulting in irreparable cell injury and concomitantly cell death, primarily through necrosis. Higher expression of electron transfer flavoprotein dehydrogenase (ETFDH) mRNA in KE mice suggests improvement of skeletal muscle mitochondria β-oxidation in the ETC (79) while an upregulation of Bcl-2-associated X protein (Bax) suggests that the KE may enhance regulation of mitochondrial morphology during apoptosis (80). Given findings that a basal amount of BAX is necessary to maintain energy production via aerobic respiration this may be important (81). Taken together, these data indicate that the KE is influencing the ETC, which is similar to what has been reported with a KD in aging mice (10).

Nutritional ketosis has been defined as a metabolic state where β-hydroxybutyrate (β-HB) blood concentrations rise
above 0.5 millimole per liter (mM/L) (82, 83). In human clinical trials, circulating ketones such as β-HB reach levels ranging from 1 to 2 mM/L and up to 5 mM/L (6, 84, 85). Comparatively, we (and others) have shown the ability to induce similar levels of ketones in rodents (15, 17, 19, 86). Exogenous ketones offer a way to induce ketone production quickly and efficiently in humans without the need for caloric restriction or a KD and have been well-studied in exercise physiology (11, 12, 87-89). While more research is needed, KE have been suggested as a novel method to treat several diseases, including perturbations that occur in aging (90–94).

A strength of this research is that we took an -omics based approach to determine how a ketone ester influences skeletal muscle, providing multiple avenues for future research. A limitation of this approach is not identifying a specific molecular mechanism by which the KE is acting—although based on our findings it seems the BD-AcAc2 is influencing several pathways in skeletal muscle that may be responsible for the physiological and metabolic responses observed. Another limitation is the lack of baseline data on FM and LBM, which was due to COVID-19 shutdowns in the UAB Animal Resources Program. Nonetheless, mice were randomized so this should equally distribute confounding variables at baseline and our previous work in this rodent and dietary model indicates a very homogenous sample in these mice. In addition, body weights of the mice were similar among groups at the start of the study and in our experience with B6 mice, FM of these animals is highly correlated with overall body mass and should not represent a confounding variable for this study.

A limitation that muddies the interpretation of our findings are the transcriptional and proteomic analysis being completed in different muscles due to insufficient tissue, so future experiments should include this outcome. The different tissue usage may explain the lack of correlation between our proteomic and mRNA data. Finally, the study is likely underpowered given the number of-omics approaches we utilized and given the lack of previous data on KE and muscle mass at the time of study initiation, we did not have sufficient data for a traditional power calculation.

In conclusion, our findings indicate that a diet containing 25% BD-AcAc2 in male aging B6 mice produces a unique skeletal muscle transcriptional, lipidomic and proteomic signature that influences signaling involved in muscle denervation as well as muscle atrophy and autophagy. Furthermore, KE may improve the satellite cell niche and β-oxidation in the ETC. Our lipidomic data and body composition data align with transcriptional findings that suggest that BD-AcAc2 may play a role in insulin sensitivity. Future studies are needed to examine whether insulin sensitivity is indeed improved in skeletal muscle or whether these findings are related to decreased adiposity and/or fat content in liver.

Data availability statement

The data presented in the study are deposited in the Open Science Framework repository (https://osf.io/e2yd5/).

Ethics statement

The animal study was reviewed and approved by UAB Institutional Animal Care and Use Committee (IACUC).

Author contributions

BR, SD, DS, and EP conceptualized the study. BR and SD conducted the experiments and collected and analyzed the data. NM contributed to the lipidomics data analysis and interpretation. JM contributed to the proteomics data analysis and interpretation. BR wrote the manuscript. All authors edited and approved the final version prior to submission for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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