Milk From Cow Fed With High Forage/Concentrate Ratio Diet: Beneficial Effect on Rat Skeletal Muscle Inflammatory State and Oxidative Stress Through Modulation of Mitochondrial Functions and AMPK Activity

Giovanna Trinchese†, Gina Cavaliere†, Eduardo Penna†, Chiara De Filippo†, Fabiano Cimmino†, Angela Catapano1,2, Nadia Musco3, Raffaella Tudisco3, Pietro Lombardi3, Federico Infascelli3, Giovanni Messina4, Laura Muredda5, Sebastiano Banni5, Marcellino Monda5, Marianna Crispino1 and Maria Pina Mollica†

†These authors have contributed equally to this work

Milk and dairy products are relevant components of daily diet and are part of dietary recommendation in many countries due to their content of key nutrients. However, the relatively high content of saturated fat of the milk and its extensive usage for every age group raises concerns about its potential negative health effects. Therefore, in the last years, several researchers dedicated their attention to milk production and quality. Milk fatty acids profile depend on cow feeding and in particular on the type of forage and concentrate and forage/concentrate ratio. It was demonstrated that feeding dairy cows with a 70/30 forage/concentrate ratio yields milk with a low $\omega_6:\omega_3$ ratio and high CLA levels. In this work, we demonstrated that the supplementation of rats diet with this high forage milk (HFM) results, in the skeletal muscle of these animals, in a reduced lipid content and inflammation levels, and an improved mitochondrial lipid oxidation, and redox status through modulation of AMPK activity.

Keywords: milk, high forage/concentrate ratio, mitochondrial functions, skeletal muscle, oxidative stress, inflammation, AMPK activation

INTRODUCTION

Skeletal muscle, the largest organ in the body, in most mammals accounts up to ~45% to 55% body mass and, consequently, is the major determinant of the basal metabolic rate (Zurlo et al., 1990, 1994; Janssen et al., 2000). Since muscle is consuming nearly 80% of insulin-stimulated glucose uptake, it plays a key role in glucose disposal (Thiebaud et al., 1982; Ferrannini et al., 1988). Moreover, it can be recruited to increase energy expenditure several folds in physical activity
acids (CLA) have beneficial properties, such as anticarcinogenic, antioxidative, and antiatherogenic effects, reduction in the body fat, and stimulation of the immune system (Pariza, 1999). Milk fatty acids profile depend on cow feeding and in particular on the type of forage and concentrate and forage/concentrate ratio (Cruz-Hernandez et al., 2007). Indeed, in previous work it was demonstrated that feeding dairy cows with a 70/30 forage/concentrate ratio yields milk with a low \( \omega_6/\omega_3 \) ratio and high CLA levels (Cavaliere et al., 2018). The administration of this high forage milk (HFM) to rats resulted in beneficial effects on lipid metabolism, inflammation and oxidative stress in the liver (Cavaliere et al., 2018). Since the skeletal muscle is a chief determinant of resting metabolic rate, we analyzed also in this tissue the effects of HFM on mitochondrial functions and redox state.

**MATERIALS AND METHODS**

**Cow Milk and Chemicals**

Chemicals were purchased by Sigma–Aldrich (St. Louis, MO, United States), unless otherwise specified. Milk was purchased from a farm that produces 2 types of commercial milk, low forage milk (LFM) and high forage milk (HFM) from Italian Friesian cows fed 2 different diets with lower or higher F:C ratio, respectively. Chemical composition, \( \omega_6/\omega_3 \) ratio and CLA content are reported in Table 1 (see Cavaliere et al., 2018 for details).

**Rat Handling and Feeding**

Male Wistar rats (Charles River, Calco, Lecco, Italy) were individually caged in a temperature-controlled room and exposed to a daily 12h–12h light–dark cycle with free access to chow and drinking water. Young animals (60 days old; about 350 g b.w.) were used; one group (\( n = 7 \)) was sacrificed at the beginning of the study to establish baseline measurements. The remaining rats, fed with standard diet, were divided into three experimental groups (\( n = 7 \) each): two groups were supplemented with equicaloric LFM or HFM (2 mL/kg) for 4 weeks; the group that did not receive milk supplement was used as control. After 4 weeks, the animals were anesthetized by intra-peritoneal injection of chloral hydrate (40 mg/100 g body weight), and blood was taken from the inferior cava. The skeletal muscle

---

**TABLE 1 | Milk chemical composition, \( \omega_6, \omega_3 \), and CLA.**

|               | LFM          | HFM          |
|---------------|--------------|--------------|
| Protein (%)   | 3.3 ± 0.2    | 3.3 ± 0.1    |
| Fat (%)       | 3.5 ± 0.3    | 3.7 ± 0.2    |
| Lactose (%)   | 4.5 ± 0.5    | 4.7 ± 0.4    |
| \( \omega_3 \) | 0.317 ± 0.01a | 1.212 ± 0.09b |
| \( \omega_6 \) | 2.207 ± 0.08  | 2.403 ± 0.03  |
| \( \omega_6/\omega_3 \) | 6.96 ± 1.00a | 1.98 ± 0.03b  |
| \( \Sigma CLA \) | 0.45 ± 0.02b | 0.79 ± 0.02a  |

Data are means ± SEM. Along the rows data with different superscripted letters are significantly different (\( P < 0.05 \)). LFM, low forage milk; HFM, high forage milk.
was removed and sub-divided; samples not immediately used for mitochondrial preparation were frozen and stored at −80°C.

**Serum and Tissue Parameters**

The serum levels of cholesterol and triglycerides were measured with standard procedures. Glucose levels were determined by glucometer (Contour next, Ascensia, Switzerland). The serum levels of insulin (Merckodia AB, Upssala, Sweden) were measured using commercially available ELISA kits. The serum and tissue levels of tumor necrosis factor-α (TNF-α) were measured with standard procedures. Glucose levels were determined by applying equation 11 by Cairns et al. (1998):$
\text{degree of coupling} = \frac{\sqrt{T - (\text{Jo})_{\text{sh}}}}{\text{(Jo)unc}}$

**Isolation of Mitochondria Skeletal Muscle and Measurements of Mitochondrial Oxidative Capacities and Degree of Coupling**

Hind leg muscles were freed of excess connective tissue, finely minced, washed in a medium containing 100 mmol/L KCl, 50 mmol/L TRIS, pH 7.5, 5 mmol/L MgCl₂, 1 mmol/L EDTA, 5 mmol/L EGTA, 0.1% (w/v) fatty acid-free BSA and treated with protease (3.6 U/g tissue) for 4 min. Tissue fragments were then homogenized with the above medium (1:8 w/v) at 500 rpm (4 strokes/min). Homogenate was centrifuged at 3,000 g for 10 min, the resulting supernatant was rapidly discarded, and the pellet was resuspended and centrifuged at 500 g for 10 min. The supernatant was then centrifuged at 3,000 g for 10 min, and the pellet was washed once and resuspended in suspension medium (250 mmol/L Sucrose, 50 mmol/L Tris, pH 7.5, 0.1% fatty acid-free BSA). In control experiments, we assured the quality of our mitochondrial preparation by checking that contamination of mitochondria by other ATPase-containing membranes was lower than 10%, and addition of cytochrome c (3 nmol/mg protein) only enhanced state 3 respiration by approximately 10%.

Oxygen consumption was measured polarographically with a Clark-type electrode (Yellow Springs Instruments, OH, United States) in a 3-mL glass cell, at a temperature of 30°C. Skeletal muscle mitochondria were incubated in a medium containing 30 mmol/L KCl, 6 mmol/L MgCl₂, 75 mmol/L sucrose, 1 mmol/L EDTA, 20 mmol/L KH₂PO₄ pH 7.0 and 0.1% (w/v) fatty acid-free BSA, pH 7.0. The substrates used were 10 mmol/L succinate + 3.8 µmol/L rotenone, 10 mmol/L glutamate + 2.5 mmol/L malate, 40 µmol/L palmitoyl-carnitine + 2.5 mmol/L malate or 10 mmol/L pyruvate + 2.5 mmol/L malate. After the addition of 0.3 mmol/L ADP, maximal ADP-stimulated oxygen consumption was measured and taken as state 3, while state 4 was obtained from oxygen consumption measurements at the end of state 3, when ADP becomes limiting. Respiratory control ratio was calculated as state 3/state 4 ratio.

The degree of coupling was determined in skeletal muscle mitochondria as previously reported (Lama et al., 2016) by applying equation 11 by Cairns et al. (1998):$
\text{degree of coupling} = \frac{\sqrt{T - (\text{Jo})_{\text{sh}}}}{\text{(Jo)unc}}$

where (Jo)sh represents the oxygen consumption rate in the presence of oligomycin that inhibits ATP synthase, and (Jo)unc is the uncoupled rate of oxygen consumption induced by FCCP, which dissipates the transmembrane proton gradient. (Jo)sh and (Jo)unc were measured as above using succinate (10 mmol/L) + rotenone (3.75 µmol/L) in the presence of oligomycin (2 µg/mL) or FCCP (1 µmol/L), respectively, both in the absence and in the presence of palmitate at a concentration of 50 µmol/L.

Carnitine palmitoyl-transferase (CPT) system, aconitase and superoxide dismutase (SOD) specific activity were measured spectrophotometrically as previously reported (Flohè and Otting, 1984; Alexson and Nedergaard, 1988; Hausladen and Fridovich, 1996). Catalase (CAT) activity was determined based on the decomposition of H₂O₂ at 25°C (Aebi, 1984).

**H₂O₂ Measurement**

Rate of mitochondrial H₂O₂ release was assayed by following the linear increase in fluorescence (ex 312 nm and em 420 nm) due to the oxidation of homovanillic acid in the presence of horseradish peroxidase (Barja, 1998).

**Lipid Content, Redox Status and Detoxifying Enzyme Activities in Skeletal Muscles**

Total lipid content in the skeletal muscle was estimated by using the Folch method. Briefly, the skeletal muscle was weighed, chopped into small pieces, thoroughly mixed, and finally homogenized with water (volumes equal to twice the skeletal muscle weight) in a Polytron homogenizer. On appropriate aliquots of the homogenate, lipid content was determined gravimetrically after extraction in chloroform–methanol and evaporation to constant weight by a rotating evaporator (Folch et al., 1957). ATP content in skeletal muscles was measured using commercially available kit (Abcam, ab83355). Reduced (GSH) and oxidized (GSSG) glutathione concentrations in skeletal muscle were measured with the dithionitrobenzoic acid (DTNB)-GSSG reductase recycling assay (Bergamo et al., 2007), the GSH/GSSG ratio was used as an oxidative stress marker. To investigate the detoxifying activity, the enzymatic activities of glutathione S-transferases (GSTs) and quinone-oxidoreductase (NQO1) were evaluated spectrophotometrically in cytoplasmic extracts prepared from rat skeletal muscle (Benson et al., 1980; Habig and Jakoby, 1981).

**ROS Assay**

The levels of ROS were determined as previously reported (Montoliu et al., 1994). An appropriate volume of freshly prepared tissue homogenate was diluted in 100 mM potassium phosphate buffer (pH 7.4) and incubated with a final concentration of 5 µM dichloro-fluorescein diacetate (Sigma–Aldrich) in dimethyl sulfoxide for 15 min at 37°C. The dye-loaded samples were centrifuged at 12,500 × g per 10 min at 4°C. The pellet was mixed at ice-cold temperatures in 5 ml of 100 mM potassium phosphate buffer (pH 7.4) and again incubated for 60 min at 37°C. The fluorescence measurements were performed with a HTS-7000 Plus-plate-reader spectrophluorometer (Perkin Elmer, Wellesley, MA,
United States) at 488 nm for excitation and 525 nm for emission wavelengths. ROS were quantified from the dichloro-fluorescein standard curve in dimethyl sulfoxide (0–1 mM).

Lipid Analyses
Aliquots of total lipid extract from tissues were mildly saponified as previously described (Banni et al., 1996) in order to obtain free fatty acids for HPLC analysis. Separation of unsaturated fatty acids was carried out with an Agilent 1100 HPLC system (Agilent, Palo Alto, CA, United States) equipped with a diode array detector as previously reported (Melis et al., 2001). ω3 HUFA score, taken as an index of the impact of the diet on the ratio between ω3 and ω6 highly polyunsaturated fatty acids (HPUFAs), was calculated as the ratio between the sum of the concentrations of ω3 HPUFAs (those with 20 or more carbon atoms and three or more double bonds of ω3 family) and the sum of total ω3 and ω6 HPUFAs: [(20:5ω3 + 22:5ω3 + 22:6ω3)/(20:5ω3 + 22:5ω3 + 22:6ω3 + 20:4ω6 + 20:3ω6 + 22:4ω6 + 22:5ω6)]∗100] (Stark, 2008).

Protein Carbonyl and Malondialdehyde Measurements
Protein Carbonyl accumulation (PC), as indicator of protein oxidative modification, was measured in the skeletal muscle as previously reported (Levine et al., 1990). Malondialdehyde (MDA) levels in the skeletal muscle were determined as indicator of lipid peroxidation (Lu et al., 2009). Tissues were homogenized in 1.15% KCl solution. An aliquot (200 µl) of the homogenate was added to a reaction mixture containing 200 µl of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid, and 600 µl of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000 × g for 10 min. The supernatant absorbance was measured by spectrophotometry at 550 nm and the concentration of MDA was expressed as µmol MDA/mg protein of cell homogenate. A standard curve was prepared using MDA bisdimethylacetal as the source of MDA.

Western Blot Analysis
Skeletal muscle was homogenized in the lysis buffer (150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM Tris–HCl pH 7.5) containing protease and phosphatase inhibitors (Sigma Aldrich). Separation of proteins and western blot analyses were performed as previously described (Chun et al., 2004).

TABLE 2 | Gross energy intake.

| Control LFM HFM |
|------------------|
| Total Energy (kJ) | 11180 ± 195\textsuperscript{a} | 12940 ± 170\textsuperscript{b} | 13203 ± 178\textsuperscript{b} |
| Chow (kJ) | 11180 ± 195 | 11076 ± 140 | 11354 ± 132 |
| Milk (kJ) | 1864 ± 78 | 1849 ± 60 | 1849 ± 60 |

Data are means ± SEM. Along the rows data with different superscripted letters are significantly different (P < 0.05). LFM, low forage milk; HFM, high forage milk.
Briefly, proteins (25 µg/lane) were separated on 10% SDS-PAGE and transferred to PVDF membranes. Filters were probed with anti-phosphorylated AMPKα antibody (pAMPK, Thr172; Cell Signaling Technology Inc. 1:500) and, after stripping, the same filters were probed with anti-AMPKα antibody (Cell Signaling Technology Inc., Beverly, MA, United States. 1:2000). Secondary antibody, directed against rabbit IgG, was HRP-conjugate (Sigma 1:20000). The signals were detected using Immobilon Western Chemiluminescent HRP Substrate (Sigma).

**Statistical Analysis**

All data are presented as means ± SEM. Differences among groups were compared by ANOVA followed by Tukey post hoc test to correct for multiple comparisons. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using the statistical software package SPSS.
Figure 3 | Effects of oral administration of Low Forage Milk (LFM) and High Forage Milk (HFM) on oxidative stress and antioxidant activity: H$_2$O$_2$ yield (A), ROS (B), malondialdehyde (MDA, C), Protein Carbonyl (PC, D) levels, basal/total aconitase ratio (E), superoxide dismutase (SOD, F) and Catalase (CAT, G) activity. Data are presented as means ± SEM from $n = 7$ animals/group. Data with different superscripted letters are significantly different ($p < 0.05$).

RESULTS

Energy Intake

As shown in Table 2, the two milk treatments provided similar gross energy intake, which was significantly higher than control. The LFM and HFM groups received about 86% of energy from chow and 14% from milk.

Body Weight, Serum Metabolites and Inflammatory Parameters

Low forage milk- and HFM-treated animals exhibited higher body weight gain compared to control (LFM: 1972 ± 89$^a$ kJ, HFM: 1896 ± 68$^a$ kJ, control: 868 ± 55$^b$ kJ; data with different superscripted letters are significantly different, $P < 0.05$), and higher percentage of body lipid compared to control (LFM: 18.1 ± 1$^a$%, HFM: 17.5 ± 0.8$^a$%, control: 10.3 ± 0.3$^b$%; data with different superscripted letters are significantly different, $P < 0.05$). Glucose, insulin, triglycerides, cholesterol serum levels and HOMA index were not significantly different in the...
three groups of animals (data not shown) indicating that these parameters are not affected by LFM or HFM administration, in agreement with previously reported data (Cavaliere et al., 2018). Interestingly, tumor necrosis factor-α (TNF-α), IL-1, IL-6, and MCP1 levels significantly decreased in serum and skeletal muscle of HFM-fed animals compared to controls and LFM-fed rats (Figures 1A–D), indicating a possible anti-inflammatory role of HFM. This hypothesis has been further supported by the fact that IL-10, an anti-inflammatory cytokine, was found to increase in the serum of LFM group and even more in the HFM group compared to controls (Figure 1E inset), although no difference was found in skeletal muscle (Figure 1E). Adiponectin levels significantly decreased in LFM, and significantly increased in HFM group compared to the other two groups (Figure 1F).

### Mitochondrial Function and Efficiency
Mitochondrial state 3 respiration, evaluated using succinate or palmitoyl-carnitine as substrates (to detect fatty acid oxidation), was increased in LFM- and HFM-fed animals in comparison with the control (Figures 2A,B). Mitochondrial state 4 respiration, using succinate as substrates was increased in HFM-fed animals in comparison with the other two groups (Figure 2A), while no change was observed using palmitoyl-carnitine as substrates (Figure 2B). The high-quality of mitochondrial preparations was tested by evaluation of RCR values (data not shown). CPT activity...
was increased in HFM-fed animals compared with the other two groups (Figure 2C).

Oligomycin state 4 respiration was significantly higher, both in the absence and in the presence of palmitate, in HFM-fed animals compared to LFM and control rats (Figure 2D), while no variation was found in maximal FCCP-stimulated respiration (Figure 2E). As a consequence, skeletal muscle mitochondrial energetic efficiency, assessed as degree of coupling, was significantly lower in HFM-fed animals compared with the other two groups, both in the absence and in the presence of palmitate (Figure 2F). We also tested the ATP level in skeletal muscle and we did not observe any difference among the three groups of animals (Control 1392 ± 57, LFM: 1495 ± 33, HFM: 1444 ± 64 pmol ATP/mg tissue).

**Oxidative Stress and Antioxidant Activity**

H$_2$O$_2$ yield significantly increased in mitochondria of LFM-fed animals compared with the other two groups (Figure 3A). ROS, MDA and PC in skeletal muscle of LFM-fed animals was significantly higher than in the other groups (Figures 3B–D). Accordingly, the ratio basal/total aconitase activity, as intramitochondrial sensor of redox status, significantly decreased in LFM-fed animals (Figure 3E). In line with this, the antioxidant SOD and CAT activity significantly increased in HFM-fed animals compared with the other two groups (Figures 3F,G).

**Detoxifying Defenses**

The lipid content in skeletal muscle of LFM-fed animals was significantly higher than in the other groups (Figure 4A). HFM treatment improved the antioxidant state and cytoprotective enzyme activities. Indeed, while GSH levels significantly increased in HFM-fed animals, compared to the other groups (Figure 4B), GSSG content didn’t change (Figure 4C), resulting in an increased GSH/GSSG ratio in the HFM animal (Figure 4D). In addition, the activities of GST and NQO1 were significantly higher in HFM-fed animals compared with the other two groups (Figures 4E,F).
FATTY ACID ANALYSIS

High forage milk-treated animals exhibit significantly increased ω3 HUFA score in liver (Figure 5A) and skeletal muscle (Figure 5C) compared to control and LFM-treated animals. CLA significantly increased in liver of LFM- and HFM-treated animals compared to control (Figure 5B), and significantly increased in skeletal muscle of HFM-treated animals compared to the other two groups (Figure 5D).

ACTIVATION OF AMPK

To elucidate the possible molecular pathway underlying the beneficial effect of milk supplementation, we analyzed the activity level of AMPK in the three groups of animals. As shown in Figure 6, in HFM group we found an enhanced AMPK phosphorylation levels, compared to LFM.

DISCUSSION

Our data demonstrated, for the first time, that the administration of HFM in rat model system, reduces lipid content and inflammation in skeletal muscle, and improves redox state modulating mitochondrial functions and increasing detoxifying enzymes activity. These protective effects of HFM may be mediated, at least in part, by activation of skeletal muscle AMPK. These results are particularly relevant in view of the crucial role played by skeletal muscle in metabolic homeostasis of the whole body.

A low grade of inflammation is associated with chronic metabolic disease, such as obesity, in both human and animals. Therefore, in our experiments, we analyzed the influence of HFM in inflammatory state. We show that HFM administration decreases TNFα, IL-1β, IL-6 and MCP1 in both serum and skeletal muscle, and increases IL-10, an anti-inflammatory interleukin, in the serum. In addition, our data provided the first indication that the anti-inflammatory effect of HFM may be, at least partially, mediated by activation of AMPK, the downstream component of a serine/threonine protein kinase cascade involved in the regulation of cellular energy homeostasis. Indeed, AMPK, recognized as an anti-inflammatory protein (Salt and Palmer, 2012), was found to inhibit the synthesis of proinflammatory cytokines and promote the expression of IL-10 (Galic et al., 2011). The decreased inflammation is associated to a decrease in oxidative stress (Spagnuolo et al., 2015; Cavaliere et al., 2016). Since the main sites of ROS production are mitochondria, we analyzed the modulation of skeletal muscle mitochondrial function and efficiency following the administration of LFM and HFM. We observed that LFM and even more HFM-fed rats exhibited increased respiratory capacity and increased fatty acid oxidation. In HFM-fed rats, this increase in fatty acid oxidation is associated to an enhancement of CPT activity, which would further increase entry of long-chain FFAs into mitochondria. Thus, the consequent increase in lipid oxidation explains the decreased load of skeletal muscle lipid content found in these rats. The enhancement of CPT activity may be modulated by AMPK. Indeed, the activation of AMPK decreases the expression of lipogenic genes and increases the phosphorylation of acetyl-CoA carboxylase (ACC), leading to a reduction in malonyl-CoA, which regulates fatty acid oxidation through the inhibition of CPT (Gaidhu et al., 2009). Moreover, AMPK signaling pathway is the main target of adiponectin in promoting fatty acid oxidation (Hernandez-Aguilera et al., 2013). Therefore, the activation of AMPK in HFM group is consistent with the increased level of adiponectin that we observed in the same group.

In HFM group was also observed a reduced mitochondrial energy efficiency that may contribute to fat burning. Indeed, in these conditions, more substrates are burned to produce the same amount of ATP, creating a thermogenic effect. Taken together, the increase in fatty acid oxidation and the decrease in mitochondrial energy efficiency may explain the decrease in lipid content and MDA levels observed in skeletal muscle of HFM-fed animals. The reduced levels of MDA, index of lipid peroxidation, is particularly relevant since the lipid peroxidation is the first step on the way to the development of insulin resistance and its associated diseases. The improvement in mitochondrial functions found in HFM-fed rats could be associated to the increase in CLA levels and ω-3 HUFA score detected in skeletal muscle of these animals. Indeed, it is well-accepted that CLA and ω-3PUFA positively affect mitochondrial activity (Mollica et al., 2014; Cavaliere et al., 2016). Other positive metabolic effects of CLA are related to its ability to increase ω-3 HUFA score in the liver of obese rats with concomitant decrease of fat deposition (Piras et al., 2015), and to increase plasma n-3 HUFA score in hypercholesterolemic subjects (Pintus et al., 2013).
The beneficial effects of HFM administration are indicated also by the increased activities of detoxifying enzymes (GST-NQO1), and by reduction of oxidative stress, indicated by a decrease in H$_2$O$_2$ production, ROS and CP levels, and an increase in aconitate, SOD and CAT activity. Accordingly, it was recently demonstrated that mitochondrial SOD upregulation leads to the activation of AMPK (Hart et al., 2015). The decreased ROS production may be associated to a decrease in mitochondrial efficiency, since the mitochondrial uncoupling plays a natural antioxidant effect, keeping the mitochondrial membrane potential below the critical threshold for ROS production (Skulachev, 1991; Mailloux and Harper, 2011).

From another point of view, it is important to consider that increased ROS production and reduced antioxidant capacity may also be responsible of altered vascular tone (Gliemann et al., 2016). Regulation of vascular tone is, at least in part, mediated by AMPK through several mechanisms, as stimulation of nitric oxide release in endothelial cells (García-Prieto et al., 2015). Therefore, the correct control of AMPK activation may play a key role in providing an appropriate blood flow to the muscle to enhance the protection from pro-oxidant stimuli.

**CONCLUSION**

In conclusion, our study highlights that rat dietary supplementation with HFM decreases inflammatory status and improves lipid oxidation through AMPK activation in skeletal muscle. Since mitochondrial metabolism and regulation are similar in skeletal and cardiac muscle, that are both striated, our data raise the possibility that consumption of HFM improves lipid oxidation through AMPK activation in skeletal muscle. Since mitochondrial metabolism and regulation are similar in skeletal and cardiac muscle, that are both striated, our data raise the possibility that consumption of HFM improves lipid oxidation through AMPK activation in skeletal muscle.

**REFERENCES**

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol.* 105, 121–126. doi: 10.1016/0076-6879(84)05016-3

Alexson, S. E., and Nedergaard, J. (1988). A novel type of short- and medium-chain acyl-CoA hydrases in brown adipose tissue mitochondria. *J. Biol. Chem.* 263, 13564–13571.

Banni, S., Carta, G., Contini, M. S., Angioni, E., Deiana, M., Dessì, M. A., et al. (1996). Characterization of conjugated diene fatty acids in milk, dairy products, and lamb tissues. *J. Nutr. Biochem.* 7, 150–155. doi: 10.1016/0955-2863(95)00193-X

Barja, G. (1998). Mitochondrial free radical production and aging in mammals and birds. *Ann. N.Y. Acad. Sci.* 854, 224–238. doi: 10.1111/j.1749-6632.1998.tb09905.x

Benson, A. M., Hunkeler, M. J., and Talalay, P. (1980). Increase of NAD(P)H: quinone reductase by dietary antioxidants: possible role in protection against carcinogenesis and toxicity. *Proc. Natl. Acad. Sci. U.S.A.* 77, 5216–5220. doi: 10.1073/pnas.77.9.5216

Bergamo, P., Maurano, F., and Rossi, M. (2007). Phase 2 enzyme induction by conjugated linoleic acid improves lupus-associated oxidative stress. *Free Radic. Biol. Med.* 43, 71–79. doi: 10.1016/j.freeradbiomed.2007.03.023

Cairns, C. B., Walther, J., Harken, A. H., and Banerjee, A. (1998). Mitochondrial oxidative phosphorylation efficiencies reflect physiological organ roles. *Am. J. Physiol.* 274, R1376–R1383. doi: 10.1152/ajpregu.1998.274.5.R1376

Cavaliere, G., Trinchese, G., Bergamo, P., De Filippo, C., Mattace Raso, G., Gifuni, G., et al. (2016). Polyunsaturated fatty acids attenuate diet induced obesity and insulin resistance, modulating mitochondrial respiratory uncoupling in rat skeletal muscle. *PLoS One* 11:e0149033. doi: 10.1371/journal.pone.0149033

Cavaliere, G., Trinchese, G., Musco, N., Infascelli, F., De Filippo, C., Mastellone, V., et al. (2018). Milk from cows fed a diet with a high forage: concentrate ratio improves inflammatory state, oxidative stress, and mitochondrial function in rats. *J. Dairy Sci.* 101, 1843–1851. doi: 10.3168/jds.2017–13550

Chun, J. T., Crispino, M., and Tocco, G. (2004). The dual response of protein kinase Fyn to neural trauma: early induction in neurons and delayed induction in reactive astrocytes. *Exp. Neurol.* 185, 109–119. doi: 10.1016/j.expneurol.2003.09.019

Cruz-Hernandez, C., Kramer, J. K. G., Kennelly, J. J., Glimm, D. R., Sorensen, B. M., Okine, E. K., et al. (2007). Evaluating the conjugated linoleic acid and trans 18:1 isomers in milk fat of dairy cows fed increasing amounts of sunflower oil and a constant level of fish oil. *J. Dairy Sci.* 90, 3786–3801. doi: 10.3168/jds.2006-698

Ferramini, E., Simonson, D. C., Katz, L. D., Reichard, G. Jr., Bevilacqua, S., Barrett, E. J., et al. (1988). The disposal of an oral glucose load in patients with non-insulin-dependent diabetes. *Metabolism* 37, 79–85. doi: 10.1016/0026-0495(88)90033-9

Flohó, L., and Otting, F. (1984). Superoxide dismutase assay. *Methods Enzymol.* 105, 93–104. doi: 10.1016/S0076-6879(84)50131-8
Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A. G., Lo Verso, F., Carnio, S., Vainshtein, A., and Sandri, M. (2014). Autophagy is not required to sustain exercise and PRKAA1/AMPK activity but is important for mitochondrial reactive oxygen species production. J. Clin. Invest. 121, 4903–4915. doi: 10.1172/JCI58577

Garcia-Prieto, C. F., Gil-Ortega, M., Aránguez, I., Ortiz-Besoino, M., Somozo, B., and Fernández-Alfonso, M. S. (2015). Vascular AMPK as an attractive target in the treatment of vascular complications of obesity. Vascul. Pharmacol. 60, 10–20. doi: 10.1016/j.vph.2015.02.017

Glemann, L., Nyberg, M., and Hellsten, Y. (2016). Effects of exercise training and resveratrol on vascular health in aging. Free Radic. Biol. Med. 98, 165–176. doi: 10.1016/j.freeradbiomed.2016.03.037

Habig, W. H., and Jakoby, W. B. (1981). Assays for determination of glutathione S-transferases. Methods Enzymol. 77, 398–405. doi: 10.1016/S0076-6879(81)77053-8

Hardie, D. G., Ross, F. A., and Hawley, S. A. (2011). Uncoupling proteins and the control of mitochondrial reactive oxygen species. FEBS Lett. 584, 158–162. doi: 10.1016/j.febslet.2010.09.042

Habib, M. P., Angioni, E., Carta, G., Murru, E., Spada, S., et al. (2001). N-acetylcysteine suppresses oxidative stress in experimental rats with subarachnoid hemorrhage. J. Clin. Neurosci. 16, 684–688. doi: 10.1016/j.jocn.2008.04.021

Mailloux, R. J., and Harper, M. E. (2011). Uncoupling proteins and the control of mitochondrial reactive oxygen species production. Free Radi. Biol. Med. 51, 1106–1115. doi: 10.1016/j.freeradbiomed.2011.06.022

Melis, M. P., Angioni, E., Carta, G., MurrU, E., ScapU, S., Spada, S., et al. (2001). Characterization of conjugated linoleic acid and its metabolites by RP-HPLC with diode array detector. Eur. J. Lipid Sci. Technol. 103, 617–621. doi: 10.1002/1438-9312(200109)103:9<617:AID-EJLT6170-3.0.CO;2-C

Merrill, G. F., Kurth, E. J., Hardie, D. G., and Winder, W. W. (1997). AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle. Am. J. Physiol. 273, E1107–E1112. doi: 10.1152/ajpendo.1997.273.6.E1107

Miller, G. D., and Jarvis, J. K. (2000). Handbook of Dairy Foods and Nutrition, Second Edn. Rosemont, IL: National Dairy Council.

Mollica, M. P., Trinchese, G., Cavaliere, G., De Filippo, C., Cocca, E., Gaita, M., et al. (2014). c911-Conjugated linoleic acid ameliorates steatosis by modulating mitochondrial uncoupling and Nrf2 pathway. J. Lipid Res. 55, 837–849. doi: 10.1194/jlr.M044032

Montolui, C., Valles, S., Renau-Piqueras, J., and Guerr, C. (1994). Ethanol-induced oxygen radical formation and lipid peroxidation in rat brain: effect of chronic alcohol consumption. J. Neurochem. 63, 1855–1862. doi: 10.1046/j.1471-4159.1994.6301855.x

Pant, M., Bal, N. C., and Periasamy, M. (2016). Sarcoplasm: a key thermogenic and metabolic regulator in skeletal muscle. Trends Endocrinol. Metab. 27, 881–892. doi: 10.1016/j.tem.2016.08.006

Pariza, M. W. (1999). “The biological activities of conjugated linoleic acid,” in Advances in Conjugated Linoleic Acid Research, Vol. 1, eds M. YuraceW, M. M. Mossoba, J. Kramer, M. Pariza, and G. Nelson (Champaign, IL: AOCS Press), 13.

Periasamy, M., Maurya, S. K., Sahoo, S. K., Singh, S., Sahoo, S. K., Reis, F. C. G., et al. (2017). Role of SERCA pump in muscle thermogenesis and metabolism. Compr. Physiol. 7, 879–890. doi: 10.1002/cphy.c160030

Pintus, S., MurrU, E., Carta, G., Cordeddu, L., Batetta, B., AccossU, S., et al. (2013). Sheep cheese naturally enriched in alpha-linolenic, conjugated linoleic and vaccenic acids improves the lipid profile and reduces anandamide in the plasma of hypercholesterolemic subjects. Br. J. Nutr. 109, 1453–1462. doi: 10.1017/S0007114510002324

Piras, A., Carta, G., MurrU, E., Lopes, P. A., Martins, S. V., Prates, J. A., et al. (2015). Effects of dietary CLA on n-3 HUFA score and N-acylalaminolides biosynthesis in the liver of obese Zucker rats. Prestaglandins Leukot. Essent. Fatty Acids 98, 15–19. doi: 10.1016/j.pla.2015.04.004

Porter, C., Herndon, D. N., Sidossis, L. S., and Borsheim, E. (2013). The impact of severe burns on skeletal muscle mitochondrial function. Burns 39, 1039–1047. doi: 10.1016/j.burns.2013.03.018

Rolfé, D. F., and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. Physiol. Rev. 77, 731–758. doi: 10.1152/physrev.1997.77.3.731

Romanello, V., and Sandri, M. (2016). Mitochondrial quality control and muscle mass maintenance. Front. Physiol. 6:422. doi: 10.3389/fphys.2015.00422

Salt, I. P., and Palmer, T. M. (2012). Exploiting the anti-inflammatory effects of AMP-activated protein kinase activation. Expert. Opin. Investig. Drugs 21, 1155–1167. doi: 10.1517/13543784.2012.696609

Sartori, R., Gregorevic, P., and Sandri, M. (2014). TGFβ and BMP signaling in skeletal muscle: potential significance for muscle-related disease. Trends Endocrinol. Metab. 25, 464–471. doi: 10.1016/j.tem.2014.06.002

Sartori, R., Schirwis, E., Blauw, B., Bortolanza, S., Zhao, J., Enzo, E., et al. (2013). BMP signaling controls muscle mass. Nat. Genet. 45, 1309–1318. doi: 10.1038/ng.2772

Skulachev, V. P. (1991). Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation. FEBS Lett. 294, 158–162. doi: 10.1016/0014-5793(91)80658-P

Sanguinuolo, M. S., Mollica, M. P., Maresco, B., Cavaliere, G., Cefaliello, C., Trinchese, G., et al. (2015). High fat diet and inflammation - modulation of haptoglobin level in rat brain. Front. Cell. Neurosci. 9:479. doi: 10.3389/fncel.2015.00479

Stark, K. D. (2008). The percentage of n-3 highly unsaturated fatty acids in total HUFA as a biomarker for omega-3 fatty acid status in tissues. Lipids 43, 45–53. doi: 10.1007/s11745-007-3128-3

Stucki, J. W. (1980). The optimal efficiency and the economic degrees of coupling of oxidative phosphorylation. J. Biol. Chem. 255, 10960–10967. doi: 10.1016/S0021-9258(80)90608-P

Sandal, I., and Palmer, T. M. (2012). Exploiting the anti-inflammatory effects of AMP-activated protein kinase activation. Expert. Opin. Investig. Drugs 21, 1155–1167. doi: 10.1517/13543784.2012.696609

Sartori, R., Gregorevic, P., and Sandri, M. (2014). TGFβ and BMP signaling in skeletal muscle: potential significance for muscle-related disease. Trends Endocrinol. Metab. 25, 464–471. doi: 10.1016/j.tem.2014.06.002

Sartori, R., Schirwis, E., Blauw, B., Bortolanza, S., Zhao, J., Enzo, E., et al. (2013). BMP signaling controls muscle mass. Nat. Genet. 45, 1309–1318. doi: 10.1038/ng.2772
oxidation, and glucose storage in man. Diabetes Metab. Res. Rev. 31, 957–963. doi: 10.2337/diacare.31.11.957
Trinchese, G., Cavaliere, G., De Filippo, C., Aceto, S., Prisco, M., Chun, J. T., et al. (2018). Human milk and donkey milk, compared to cow milk, reduce inflammatory mediators and modulate glucose and lipid metabolism, acting on mitochondrial function and oleylethanolamide levels in rat skeletal muscle. Front. Physiol. 9:32. doi: 10.3389/fphys.2018.00032
Trinchese, G., Cavaliere, G., De Filippo, C., Aceto, S., Prisco, M., Chun, J. T., et al. (2018). Human milk and donkey milk, compared to cow milk, reduce inflammatory mediators and modulate glucose and lipid metabolism, acting on mitochondrial function and oleylethanolamide levels in rat skeletal muscle. Front. Physiol. 9:32. doi: 10.3389/fphys.2018.00032
Tudisco, R., CalabroÌ, S., Cutrignelli, M. I., Moniello, G., Grossi, M., Gonzalez, O. J., et al. (2012). Influence of organic systems on stearoyl-CoA desaturase gene expression in goat milk. Small Rumin. Res. 106, S37–S42. doi: 10.1016/j.smallrumres.2012.04.031
Tudisco, R., Cutrignelli, M. I., CalabroÌ, S., Piccolo, G., Bovera, F., Guglielmelli, A., et al. (2010). Influence of organic systems on milk fatty acid profile and CLA in goats. Small Rumin. Res. 88, 151–155. doi: 10.1016/j.smallrumres.2009.12.023
Tudisco, R., Grossi, M., CalabroÌ, S., Cutrignelli, M. I., Musco, N., Addi, L., et al. (2014). Influence of pasture on goat milk fatty acids and stearoyl-CoA desaturase expression in milk somatic cells. Small Rumin. Res. 122, 38–43. doi: 10.1016/j.smallrumres.2014.07.016
Turner, N., Cooney, G. J., Kraegen, E. W., and Bruce, C. R. (2014). Fatty acid metabolism, energy expenditure and insulin resistance in muscle. J. Endocrinol. 220, T61–T79. doi: 10.1530/JEO-13-0397
Wiking, L., Theil, P. K., Nielsen, J. H., and Sørensen, M. T. (2010). Effect of grazing fresh legumes or feeding silage on fatty acids and enzymes involved in the synthesis of milk fat in dairy cows. J. Dairy Res. 77, 337–342. doi: 10.1017/S002202991000021X
Winder, W. W., Holmes, B. F., Rubink, D. S., Jensen, E. B., Chen, M., and Holloszy, J. O. (2000). Activation of AMP-activated protein kinase increases mitochondrial enzymes in skeletal muscle. J. Appl. Physiol. 88, 2219–2226. doi: 10.1152/jappl.2000.88.6.2219
Zurlo, F., Larson, K., Bogardus, C., and Ravussin, E. (1990). Skeletal muscle metabolism is a major determinant of resting energy expenditure. J. Clin. Invest. 86, 1423–1427. doi: 10.1172/JCI114857
Zurlo, F., Nemeth, P. M., Choksi, R. M., Sesodia, S., and Ravussin, E. (1994). Whole-body energy metabolism and skeletal muscle biochemical characteristics. Metabolism 43, 481–486. doi: 10.1016/0026-0495(94)90081-7
Conflict of Interest Statement: 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.
Copyright © 2019 Trinchese, Cavaliere, Penna, De Filippo, Cimmino, Catapano, Musco, Tudisco, Lombardi, Infascelli, Messina, Muredda, Banni, Monda, Crispino and Mollica. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.