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The influence of L-methionine, DL-methionine, and a methionine hydroxy analog on proliferation and differentiation potential of avian myoblasts

Ronald J. Savino[1], Mercedes Vazquez-Anon[2], Katarzyna Stefańska[3], James N. Petitte[1], Paul Mozdziak[1]

Abstract

Methionine is an essential amino critical to many cell functions including the synthesis of proteins. Supplementation of methionine in vivo is typically through L-methionine, DL-methionine, or a methionine hydroxy analog (MHA). The goal of this study was to compare the function of L-methionine, DL-methionine, and an MHA as a source of methionine to myoblasts in vitro. Avian myoblasts isolated from turkey embryos were plated in media containing varying concentrations of DL-methionine (DLM; 1.125 mg/mL or 0.56 mg/mL) or methionine hydroxy analog (MHA; 1.28 mg/mL or 0.64 mg/mL) as well as a methionine deficient negative control group and an L-methionine supplemented positive control group. The results of the proliferation assay exhibited cell division in the absence of methionine which was not significantly different than the positive control group. Results from the myoblast fusion assay revealed significantly greater myotube diameter between methionine supplemented groups compared to the methionine deficient negative control. The findings of this study show an ability for avian myoblasts to proliferate in the absence of methionine, the significance of which is discussed. Additionally, findings from the fusion assay suggest that DL-methionine and MHA are potential cost-effective substitutes for methionine supplementation during terminal differentiation of avian myoblasts.

Running title: Alternative methionine sources impacting myoblast proliferation and differentiation

Keywords: methionine, in vitro, myoblast, avian, proliferation, fusion, myotube diameter, differentiation

[1]Prestage Department of Poultry Science, College of Agriculture and Life Sciences, North Carolina State University Raleigh, NC, USA
[2]Novus International, Inc., Saint Charles, MO, USA
[3]Department of Histology, Poznan University of Medical Sciences, Poznań, Poland
Correspondence: rjsavino@ncsu.edu
Full list of author information is available at the end of article

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Introduction

Methionine is an essential amino acid that serves an array of roles in both eukaryotes and prokaryotes. It is commonly known as the initiator of protein synthesis and plays an essential role as a methyl donor during the synthesis of fatty acids, polyamines, and biotin [1]. Its importance in cellular function makes its supplementation essential in animal feed as well as cell culture media. Three forms of methionine are typically used for supplementation *in vivo*: L-methionine (LM), DL-methionine (DLM), or liquid DL-methionine hydroxy analog-free acid (MHA). L-methionine is biologically active and commonly found in most tissues and is, therefore, the common form used for supplementation [2].

DL-methionine is a racemic mixture of D-methionine and L-methionine. Although D-methionine is the biologically inactive form, birds have the ability to utilize both D- and L-methionine [3]. Therefore, birds may be supplemented with either powdered DL-methionine, which is typically 99% pure DLM, or liquid DL-methionine hydroxy analog-free acid, which typically contains 88% DL-hydroxy-4- (methylthio) butanoic acid (DL-HMB). Chemically, LM

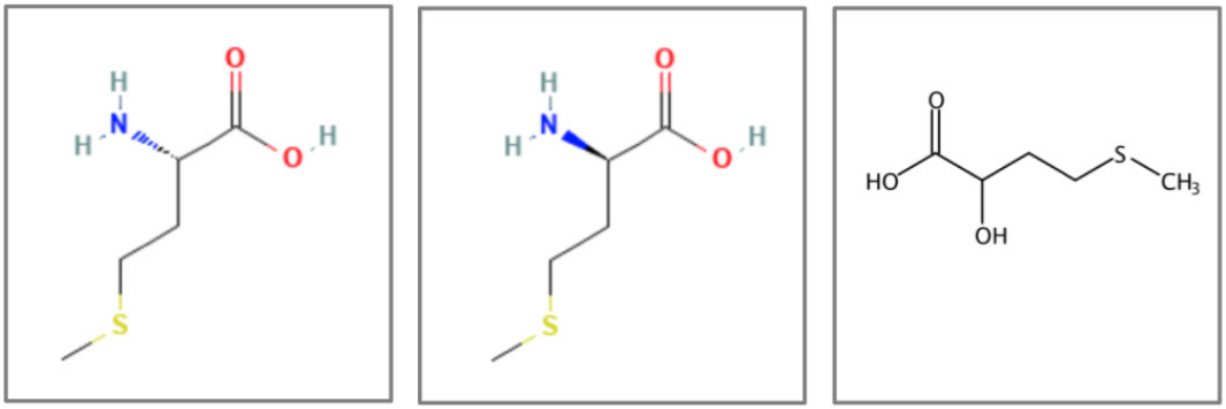

FIGURE 1 The images above represent the chemical structures of the three forms of methionine used in this experiment. From left to right: L-methionine (PubChem CID 6137), D-methionine (PubChem CID 84815), and methionine hydroxy analog (Alimet, Novus International, St. Charles, MO)

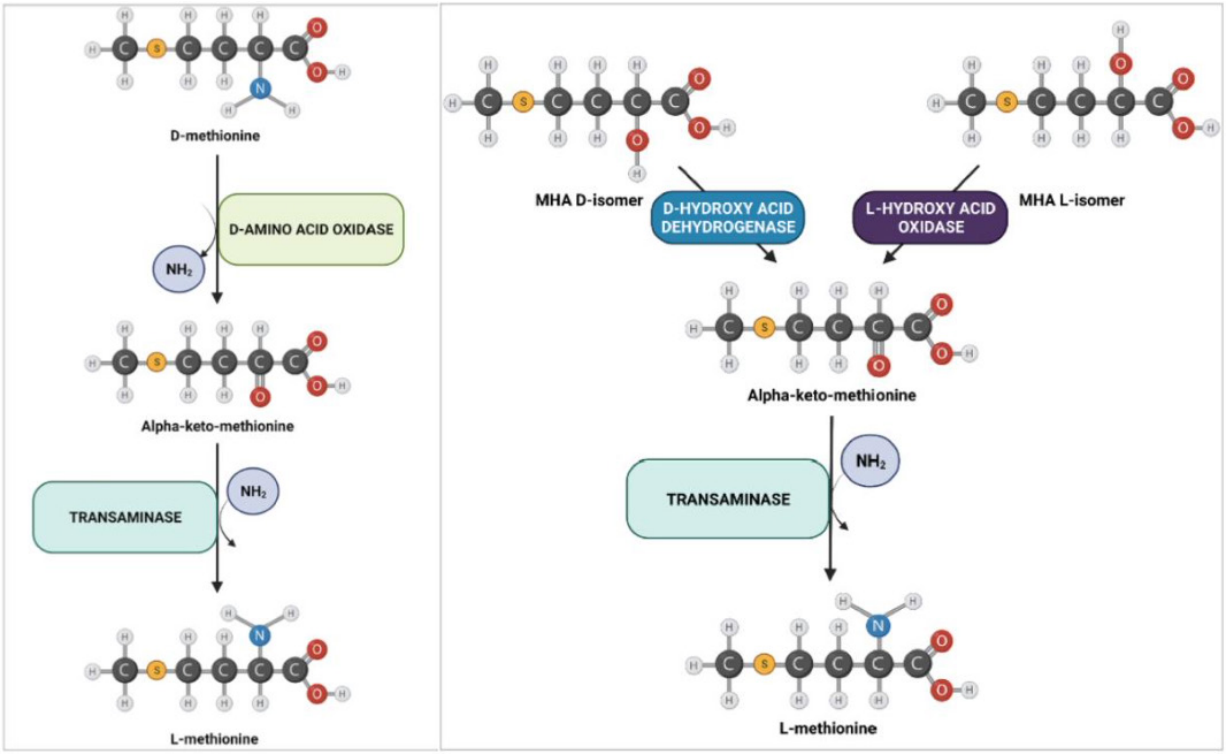

FIGURE 2 The image on the left-hand side of the figure describes the pathway of the conversion of D-methionine to L-methionine. The image on the right-hand side of the figure describes the conversion of both the D-isomer and L-isomer of methionine hydroxy analog (MHA) to L-methionine

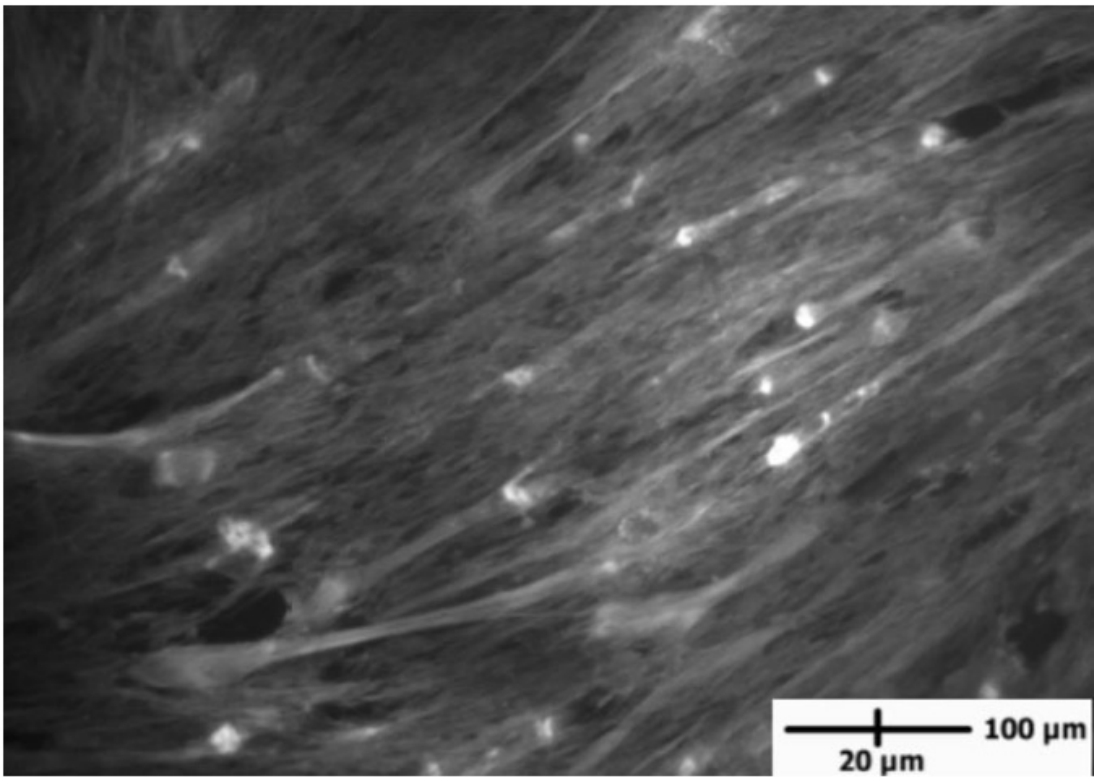

FIGURE 3 Photomicrograph of phalloidin-stained myotubes. Myoblasts were seeded at high density and incubated for 10 days until 100% confluency and differentiation were observed. Phalloidin-stained myotubes show fluorescent actin protein confirming myogenicity of cells

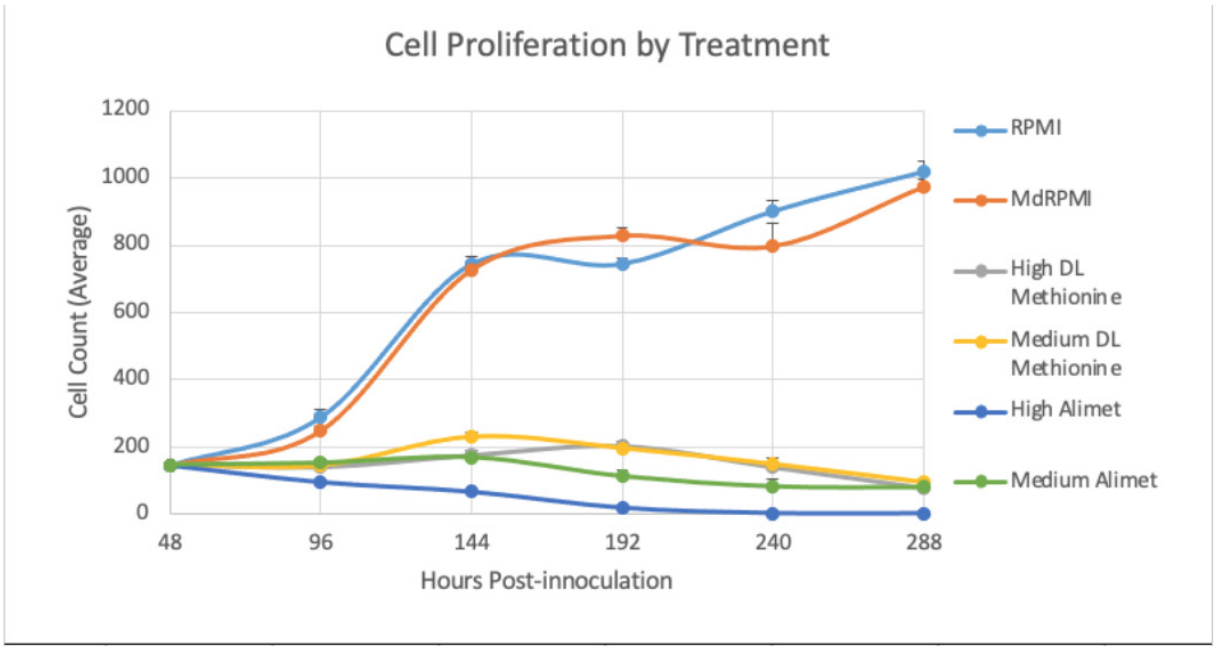

FIGURE 4 Proliferation of myoblasts isolated from the *pectoralis major* from embryonic day 21 turkey embryos. Average cell (±SEM) count by treatment 48 h post-inoculation. High Alimet (1.125 mg/mL Alimet), High DL-Methionine (1.28 mg/mL DLM), Medium Alimet (0.64 mg/mL Alimet), Medium DL-Methionine (0.56 mg/mL DLM), MdRPMI (negative control Methionine-deficient RPMI), and RPMI (15 mg/L positive control LM RPMI)

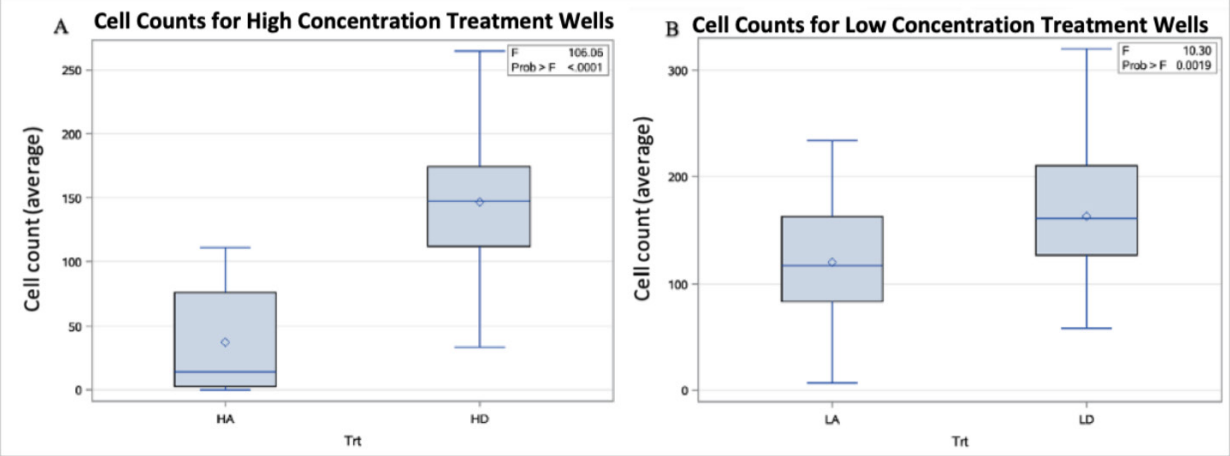

FIGURE 5 Proliferation of myoblasts isolated from the *pectoralis major* from embryonic day 21 turkey embryos. Average cell count combined data from 96 h post-inoculation to 288 h post-inoculation. (A) Comparing only treatments that received either Alimet at 1.125 mg/mL (HA) or DLM at 1.28 mg/mL (HD). (B) Comparing only treatments that received either Alimet at 0.56 mg/mL (LA) or DLM at 0.64 mg/mL (LD). Statistical significance is indicated by Prob > F (a < .05). Mean, upper quartile, and lower quartile of cell counts were calculated for each treatment. Standard errors and outliers included. Proc GLM 1-way ANOVA was conducted using SAS Studio 3.8 (SAS Institute Inc, Cary, NC)

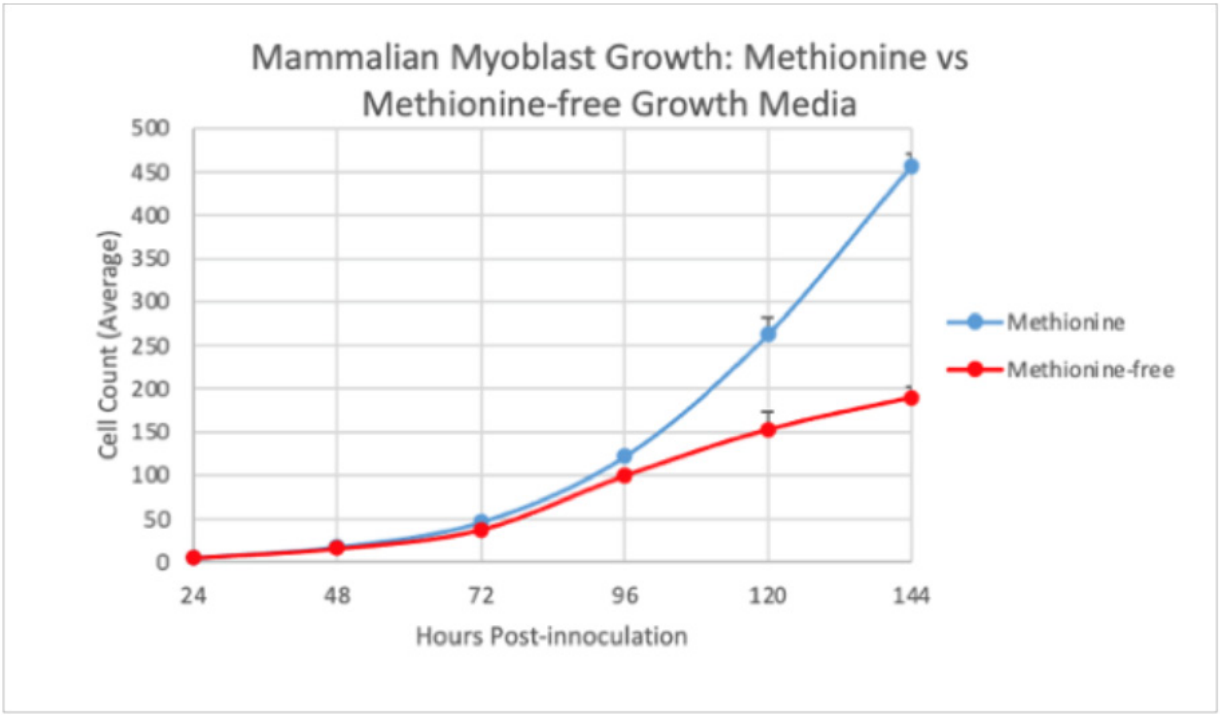

FIGURE 6 Proliferation of mammalian C2C12 cell line sourced from frozen stock. Average cell (±SEM) count by treatment 24 h post-inoculation. Treatments include Methionine-free (Methionine-deficient RPMI), and Methionine (15 mg/L LM RPMI)

and DM are identical with differences only in their chemical structure based around their central carbons as seen in **figure 1**. An MHA on the other hand differs chemically from L-methionine in that it contains a hydroxyl group in place of the amine group (**Fig. 1**) and contains one asymmetrical carbon which means contains a mixture of 50% L-isomer and 50%-D-isomer. Converting D-methionine to L-methionine requires the enzyme D-amino acid oxidase to remove the amino group forming an alpha-keto-methionine intermediate which is finally converted to L-methionine by the addition of an amino-group via a transaminase (**Fig. 2**).

The conversion of an MHA consists of converting both the L-isomer and D-isomer and therefore involves additional enzymes. The conversion of the L-isomer utilizes L-hydroxy acid oxidase while the D-isomer utilizes D-hydroxy acid dehydrogenase, both of which converts the hydroxyl group of their respective isomers into a keto group (**Fig. 2**). This

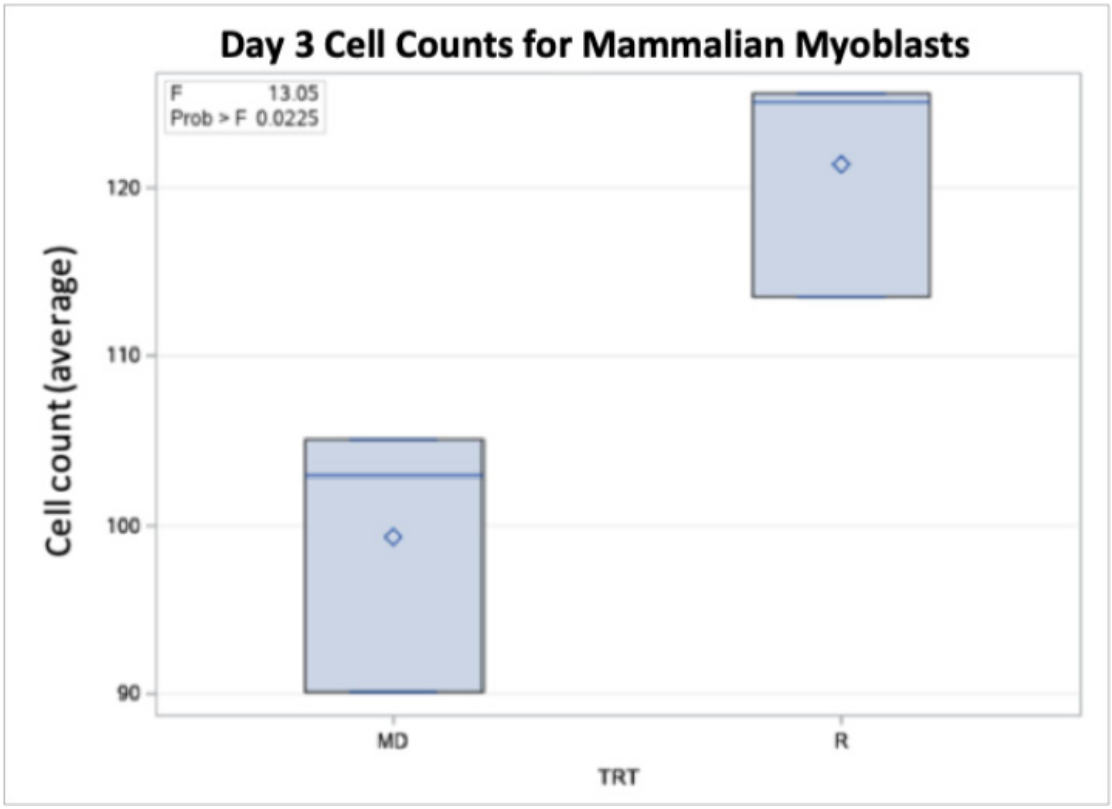

FIGURE 7 Proliferation of mammalian C2C12 cell line sourced from frozen stock. Average cell count of myoblasts 72 hours post-inoculation. Comparing cell counts from myoblasts treated with RPMI (R, 15 mg/L methionine) and MdRPMI (MD, methionine deficient)

conversion is interesting in that it forms the same intermediate as the conversion of D-methionine: alpha-keto methionine. Finally, much like in D-methionine, a transaminase attaches an amino group which forms L-methionine from this intermediate [4].

A commonly used methionine supplement, Alimet (Novus International, St. Charles, MO), is one of these liquid MHAs that serves as a feed additive in livestock. Previous studies have shown that the use of MHA, such as Alimet, shows no difference in the biological response in methionine bioactivity and growth efficiency compared to supplementation with DL-methionine or L-methionine in birds [4-8]. Though the difference of these supplements on growth efficiency and methionine activity is negligible *in vivo*, supplementation with DL-methionine and MHAs *in vitro* has not been previously investigated. The aim of this study was to analyze the effect of supplementation with DL-methionine hydroxy analog-free acid and crystalline DL-methionine on the proliferation and differentiation of avian myoblasts *in vitro*.

Materials and Methods

Standard plating and proliferation media

Plating media was made containing 94% Dulbecco's Modified Eagle's Medium (Gibco Burlington, ON, Canada), 5% horse serum (Gibco, Auckland, New Zealand), and 1% antibiotic/antimycotic (A5955 Sigma-Aldrich). Complete proliferation media was made containing 84% RPMI medium 1640 from powder (Gibco), 15% chicken serum (Gibco, Auckland, New Zealand), and 1% antibiotic/antimycotic (A5955, Sigma-Aldrich, St. Louis, MO). Media was made based on previous formulation by MacFarland et al [9].

Media for proliferation assay

Media used as the positive control was RPMI complete proliferation media containing L-methionine. Media which served as the negative control for the proliferation assay was made with 84% methionine deficient RPMI (Gibco Burlington, ON, Canada), 15% chicken serum (Gibco, Auckland, NZ), and 1% antibiotic/antimycotic (A5955 Sigma-Aldrich). DL--methionine media was made using 84% methionine deficient RPMI (Gibco), 15% chicken serum (Gibco, NZ), 1% antibiotic/antimycotic (Sigma-Aldrich), and 1.125 mg/mL DL-methionine (Novus International, St. Charles, MO). DL-methionine media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as 0.56 mg/mL DL-methionine treatment. Media with Alimet for the proliferation assay was made using 84% methionine deficient RPMI (Gibco), 15% chicken serum (Gibco, NZ), 1% antibiotic/antimycotic (A5955, Sigma-Aldrich), and 1.28 mg/mL liquid Alimet (Novus International). Ali-

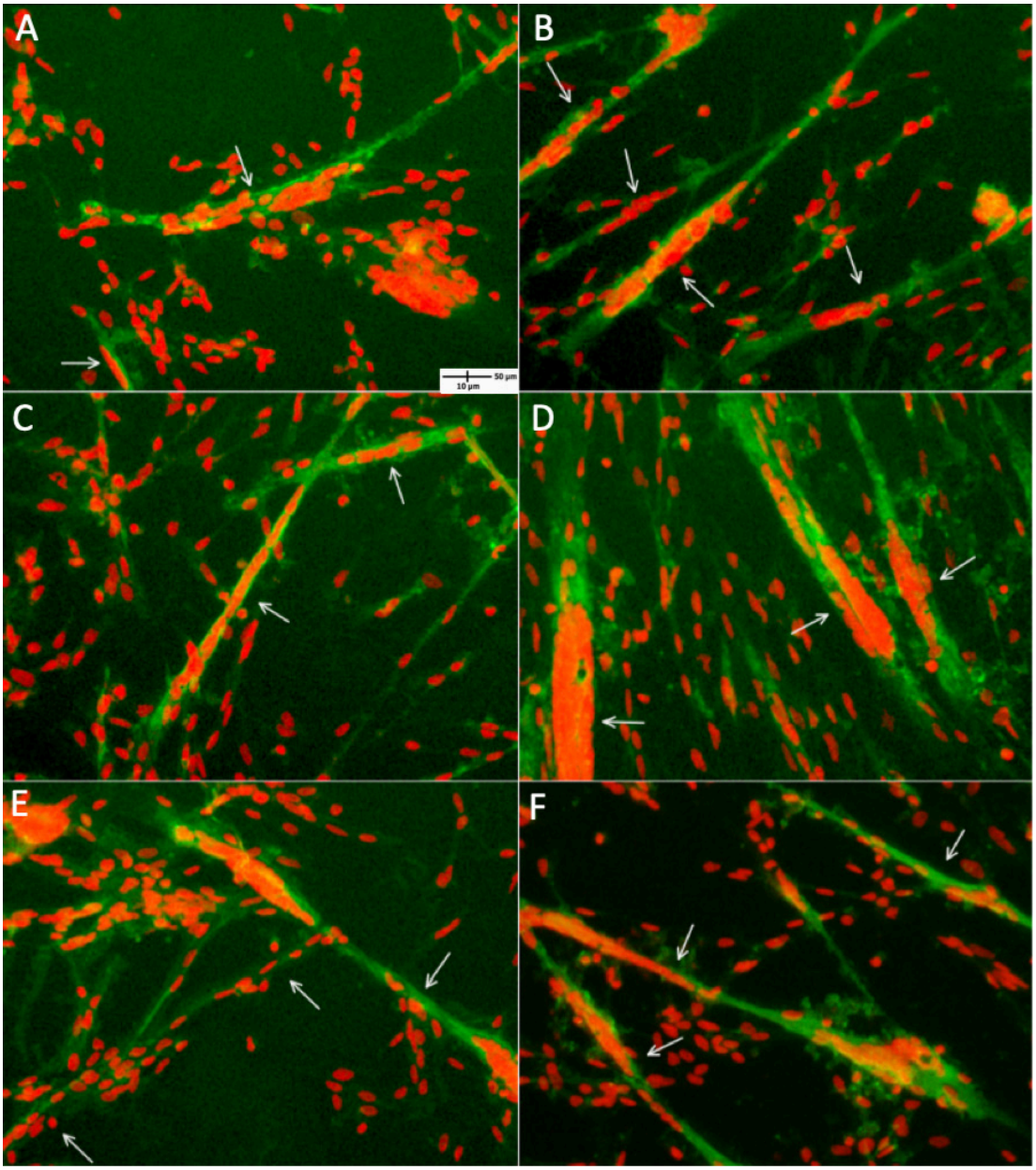

FIGURE 8 Images of differentiated myotubes formed from myoblasts isolated from the *pectoralis major* of embryonic day 21 turkey embryos. Images were taken from the day 4 plate of the differentiation assay using an inverted microscope under a 20x objective lens using SPOT imaging software (SPOT imaging, Sterling Heights, MI) Myotubes were incubated with MF 20-c myosin heavy chain antibody, IgG Goat anti-mouse secondary antibody conjugated with FITC, and counter-stained with propidium iodide. Images taken from each of the six control and six treatment columns: (A) positive control of RPMI with LM, (B) methionine deficient negative control, (C) 1.28 mg/mL DLM, (D) 0.56 mg/mL DLM (E) 1.125 mg/mL Alimet, (F) 0.64 mg/mL Alimet

met media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as the 0.64 mg/mL Alimet treatment. Alimet and DL--methionine treatment media were formulated to have equivalent molar concentrations of methionine.

Differentiation media used for fusion and myotube diameter assay

Negative control media was made with 96% methionine deficient RPMI (Gibco Burlington, ON, Canada), 3% horse serum (Gibco, Auckland, New Zealand), and 1% antibiotic/antimycotic (A5955 Sigma-Aldrich St. Louis, MO). Treatment media with DL-methionine was made using 96% methionine deficient RPMI (Gibco), 3% horse serum (Gibco, NZ), 1% antibiotic/antimycotic (A5955, Sigma-Aldrich), and 1.125 mg/mL DL-methionine (Novus International, St. Charles, MO). DL-methionine media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as 0.56 mg/mL DL-methionine treatment. Treatment media with Alimet was made using 96% methionine deficient

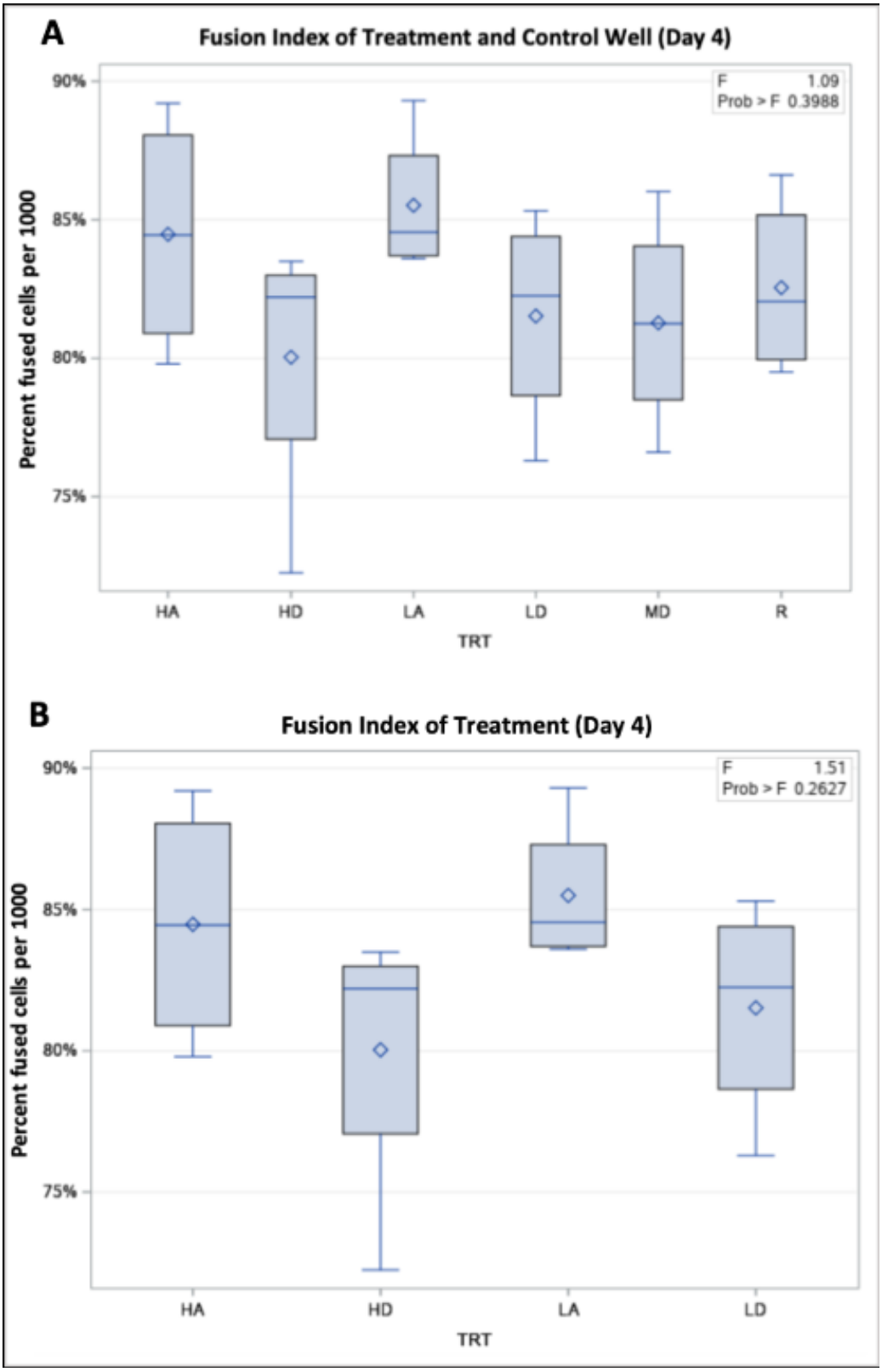

FIGURE 9 Percent of fused myoblasts per 1000 cells counted separated by treatment. Data includes fusion index from the day 4 plate of the myotube differentiation assay. (A) Fusion indices separated by treatment with 1.125 mg/mL Alimet (HA), 1.28 mg/mL DLM (HD), 0.64 mg/mL Alimet (LA), 0.56 mg/mL DLM (LD), negative control methionine deficient RPMI (MD), and positive control LM RPMI (R). (B) Fusion indices comparing only treatment with 1.125 mg/mL Alimet (HA), 1.28 mg/mL DLM (HD), 0.64 mg/mL Alimet (LA), and 0.56 mg/mL DLM (LD). Statistical significance is indicated by Prob > F (a < .05). Mean, upper quartile, and lower quartile of cell counts were calculated for each treatment. Standard errors and outliers included. Proc GLM 1-way ANOVA was conducted using SAS Studio 3.8 (SAS Institute Inc, Cary, NC)

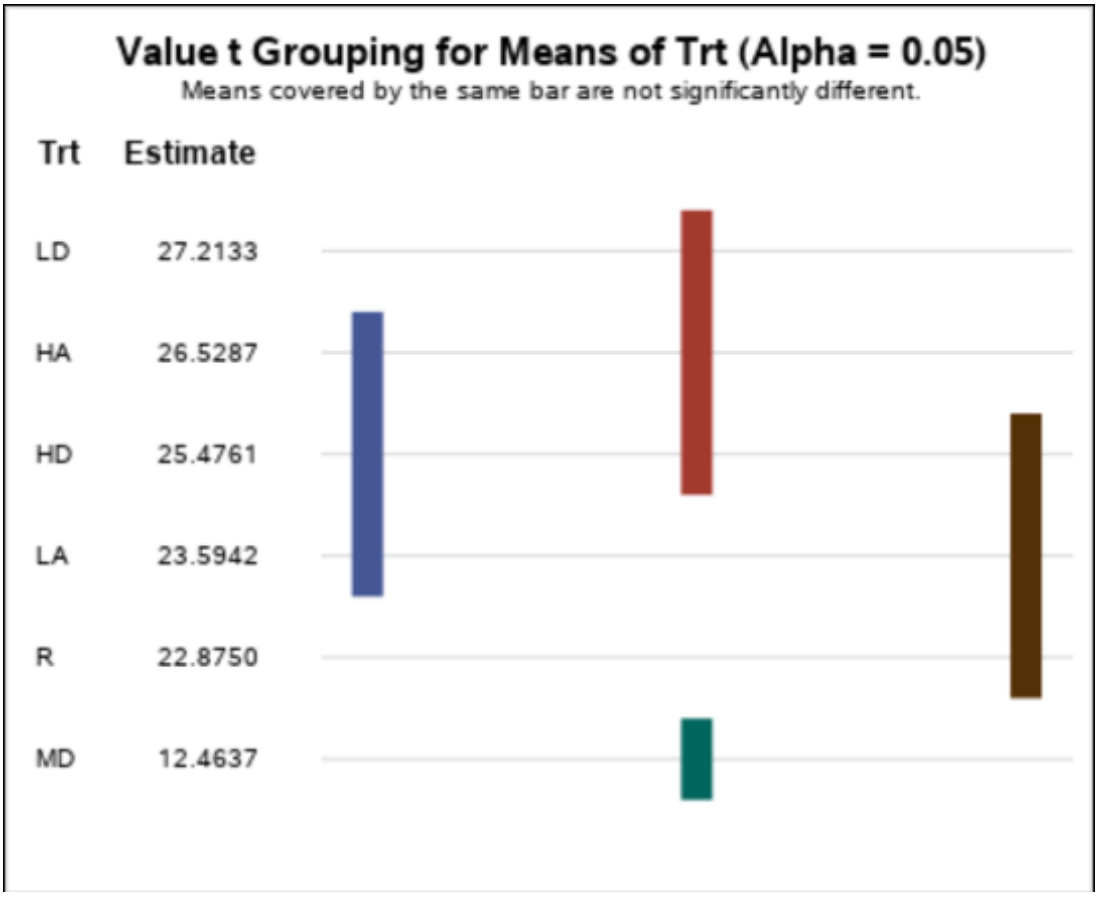

FIGURE 10 Average myotube diameter averaged from three randomly selected myotubes. Myotubes were selected from the day four and day five plates of the differentiation assay. Measurements were taken from positive control RPMI + LM (R), methionine deficient negative control (MD), and wells treated with 1.125 mg/mL Alimet (HA), 1.28 mg/mL DLM (HD), 0.64 mg/mL Alimet (LA), and 0.56 mg/mL DLM (LD). Estimates represent the average myotube diameter in μm from each treatment and control group. Treatments covered by the same color line represent myotube diameters that are not significantly different while treatments covered with bars of different colors are myotube diameters that are significantly different (a < .05). A least significant difference test was conducted using SAS Studio 3.8 (SAS Institute Inc, Cary, NC)

RPMI (Gibco), 3% horse serum (Gibco, NZ), 1% antibiotic/antimycotic (A5955, Sigma-Aldrich), and 1.28 mg/mL liquid Alimet (Novus International). Alimet media was diluted 1:1 with methionine deficient RPMI negative control media. This media served as the 0.64 mg/mL Alimet treatment. To avoid mature myotubes contracting and detaching from the culture dish, .01 mg/mL gelatin and 1 mg/mL bovine serum albumin (BSA fraction V, Fisher Scientific, Pittsburgh, PA) were added to all differentiation media based on a previously described protocol [10].

Avian myoblast isolation

Fertilized turkey embryos were incubated at 37.5˚C and 55% relative humidity (RH) until embryonic day (ED) 21. The egg was sprayed with 70% EtOH after which the embryo was placed in a dish and euthanized by cervical dislocation. A small sample of muscle was dissected from the *pectoralis major* and placed into 2 mL of Hanks Balanced Salt Solution (HBSS, Sigma-Aldrich, pH 7.4). The muscle sample was mechanically disassociated while still suspended in HBSS using sterile scissors. Once minced, 8 mL of a 0.17% trypsin (Sigma-Aldrich, St. Louis, MO) and 0.085% collagenase (Sigma-Aldrich) solution were added to the muscle in HBSS. The sample of muscle was then incubated at 37˚C and 5% CO_2 for 20 minutes to enzymatically dissociate muscle tissue and myoblast cells. Following incubation, the muscle homogenate was transferred to a snap cap tube and centrifuged for 5 minutes at 1,500 RPM. Following centrifugation, the supernatant was aspirated, and the pellet was resuspended in proliferation media. The cell homogenate was passed through a 100 μm Swinnex filter (Millipore Corporation, Darmstadt, Germany) where large tissue debris was removed, and myoblast cell isolate was collected. A complete cell count was conducted using a hemacytometer to calculate the appropriate seeding density for each assay.

Avian myoblast proliferation assay

Primary turkey myoblasts were plated into 12 .1% gelatin-coated 24-well cell culture plates (Pri-

maria, Corning, Durham, NC) at a density of 11,400 cells per well in 2 mL of media. One additional plate was seeded with 114,000 cells/well in 2 mL of media. This high-density plate served to verify the myogenicity of the isolate. All cultures were placed in an incubator at 37˚C and 5% CO_2. After 48 h, media was completely aspirated from two plates and the cells were fixed with 70% EtOH to serve as the day 0 control. Of the remaining plates, 1 mL of spent media was replaced in 4 wells per plate with 1 mL of positive control, negative control, or the associated methionine treatment media. This process was repeated every 48 h with two plates fixed each time until the final two plates were fixed. This provided a total of n=8 replicates per treatment for each day. The high-density plate was fixed with a paraformaldehyde and phosphate-buffered saline solution once confluency was reached for later visualization of myotubes. After plate fixation, wells were rinsed with phosphate-buffered saline (PBS) to rehydrate cells before staining. PBS was aspirated and replaced with propidium iodide (PI, Invitrogen, Washington, DC) at a concentration of 50 µg/mL as described previously [10]. The high-density plate fixed was stained with a phalloidin solution following a protocol provided by the manufacturer (Sigma-Aldrich, St. Louis, MO). Stained wells were placed under a fluorescent inverted microscope under a 20x objective lens with a rotamine filter set. Using SPOT imaging software (SPOT imaging, Sterling Heights, MI), 10 images were taken at random from each well. Using Image-Pro Plus image analysis software (Media Cybernetics, Rockville, MD), illuminated nuclei were segmented and counted from each image and the average of all 10 images was calculated to determine average cell count per well. Images of the differentiated cells stained with phalloidin confirmed myogenicity of cells and showed fluorescent actin (**Fig. 3**).

Mammalian myoblast proliferation assay

Mammalian myoblast proliferation assay C2C12 cells (ATCC; CRL-1772) derived from frozen stock were plated onto 6 .1% gelatin coated 6-well culture plates (Primaria, Corning, Durham, NC) at a density of 30,000 cells/well in 3 mL of media. All cultures were placed in an incubator at 37˚C and 5% CO_2. After 24 h, media was completely aspirated from one plate and cells were fixed with 70% EtOH to serve as the day 0 control. Of the remaining plates, 2 mL of spent media was replaced with either methionine supplemented or methionine deficient media. This process was repeated every 24 h until the final plate was fixed. This provided a total of n=3 replicates per treatment for each day. After fixation of the final plate, cells were rinsed with PBS for rehydration before staining. PBS was aspirated and replaced with PI at a concentration of .3 µg/mL according to a protocol provided by the manufacturer (ThermoFisher Scientific, Waltham, MA). Stained wells were placed under a fluorescent inverted microscope under a 20x objective lens with a rotamine filter set. Using SPOT imaging software (SPOT imaging, Sterling Heights, MI), 10 images were taken at random from each well. Using ImageJ analysis software (https://imagej.nih.gov/ij/index.html), illuminated nuclei were segmented and counted from each image and the average of all 10 images was calculated to determine average cell count per well.

Myotube fusion assay

Primary myoblasts isolated from turkey embryos were plated into 6.1% gelatin-coated 24-well cell culture plates (Primaria, Corning, Durham, NC). Each well was seeded with 19,000 cells and 2 mL of plating media and placed into a 37˚C, 5% CO_2 incubator. Spent media was replaced with 1 mL of complete proliferation media every 48 h until cells were nearing 100% confluency. Once confluent, media was aspirated completely from one plate and cells were fixed with 70% EtOH to serve as the day 0 control. For each remaining plate 1 mL of spent media was replaced with 1 mL of positive control, negative control, and treatment differentiation media with four replicate wells per plate. Media was replaced every 24 h with one plate fixed with 70% EtOH each time for a total of n=4 replicates per treatment for each day fixed. This process was repeated until the final plate was fixed. Following fixation, cells from the day 4 plate were permeabilized with phosphate buffered saline (PBS) containing .25% Tween 20 (Fisher Bioreagents, Pittsburgh, PA). Cells were then blocked using PBS with Tween 20 and 3% goat serum (Gibco, Auckland, NZ) before being incubated with MF 20-c antibodies (DSHB, Iowa City, Iowa) at 4˚C overnight. Cells were then incubated with IgG Goat anti-mouse secondary antibodies conjugated with FITC (Invitrogen, Washington, DC) after which a counter stain using propidium iodide (PI, Invitrogen, Washington, DC) was performed. Stained wells were placed under a fluorescent inverted microscope and viewed using a 20x objective lens and a rotamine filter set. Using SPOT imaging software (SPOT imaging, Sterling Heights, MI), 10 images were taken at random from each well. To determine the percent of myoblasts fused into myotubes a fusion index was completed on each image. For every 1000 cells counted, fused myoblasts were determined as three or more myoblasts aligned and encompassed within the same membrane. The number of fused myoblasts per 1000 cells counted was used as the percent fusion index. To determine myotube diameter, 5 myotubes were chosen at random from each well of treatment of images taken from the day 4 plate. Chosen myotubes were measured from each end and center using ImageJ (https://imagej.nih.gov/ij/index.html) and the average diameter was determined from the three measurements of each myotube following a method previously described by Thapaliya et al. [11].

Statistical analysis

Results of the avian and mammalian proliferation assays were determined by calculating the mean cell count and standard error by day per treatment from the mean cell count determined from each well. Results from the avian proliferation assay are expressed as mean fold-changes in cell count by day relative to both the LM supplemented positive and methionine deficient negative controls, ±SEM (n=8 replicates per treatment). Overall differences in mean cell count of 80 replicates comparing cell count by treatment against both LM supplemented positive control and methionine deficient negative control groups was determined using a Proc GLM 1-way ANOVA test (α= 0.05). Additional Proc GLM 1-way ANOVA tests were completed which compared the mean cell count of the high concentration treatments against one another and the low concentrations against one another (α= 0.05). For the mammalian myoblast proliferation assay, mean cell count for RPMI and MdRPMI treatments were compared by day using a Proc GLM 1-way ANOVA test (α= 0.05). For both the avian and mammalian proliferation assays, statistical significance of data is given as Prob > F.

Fusion index data was quantified using a Proc GLM 1-way ANOVA to determine differences in percent fusion among all treatments and controls (α= 0.05). An additional Proc GLM 1-way ANOVA test was completed using the mean fusion percentage of the four wells that received treatment media (α= 0.05) with significance given as Prob > F.

Myotube diameter data was compared using a least significant differences (LSD) test to determine differences between the mean myotube diameter from each treatment and control group. Means that are not significantly different (α= 0.05) between groups were covered by the same bar while means that are significantly different were covered by separate bars.

All statistical analysis tests were completed using either SAS Studio 3.8 (SAS Institute Inc, Cary, NC), Microsoft Excel 16.50 (Microsoft, King County, WA), or ImageJ 1.53a (National Institute of Health, USA).

Ethical approval

All procedures were approved by IACUC 19-717.

Results

Supplemental methionine and myoblast proliferation

The avian myoblast proliferation curves are shown in **figure 4.** Over the 10 days of the avian myoblast proliferation assay, the average cell counts of both RPMI positive control and MdRPMI negative control wells were significantly higher than the cell count of wells treated with supplemental methionine (P < .0001). Further comparison of cell counts by changes every 48 h showed wells containing LM supplemented positive and methionine deficient negative controls had increasing cell counts over the course of the experiment while wells receiving supplemental methionine treatments all showed decreasing cell counts. In comparing only wells treated with high concentrations of DL-methionine versus treatment with high concentrations of Alimet (**Fig. 5A**) wells treated with DL-methionine showed significantly higher cell count overall (P < .0001). Comparison of low concentration wells for DL-methionine and Alimet (**Fig. 5B**) yielded the same result with significantly higher cell counts in wells treated with DL-methionine than wells treated with Alimet (P < .0019).

Mammalian myoblast proliferation in the presence and absence of supplemental methionine

Results of the mammalian myoblast proliferation curve is shown in **figure 6**. Over the course of the 5 days of the mammalian myoblast proliferation assay, cell counts in wells supplemented with methionine and those in methionine deficient media showed growth rates that were not significantly different until day 3. Beginning at day 3, wells treated with methionine supplemented RPMI showed significantly higher rates of growth compared to wells treated with MdRPMI (P < .0225) as shown in **figure 7**. The difference in cell counts between the two treatment groups continually increased throughout the remainder of the assay.

Fusion index shows no significant difference across all wells

The percent of myoblasts fused into myotubes was calculated for wells of each treatment from which the average fusion index was determined for each plate by treatment (**Fig. 8A-F**). As shown by **figure 9A**, no significant difference in fusion was found across all treatments and controls. Additional comparisons of fusion indices of only treatment wells (**Fig. 9B**) again revealed no differences in the rate of fusion at any concentration with either DL-methionine or Alimet supplements.

Supplemental methionine sources yielded larger myotube diameter than methionine deficient control

Myotube diameter was measured from the day 4 control and treatment wells and the average diameter was determined from the four wells of each treatment and control columns. Grouping of mean myotube diameter by treatment (**Fig. 10**) showed that wells receiving the positive control containing LM and wells receiving treatment methionine supplements were significantly larger than the methionine deficient negative control myotubes (P<.0001). Wells receiving DL-methionine had the greatest myotube diameter and were significantly larger than wells receiving positive control LM and LA treatment. The HA treatment group also contained myotube diameters signi-

ficantly larger than positive control LM wells but not significantly larger than any other treatment.

Discussion

Previous studies have concluded that the efficacy of DLM and MHA as sources of L-methionine in birds is very similar [4-8]. However, these experiments focus on the *in vivo* ability of birds to metabolize these forms of methionine rather than *in vitro*. The efficacy of these sources of methionine to be converted and utilized by cells *in vitro* is still unknown. Alimet, being an MHA, contains a precursor to L-methionine called DL-hydroxy-4- (methylthio) butanoic acid (DL-HMB) which differs from LM in that it contains a hydroxy group on the alpha carbon instead of an amino group. The conversion of DL-HMB into LM is a previously studied process [12] which requires the enzymes L-hydroxy acid oxidase (L-HOAX), D-Hydroxy acid dehydrogenase (D-HADH), and D-amino acid oxidase (D-AAOX) to initiate the conversion of L-HMB isomer, D-HMB isomer, and D-methionine respectively. The enzymes L-HOAX and D-AAOX are both found in the peroxisomes of cells, and in birds, are found in high concentrations in the liver and kidneys and in low concentrations elsewhere in the organism [13]. L-HOAX functions to convert L-HMB into 2-keto-4-(methylthio) butanoic acid; an intermediate in the conversion to LM [14]. Interestingly, D-AAOX converts D-methionine into the same intermediate: 2-keto-4-(methylthio) butanoic acid [15] with both steps being the first conversion of L-HMB and D-methionine. D-HADH differs from L-HOAX and D-AAOX since it is a dehydrogenase rather than an oxidase and it is a mitochondrial enzyme that has been found in all tissues including skeletal muscle [16]. The known disparity between the cell types in which these enzymes are found may have an impact on the ability of an *in vitro* culture of myoblasts to process these various sources of methionine.

Data collected from the proliferation of myoblasts assay has shown that in both treatments containing varying concentrations of Alimet and DL-methionine, cell count continually decreased throughout the course of the experiment. Meanwhile, myoblasts cultured with methionine provided in the LM form in positive control wells saw cell count steadily increasing. A likely explanation for this is that methionine supplemented to treatment groups reached a concentration inhibitory to myoblast proliferation. A previous study using fibroblasts derived from mice found that the optimal concentration of methionine for cell proliferation was .05 μmoles/mL with proliferation decreasing with increased methionine concentration past this point [17]. Additionally, a study using CHO cells concluded that the maximum methionine concentration for animal cell culture media is 153 mg/L [18]. In this experiment, Alimet was supplemented at a concentration of 1.28 mg/mL (7.5 mM) and .64 mg/mL (3.8 mM) for high and low concentration treatments respectively. Additionally, DLM was supplemented at a concentration of 1.125 mg/mL (7.5 mM) and .56 mg/mL (3.8 mM) for high and low concentrations respectively.

It has been suggested that the transaminative pathway utilized by cells at high intracellular methionine concentrations produces methanethiol as a breakdown product [19]. Methanethiol is highly toxic to cells and may be partly responsible for the significant reduction in cell proliferation witnessed in DLM and Alimet treatment groups. While it is clear that methionine concentration was inhibitory for normal myoblast proliferation, rates of proliferation between DLM and Alimet treatment groups of surviving cells revealed a significantly higher rate of proliferation in DLM treated myoblasts than Alimet treated myoblasts. A possible explanation for this difference is that supplemented DLM was in a 99% racemic mixture with 50% of the mixture being LM. Comparing this to Alimet which contains 88% DL-HMB where myoblasts only can convert D-HMB which comprises only half of the available DL-HMB. Conversion efficiency of D-HMB into LM may be worth exploring which may have further contributed to the difference in proliferation.

Data collected from the methionine deficient negative control wells did yield un-expected results. It was found that myoblast proliferation in these wells was significantly higher than all treatment wells and was not significantly different from the LM sup-plemented positive control. A study previously conducted by Reidy et al. [20] using older humans supplemented with essential amino acids (EAA) or non-supplemented found that supplemented groups saw increased satellite cell proliferation in type I muscle fibers but no difference in type II fibers. Though this study partially suggests that supplementation with essential amino acids does not impact proliferative ability, the effects of specific essential amino acids were not explored and therefore methionine's impact could not be determined. There have been reported cases of normal rates of proliferation of cells in the absence of methionine, though the supplementation of homocysteine was required [21,22]. To determine whether this methionine-free proliferation was specific to avian myoblasts, a similar assay was completed using mammalian C2C12 cells. Data collected from this experiment revealed a significantly greater rate of proliferation in wells sup-plemented with methionine revealing a methionine dependence. As previously men-tioned, the in vitro ability for birds to utilize varying sources of methionine as well as the ability to utilize homocysteine as a methionine precursor highlights the phenotypic adaptability of myoblasts during proliferation, though the exact mechanism utilized in the absence of methionine is unknown. A number of mechanisms have been identified in more recent studies that show protein synthesis initiation using alternative start codons. In prokaryotes,

successful protein synthesis has been initiated after the replacement of the methionine anti-codon CAU with alternative anticodons allowing for initiation by valine, phenylalanine, isoleucine, tryptophan, and glutamine [23-26]. Further evidence shows mis-acetylated formyl methionine tRNAs were also able to initiate protein synthesis with non-canonical amino acids [27,28]. In eukaryotes, mutant initiator tRNAs for methionine and valine have also been identified where no detrimental downstream effects on protein synthesis were observed [29]. Though mutagenesis of RNAs in this experiment was conducted by the researchers, all of the studies listed showcase impressive plasticity of translation machinery that may be used by cells in methionine-stressed environments for survivability. Other reports have found the complete circumvention of traditional protein synthesis initiation. In a study by Malarkannan et al., MHC class I molecules were found to present cryptic peptides that had been synthesized both using leucine as the initiating amino acid and independent of upstream translation initiation machinery [30]. Whether avian myoblasts have the ability to utilize alternative start codons or bypass the initiation phase is unknown, but it does seem that they are able to proliferate in a methionine deficient environment.

The impact of methionine availability on myoblast differentiation is known for several species. Previous studies have shown that deprivation of methionine in mammalian myoblasts [31] maintained a state of satellite cell quiescence with MyoD being downregulated and Myf-5 expressed at a high level. When reintroducing methionine to quiescent satellite cells, the levels of MyoD and Myf-5 were inverted, and cells began to differentiate showing methionine's direct role in the process of differentiation. Additional studies looking at methionine's role in satellite cell differentiation in fish [32] concluded similar results that a lack of methionine resulted in lower levels of myogenin and myod1, which are known as the important markers of myogenesis. Introduction of methionine in fish satellite cells also showed the restoration of differentiation activity, again presenting methionine's direct role in the process. This direct relationship between methionine and induction of differentiation in avian systems however has not been as well documented. It has been observed that chicks supplemented in-ovo with methionine have increased body weights and muscle production of post--hatch [33-35]. These findings suggest that methionine plays a role during the early stages of myofiber development though none of the studies determined the impact to be on differentiation specifically. Differentiation data collected in the current experiment however did not concur with these studies. A fusion index (FI) of three or more nuclei contained within one membrane was used to determine rates of differentiation among the different treatment groups. The data collected showed no significant differences in the rates of fusion across all treatments and both LM supplemented positive control and methionine deficient negative control. Since differentiation was able to occur at rates equal to cells supplemented with methionine it is possible that methionine may not be necessary to trigger the onset of differentiation in avian myoblasts. Differentiation is known to begin when myoblasts exit the cell proliferation cycle and begin to fuse into multinucleated myotubes with the expression of myogenic regulatory factor (MRF) proteins. MRF proteins include Myf5 and MyoD which are expressed during active proliferation of myoblasts while myogenin and MRF4 are expressed during and post-differentiation respectively [36,37]. As mentioned previously, the introduction and removal of methionine have had a direct impact on the concentration of these MRFs and therefore have been seen to have a direct correlation with rates of differentiation. With the results of the methionine deficient control group in this experiment still showing high rates of differentiation, it is possible additional factors play a role in regulating differentiation.

The results from the myotube diameter data are interesting in that the methionine deficient negative control had a significantly smaller average size, clearly demonstrating that while methionine may not be essential for satellite cell proliferation, it has an essential role in protein synthesis. Hypertrophy of myotubes is a result of increased RNA synthesis and protein synthesis by existing nuclei or by adding additional nuclei. As this relates to methionine, a lack of sufficient methionine source would limit the rate of protein within growing myotubes as methionine is essential as the initiating amino acid of all protein synthesis. As evident by the data, the rates of myotube hypertrophy differed between the different methionine supplements and concentrations at which they were supplemented. Wells receiving treatments were generally larger than myotubes from the LM positive control wells with both LD and HA treatments having significantly larger diameters. It is possible that the reason for this is because treatment methionine supplementation was at a much higher concentration than the positive control. As methionine is often a limiting amino acid of protein synthesis; greater methionine supplementation would likely allow for greater protein accretion and therefore larger myotubes. It was also found that LD treatments had significantly larger myotube diameters than LA treatments. The reason for this may have to do with the bioavailability of DL-methionine compared to Alimet as previously mentioned, but the exact reasoning is unknown. What this data does show is that Alimet supplementation may serve as a functional replacement for traditional methionine sources during the terminal differentiation stages of myotube development *in vitro.* Further research into the functionality of Alimet as a methionine supplement to improve protein synthesis in myotube cultures is warranted.

Conclusions

In summary, data collected during this study presents evidence that DL-methionine (DLM) and liquid DL-methionine hydroxy analog-free acid (MHA) may serve as viable alternatives to L-methionine during differentiation of *in vitro* culturing of myoblasts. Furthermore, the results obtained from the myotube fusion assay suggest the involvement of an alternative factor other than methionine influencing rates of differentiation. Finally, the proliferation of avian myoblasts in the absence of methionine suggests the utilization of an alternative source of methionine or the ability to proliferate through the use of alternative translational start codons or complete circumvention of translation initiation events. These findings merit further investigation into the mechanism behind methionine-free proliferation of avian myoblasts and methionine's direct role in the proliferative function of satellite cell and myoblast proliferation and differentiation.

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Corresponding author

Ronald J. Savino, Prestage Department of Poultry Science, College of Agriculture and Life Sciences, North Carolina State University, Raleigh, NC 27695, USA, e-mail: rjsavino@ncsu.edu.

Conflicts of interest

The authors declare no conflict of interest.

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