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Oral docosahexaenoic acid supplementation alters alveolar macrophage metabolism and protein and lipid profiles in the lower airway of healthy horses

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Objective
To determine the impact of a commercially available docosahexaeonic acid (DHA) supplement (DHA, methylslfonylmethane, and mushroom blend) on equine alveolar macrophage metabolism and lipid and protein profiles in bronchoalveolar lavage (BAL) supernatant.

Methods
This was a prospective, sequential, placebo-controlled study using 10 healthy adult horses. Bronchoalveolar lavage fluid was collected at baseline, following 45 days of oral placebo administration, and following 45 days of oral commercially available DHA supplement, with a 14-day washout. Whole blood was collected following placebo and DHA supplementation. Bronchoalveolar lavage samples were collected for airway cytology. Adherent cells were isolated from BAL cell pellets and used to measure alveolar macrophage oxygen consumption rate and extracellular acidification rate. Protein and lipid profiles were measured in BAL supernatant.

Results
7 horses completed the study. The DHA supplementation significantly decreased the whole-blood ratio of omega-6 to omega-3 fatty acids and significantly increased the ratio of DHA to arachidonic acid. Following DHA supplementation, mean alveolar macrophage basal and maximal respiratory capacity increased, and protein and lipid profiles in BAL supernatant were altered. There was no significant change in BAL cytology during any study period.

Conclusions
Oral administration of a DHA supplement increased alveolar macrophage oxygen consumption rate and altered lipid and protein profiles in BAL supernatant. Changes in alveolar macrophage metabolism may indicate a greater population of M2 (anti-inflammatory) alveolar macrophages.

Clinical Relevance
These findings suggest that oral DHA supplementation may promote an anti-inflammatory profile in the lower airway, which could be beneficial for horses with subclinical airway inflammation and horses routinely exposed to airway-triggering environments.

Keywords: DHA, fatty acids, airway, equine asthma, alveolar macrophage

Omega-3 polyunsaturated fatty acids (PUFAs) play a key role in many physiological functions and have been proven beneficial for numerous inflammatory diseases, including arthritis, dermatitis, exertional rhabdomyolysis, and asthma.[1–6] Oral omega-3 PUFA supplementation (Aleira; Arenus), in conjunction with a low-dust diet, has been demonstrated to decrease clinical signs and inflammation in asthmatic horses.[7] Docosahexaeonic acid (DHA) is an omega-3 PUFA that is incorporated into cell membranes in a dose-dependent fashion.[8] Arachidonic acid (AA) is an omega-6 PUFA that serves to synthesize proinflammatory eicosanoids. These PUFAs act in a competitive manner. As DHA levels increase,

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proinflammatory eicosanoids decrease, thus resulting in an anti-inflammatory effect. Mechanistically, omega-3 PUFAs, such as DHA, alter the composition and fluidity of the plasma membrane, modify bioenergetics (cellular metabolism), and affect gene expression.[9,10] However, the effect(s) of omega-3 PUFA supplementation specifically on equine lower airway leukocyte metabolism has yet to be investigated.

Alveolar macrophages play a crucial role in the lower airway, maintaining homeostasis of the airways by recognizing foreign substances, including pathogens, particulates, and environmental antigens, and coordinating an immune response.[11,12] Macrophages have the unique ability to dynamically respond to local stimuli and polarize toward a proinflammatory (M1) state or a proresolution (M2) state depending on physiological needs. Polarization exists on a spectrum, and macrophages can exhibit characteristics of both phenotypes simultaneously in the airways.[13,14] The M1 macrophages are induced by proinflammatory cytokines and bacterial products (eg, lipopolysaccharide) and are essential for bacterial clearance; however, they can contribute to an ongoing cycle of inflammation. The M2 macrophages are responsible for resolving inflammatory events and promoting tissue repair. Macrophages in an M1 physiological state rely primarily on glycolysis for energy production, whereas macrophages in an M2 physiological state rely primarily on oxidative phosphorylation.[11,15,16] The M2 polarization of adipose tissue macrophages was enhanced in mice with administration of omega-3 PUFAs.[17]

Macrophage phenotype is linked directly to metabolic activity. Glycolysis is measured as the extracellular acidification rate (ECAR), which can be monitored by measurement of pH in the extracellular medium. The oxygen consumption rate (OCR) is derived from measurement of oxygen utilized by cells and is a direct indicator of aerobic metabolic activity and energy production. During homeostasis, alveolar macrophages typically maintain low levels of glycolysis due to the low glucose concentrations in their environment.[18] Measurement of metabolic activity (OCR and ECAR) provides insight into macrophage physiological state and functional phenotype.

Lipids have many roles in the lower airway, including protein modulation, structural composition of cell membranes, and acting as energy sources.[19] In humans, lipid profiles are altered in patients with asthma, cystic fibrosis, bacterial pneumonia, and lung injury caused by smoke inhalation; however, equine-specific lower airway lipidomic research is more limited.[20–23] Recent investigations of the equine bronchoalveolar lavage (BAL) fluid (BALF) surfactant lipid profile revealed significant compositional changes in asthmatic horses following hay exposure.[24,25] Additionally, supplementation of DHA and eicosapentaenoic acid (EPA) leads to incorporation of these fatty acids into lipids, specifically glycerophosphocholines (GPCs), in BAL surfactant.[3] Altered lipid composition with DHA supplementation may provide insight for understanding how omega-3 PUFAs modulate lower airway inflammation.

Proteomics allows for a broad investigation of protein expression and provides quantitative data, allowing for comparison between treatment groups. The proteome of equine BALF has been evaluated in both healthy horses and horses with severe equine asthma (sEA). Previous studies[26] identified 190 differentially expressed BALF proteins between healthy horses and horses with sEA. Understanding the differentially expressed proteins in BALF following administration of a DHA supplement provides further insight into mechanism(s) of the anti-inflammatory effect of DHA in horses with sEA.

For this study, we hypothesized that a DHA supplement would decrease the plasma omega-6-to-omega-3 ratio, alter alveolar macrophage metabolism, and alter protein and lipid profiles in BAL supernatant in healthy horses.

Methods

This was a prospective sequential crossover study investigating 45 days of DHA supplementation versus 45 days of placebo supplementation with a 14-day washout period in between. Whole blood was collected following placebo and DHA supplementation for fatty acid analysis. Bronchoalveolar lavage fluid was collected at baseline following placebo and DHA supplementation for BAL cytology, alveolar macrophage bioenergetics, and lipidomic and proteomic analyses of supernatant. All samples were collected from December 2022 through March 2023 to minimize seasonal effects.

Horses

All procedures were approved by the North Carolina State University IACUC (No. 22–456). Ten horses were included in the study; however, 3 were removed from the study because anti-inflammatory medication administration was indicated during the study period for unrelated conditions. Horses were university-owned research and teaching animals with no history of respiratory disease. Horses ranged in age from 8 to 24 years and were of mixed breeds and sexes. All horses weighed between 800 and 1,300 lb and received the same dose of DHA supplement (30 g as recommended by the manufacturer and used in previous studies). Horses were maintained on pastures in central North Carolina, fed supplemental hay (timothy and alfalfa) and ration balancer, and received no medications throughout the duration of the study. Each horse received a physical examination, 23-point clinical score, and BAL at baseline following the placebo administration period and DHA supplementation period.[26] Whole blood was collected via jugular venipuncture following placebo administration and DHA supplementation periods.

Horses were fed a placebo (provided by the manufacturer of the DHA supplement and used for previous studies) containing alfalfa meal, ground peppermint, peppermint extract, mixed tocopherols, and propionic acid for 45 days followed by a 14-day washout period. Horses were then fed DHA supplement (30 g daily) for 45 days. Thirty grams of the commercial DHA supplement contains

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1,500 mg docosahexaenoic acid, 5,000 mg methylsulfonylmethane, 2,000 mg mushroom blend, 1,000 mg ascorbic acid, alfalfa meal, ground peppermint, peppermint extract, mixed tocopherols, and propionic acid. Previous studies[7] have shown that plasma DHA peaks after 4 weeks of DHA supplementation, then reaches a plateau. Additionally, plasma DHA demonstrates a significant decline by 9 days after cessation of supplementation.[19] Our experimental timelines exceeded these minimums in order to account for the potential for individual animal variability.

Clinical scoring

Horses were assigned a 23-point weighted clinical score at baseline following placebo and DHA administration. The previously established 23-point clinical score evaluates respiratory rate, nasal discharge, nasal flaring, abdominal lift, tracheal sounds, bronchial tones, presence of crackles, wheezes, and cough.[27]

BALF collection and sample processing

Horses were sedated with detomidine (0.005 to 0.01 mg/kg, IV) and butorphanol (0.02 to 0.04 mg/kg, IV). Topical lidocaine gel was applied to the nares. A cuffed catheter was passed nasotracheally until wedged in a secondary or tertiary bronchus. Three hundred milliliters of sterile isotonic saline solution was infused and reaspirated in two 150-mL aliquots. All BALs achieved at least 50% recovery of infused fluid. The aspirated fluid was pooled and submitted to the North Carolina State University Clinical Pathology Laboratory for a 300-cell-count differential of BAL cells performed by board-certified veterinary clinical pathologists who were blinded to the experimental groups.

Bronchoalveolar lavage fluid was filtered through standard gauze squares to remove excess mucus. A total cell count was performed with trypan blue and a manual hemocytometer. The differential cytology was used to calculate the total alveolar macrophage number. The samples were centrifuged at 600 X *g* for 10 minutes. Bronchoalveolar lavage supernatant was harvested and stored at −80 °C until further analyses (proteomics and lipidomics). Bronchoalveolar lavage cell pellets were resuspended in cell culture media (RPMI + 10% heat-inactivated fetal bovine serum + 0.1 mg/mL streptomycin + 100 IU/mL penicillin) for alveolar macrophage metabolism assays.

Whole-blood fatty acid analysis

Whole blood was collected from the jugular vein in syringes following placebo and DHA supplementation. Blood was spotted directly onto whole-blood fatty analysis dried blood spot cards (Lipid Technologies), and the cards were frozen at −20 °C. A complete fatty acid profile analysis was performed by Lipid Technologies LLC. Fatty acids were extracted and analyzed using gas chromatography-mass spectrometry.

Alveolar macrophage seahorse assay (Mito Stress Test)

Alveolar macrophages were isolated using adhesion as previously described by our lab.[28] This isolation method routinely yields 96% to 98% macrophages based on Wright-Giemsa staining. Based on the calculated total alveolar macrophage count, mixed BAL cells were resuspended in RPMI + 10% heat-inactivated fetal bovine serum + 0.1 mg/mL streptomycin + 100 IU/mL penicillin. Cells were seeded into wells at equivalent densities in order to yield 60,000 alveolar macrophages/well in an XF cell culture miniplate (Agilent Seahorse) and cultured overnight in a 37 °C incubator. The sensor cartridge was prepared according to manufacturer instructions. One hour prior to the assay, a monolayer was confirmed microscopically, and the wells were washed with assay media (9.7 mL pH 7.4 Dulbecco's modified Eagle medium, 100 µL 1.0M glucose, 100 µL 200mM glutamine, and 100 µL 100mM pyruvate), removing any nonadherent cells. The Mito Stress Test inhibitor stock solutions (Agilent) were diluted according to manufacturer's instructions and pipetted into the ports of the sensor cartridge. A Seahorse XF HS Mini Analyzer was used to measure OCR and ECAR every 3 minutes for approximately 5 hours. Basal OCR represents the initial steady-state respiration of the cells prior to injection of the stressor compounds and is measured over 3 baseline cycles. Maximum respiratory capacity is the maximum OCR cells can achieve and represents the cell's potential for energy production due to increased demand. Specifically, cells are exposed to an uncoupling agent that allows for uninhibited electron flow through the electron transport chain.

Proteomics

Bronchoalveolar lavage supernatant samples were concentrated using a 3-kD Nanosep centrifugal filter (Pall; OD003C33) at 14,000 X *g* for 50 minutes at 25 °C. Total protein was quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific) per the manufacturer's instructions. Proteins were conjugated into 100-µg magnetic beads (10 µg protein captured; Sera-Mag Carboxylatre-Modified Magnetic Particles; Cytiva), digested on beads with 0.8 µg of a trypsin and Lys-C protease mixture (Promega; V5073), and resuspended in 80 µL of 0.1M ammonium bicarbonate buffer. Peptides (20 µL) were analyzed via nano-LC-MS-MS (Orbitrap Eclipse; Thermo Fisher Scientific) using data-independent acquisition. For nano-LC-MS-MS–based proteomic analysis, all measurements were conducted at the Molecular Education, Technology, and Research Innovation Center at North Carolina State University. Additional experiment details are provided in **Supplementary Material S1**.

Lipidomics

Bronchoalveolar lavage supernatant (300 µL) from each horse/condition was processed using a liquid-liquid lipid extraction protocol. After adding 300 µL of methanol, samples were incubated overnight at −20 °C prior to the addition of 1 mL of methyl tert-butyl ether. Samples were vortexed thoroughly on a Thermomixer (catalog No. SI-0236; Eppendorf) at 1.68 X *g* for 1 hour at room temperature for phase separation. After centrifugation at

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18,000 X *g* for 5 minutes at 4 °C, the upper phase was collected and concentrated to 100 µL under vacuum. Extracted lipids were then separated on a Vanquish Horizon UHPLC system equipped with ACQUITY CSH C18 column (1.7 µm, 2.1 X 100 mm; Waters) and analyzed via an Orbitrap Exploris 480 mass spectrometry (Thermo Fisher Scientific) coupled with a heated electrospray ionization source. Samples were analyzed in positive mode and negative mode as different lipids can be detected in each of the modes. Additional experiment details are provided in Supplementary Material S1.

All raw files acquired from Orbitrap Exploris 480 mass spectrometry were analyzed using MS-DIAL (version 4.9, Systemsomics Labs), a high-throughput lipid-identifying software. Chromatographic peaks and mass spectrum fragments that represented substances in BAL fluid were processed through detection, deconvolution, identification, and alignment using this software. Features were identified through matching the accurate mass and MS-MS spectra. Lipid annotations were performed following the lipidomic standard initiative guidelines (https://lipidomics-standards-initiative.org/). The exported data were processed using an open-source web-mass spectral feature list optimizer (MS-FLO; https://msflo.fiehnlab.ucdavis.edu/), a critical step in minimizing false positive peak reports widely applied in untargeted LC-MS data processing. This process involves removing isotopes, duplicates, and contaminant ions, such as reference mass and background ions from solvents, joining the peak height of multiple ion adducts generated from the same lipid.

Statistical analysis

Data were assessed for normality using Shapiro-Wilk testing. Parametric data were evaluated using 1-way ANOVA or Student *t* tests. Nonparametric data were evaluated using Wilcoxon matched-pairs signed-rank tests or Kruskal-Wallis tests. $P < .05$ was considered significant.

For alveolar macrophage metabolic data, percentage of change was calculated from baseline to placebo and baseline to DHA supplementation to account for individual variability. Percentage of change was used for both OCR analysis and ECAR analysis.

For proteomics data, percentage of raw nano-LC-MS-MS data were processed using Proteome Discoverer, version 3.1 (Thermo Fisher Scientific). Searching was performed using the Chimerys (version 2.0) search engine with a 20-ppm fragment mass tolerance with the *Equus caballus* (Horse) proteome database (downloaded from Uniprot database, 2024; 21,429 entries) and 298 commonly used contaminant database embedded in Proteome Discoverer, version 3.1. The database search settings were specific for trypsin digestion. Specified modifications included dynamic methionine oxidation and static cysteine carbamidomethylation, and the maximum dynamic modification per peptide was set as 3. Identifications were filtered to a strict protein false discovery rate of 1% and relaxed false discovery rate of 5% using the Chimerys engine.

For lipidomic data, following lipid annotation, additional filtering and normalization steps were performed to ensure high-confidence feature inclusion and robust statistical interpretation. Lipids lacking MS-MS fragmentation data, assigned a low confidence score, or identified as "unknown" by MS-DIAL were excluded from the dataset. Compounds with retention times under 1 minute or those detected in fewer than 60% of the samples were also removed to minimize noise. For normalization, zero intensities were imputed with the minimum detected intensity divided by $\sqrt{2}$ and subsequently $\log_2$ transformed to stabilize variance across features. Outlier detection was carried out using principal component analysis and hierarchical clustering to identify samples with atypical distributions; 1 baseline sample was removed as a result. To identify differentially abundant lipids across conditions, a mixed-effects model was used, where lipid intensity was modeled as a function of treatment group (baseline, placebo, treatment) while accounting for repeated measurements from the same horse. *P* values were adjusted using the Benjamini-Hochberg procedure, and lipids were considered significant if the adjusted *P* value was < .05 and the absolute $\log_2$ fold change compared to baseline was > 1. A χ^2 test was used to determine whether any lipid classes were over- or underrepresented in the differential abundance analysis in comparison with the overall lipidomic composition of the samples. The null hypothesis was that the proportion of lipids in each class was the same in the list of all lipids versus in the list of differentially abundant lipids from each comparison (DHA vs placebo, DHA vs baseline).

Results

All 7 horses included in the analyses had normal physical examinations and normal respiratory clinical scores (< 5) at all 3 time points (baseline, following placebo administration, and following DHA supplementation; **Supplementary Table S1**).

Bronchoalveolar lavage cytology

Three of 7 horses had elevated mast cells (> 2%) in BAL cytology at baseline. One of these horses' mast cell percentage normalized during placebo administration. Two of these horses' mast cell percentage normalized after DHA. Another horse showed an increase in BAL mast cell percentage during placebo and normalized following DHA. All horses had normal BAL cytology mast cell percentage following DHA supplementation. However, changes in median BAL mast cell percentage after DHA supplementation were not significant ($P = .07$; **Supplementary Table S2**).

Whole-blood fatty acid

The mean omega-6-to-omega-3 fatty acid ratio was significantly lower in whole blood collected after DHA supplementation compared to placebo administration. This was due to increased plasma levels of omega 3 ($P < .05$; **Figure 1**). The mean DHA:AA ratio was significantly higher in whole blood collected

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after DHA supplementation compared to placebo administration. This was due to increased plasma levels of DHA ($P < .01$).

Alveolar macrophage OCR

The alveolar macrophage mean basal OCR was significantly higher following DHA supplementation compared to placebo administration ($P < .05$; **Figure 2**). The alveolar macrophage mean maximal respiratory capacity was significantly increased following DHA supplementation compared to placebo administration ($P < .05$). The alveolar macrophage mean spare respiratory capacity was not significantly different following DHA supplementation compared to placebo but was trending higher following DHA supplementation ($P = .06$).

Alveolar macrophage ECAR

The mean basal ECAR of alveolar macrophages was not significantly different following placebo or DHA administration. The alveolar macrophage glycolytic capacity, or mean maximal acidification, was significantly lower following DHA supplementation compared to placebo ($P < .05$; **Figure 3**).

Proteomics

PD3.1 identified 3,009 equine proteins with high confidence. Differential expression analysis was conducted across 3 comparisons: placebo versus baseline, DHA supplementation versus baseline, and DHA supplementation versus placebo. Three hundred and thirty-nine of those identified proteins had significantly higher abundance in bronchoalveolar lavage supernatant following DHA supplementation compared to placebo supplementation. One hundred and fifty proteins had significantly lower abundance in bronchoalveolar lavage supernatant following DHA supplementation compared to placebo supplementation.

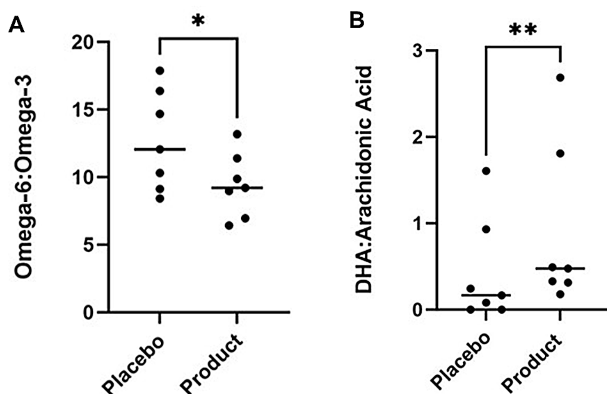

Figure 1—Whole-blood changes in polyunsaturated fatty acid composition following docosahexaenoic acid (DHA) supplementation (product) compared to placebo. A—The mean omega-6-to-omega-3 ratio is significantly lower following DHA supplementation compared to placebo administration ($P < .05$). B—The mean DHA-to-arachidonic acid ratio is significantly higher following DHA supplementation compared to placebo administration ($P < .01$). Whole blood was spotted onto whole-blood fatty analysis dried blood spot cards (Lipid Technologies). A complete fatty acid profile analysis was performed by Lipid Technologies LLC. N = 7; paired *t* test.

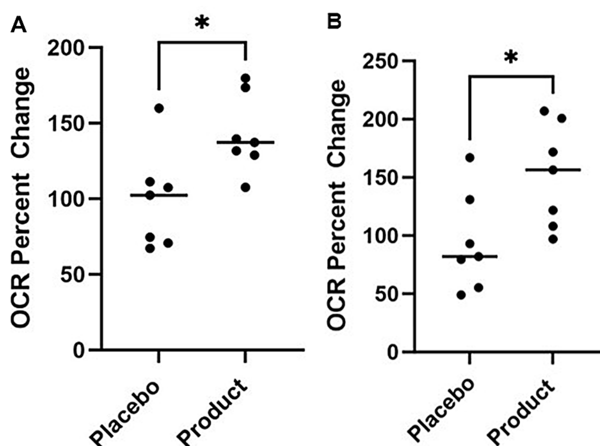

Figure 2—Alveolar macrophage aerobic metabolism as measured by basal oxygen consumption rate (OCR; A) and maximal respiratory capacity OCR (B). A—The mean alveolar macrophage basal OCR percentage of change is significantly increased following DHA supplementation (baseline vs DHA) compared to placebo administration (baseline vs placebo; $P < .05$). B—The mean alveolar macrophage maximal respiratory capacity percentage of change is significantly increased following DHA supplementation (baseline vs DHA) compared to placebo administration (baseline vs placebo; $P < .05$). Alveolar macrophages were isolated via adherence and analyzed with the Agilent Seahorse XF and Mito Stress Test kit. N = 7; paired *t* test.

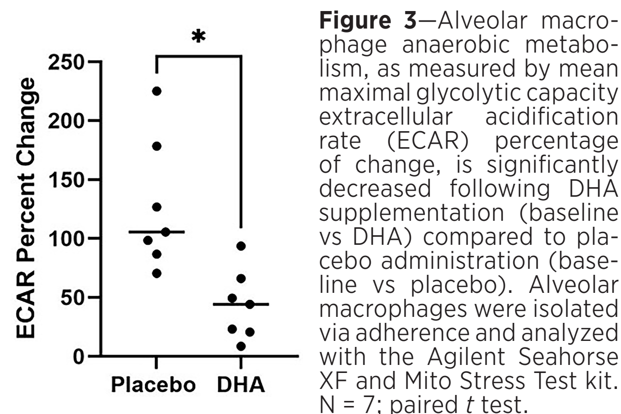

Figure 3—Alveolar macrophage anaerobic metabolism, as measured by mean maximal glycolytic capacity extracellular acidification rate (ECAR) percentage of change, is significantly decreased following DHA supplementation (baseline vs DHA) compared to placebo administration (baseline vs placebo). Alveolar macrophages were isolated via adherence and analyzed with the Agilent Seahorse XF and Mito Stress Test kit. N = 7; paired *t* test.

Of the 339 proteins with significantly higher abundance following DHA supplementation, 314 were successfully mapped to equine genes and associated Gene Ontology terms. The most common biological process was the metabolic process (173 genes), with many subdescriptions, including but not limited to cellular metabolic process, mRNA metabolic process, regulation of cellular catabolic process, and macromolecule metabolic process.

Of the 145 proteins with significantly lower abundance following DHA supplementation, 137 were successfully mapped to equine genes associated with Gene Ontology terms. The most common biological process was localization (55 genes), with

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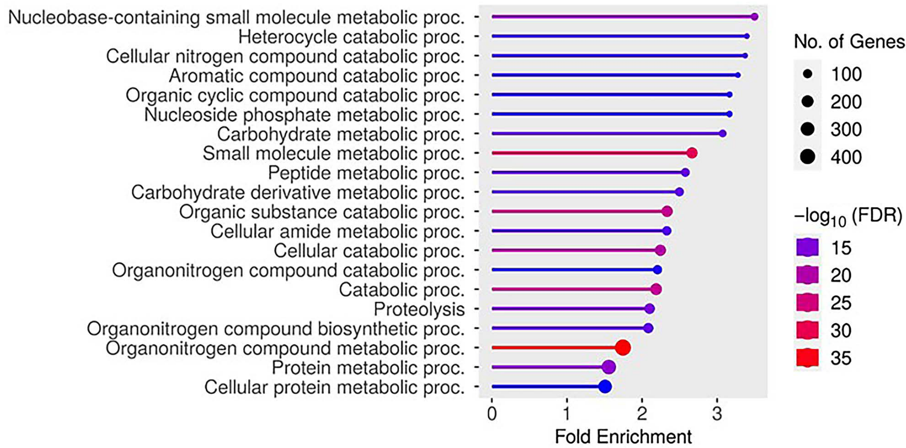

Figure 4—Biological processes (proc) associated with differentially expressed BAL proteins that had higher abundance following Aleira supplementation compared to placebo administration. Figure generated with ShinyGo.

Figure 5—Lipid differential abundance analysis. A—Volcano plot of differentially expressed lipids following DHA supplementation compared to placebo. Lipids were considered differentially expressed with a $\log_2$ fold change greater than 1 or less than −1 and an adjusted *P* value < .05. Individual points are colored according to lipid class for significantly differentially abundant lipids, with gray dots showing nonsignificant lipids. B—Stacked bar chart showing the distribution of lipid classes that were detected (left; entire lipidomic signature) and the distribution of lipid classes that were significantly differentially expressed in DHA supplementation compared to placebo (right). Adj = Adjusted.

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subdescriptions including but not limited to cellular localization, protein localization, and macromolecule localization **(Figure 4)**.

Lipidomics

Following filters, a total of 1,210 unique lipids were detected. Differential expression analysis was conducted across 3 comparisons: placebo versus baseline, DHA supplementation versus baseline, and DHA supplementation versus placebo. No significant lipid differences were identified in the placebo-versus-baseline comparison. In the DHA-versus-baseline comparison, 10 lipids were found to be significantly altered, with 6 increased and 4 decreased. The most pronounced differences were observed in the comparison between lipid abundance following DHA versus placebo, which showed that 177 lipids were significantly different, including 13 lipids that were increased and 164 lipids that were decreased after DHA supplementation relative to placebo administration **(Figure 5)**. To evaluate lipid class-level enrichment, lipids were grouped by structural class. Glycerophosphocholines were significantly enriched in both DHA-versus-baseline and DHA-versus-placebo comparisons. Lipid species were also categorized by saturation level to examine differences in fatty acid chain composition. The PUFAs were significantly enriched in the DHA-versus-placebo group.

Discussion

This study used clinically healthy university-owned horses to evaluate the impact of a commercially available DHA-enriched respiratory supplement (Aleira) on lower airway parameters, including alveolar macrophage metabolism, and BAL supernatant protein and lipid profiles. As expected, all horses had normal physical examinations and normal respiratory clinical scores at baseline. Three horses had increased mast cell percentages (> 2%) at baseline. All of these horses had normal mast cell percentages following DHA supplementation. Median mast cell percentage did not change significantly for any study time point. Median BAL neutrophil percentage was normal throughout the study time points. Although the study was completed within 1 season (December 2022 through March 2023), we cannot eliminate the possibility of environmental or seasonal impacts on variability in BAL cytology.[29]

Consistent with our hypothesis, oral administration of a commercially available algae-sourced DHA respiratory supplement for 45 days resulted in decreased plasma omega-6-to-omega-3 ratios (due to increased omega-3 plasma levels) and increased plasma DHA:AA ratios (due to increased plasma DHA levels). Polyunsaturated fatty acids are incorporated into cell membranes in a competitive manner, so increases in plasma DHA concentrations are expected to increase cell membrane DHA content and decrease cell membrane AA content, thus decreasing the available AA for synthesis of proinflammatory eicosanoids.[3,30]

To our knowledge, this is the first report to use the Seahorse XF analyzer to measure equine alveolar macrophage metabolism. Individual horse variation of alveolar macrophage metabolism was accounted for by analyzing the percentage of change in OCR and ECAR after placebo or Aleira supplementation to each animal's baseline OCR and ECAR. Oxygen consumption rate is a measure of the amount of oxygen that a cell is consuming to produce energy, which provides insight into the aerobic metabolic activity of the cell. Extracellular acidification rate is an indicator of glycolysis; however, it is known that alveolar macrophages do not utilize glycolysis as freely as blood monocyte-derived macrophages due to the glucose-poor environment in the airways.[18] It has been suggested that alveolar macrophages rely on oxidized fatty acids as a substrate for oxidative phosphorylation.[11] The basal alveolar macrophage OCR was increased following DHA supplementation compared to placebo administration, suggesting increased aerobic metabolism. Changes in basal OCR may also reflect changes in mitochondrial health and/or density and cellular energy demand.[31] The M2 macrophages tend to exhibit higher basal OCR due to increased mitochondrial oxidative phosphorylation and lipolysis, suggesting the use of fatty acids as a fuel source that drives mitochondrial activity.[32,33] Increased mitochondrial metabolism is required for the anti-inflammatory responses of M2 macrophages.[16] The maximal respiratory capacity is indicative of the cells' ability to produce ATP under high demand and is representative of the cells' overall metabolic health. Alveolar macrophages with high maximal respiratory capacity can respond robustly to infection or environmental stressors, whereas alveolar macrophages from patients with disease, such as chronic obstructive pulmonary disease, have lowered maximal respiratory capacity and show a decreased ability to respond to challenge.[34] Our results show that maximal respiratory capacity was increased following DHA supplementation compared to placebo administration, suggesting that alveolar macrophages may have a higher capacity to respond to higher demand, such as infection or oxidative stress, with DHA supplementation.

Proteomic analysis successfully identified numerous proteins that were more abundant following DHA supplementation compared to placebo. The most common biological process associated with these proteins was metabolism, consistent with the alveolar macrophage metabolism findings. The most common biological processes associated with proteins that were less abundant following DHA supplementation were cellular localization and protein localization. This could be relevant to important airway processes, such as leukocyte migration, trafficking of adhesion molecules, nuclear localization of nuclear factor κ B, and mucus hypersection. These increases and decreases in protein abundance provide insight into the molecular mechanisms by which DHA exerts its effects on the lower airway.

Polyunsaturated fatty acids, such as DHA, become incorporated into GPCs within the cell membrane to create a reservoir for these beneficial fatty acids.[3] Glycerophosphocholines serve as

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building blocks for cell membranes and contribute to the mechanical stability and elasticity of the alveoli.[29] Phosphatidylcholine is a GPC that makes up approximately 80% of lipid content in lung surfactant.[3,29] Glycerophosphocholines have a protective effect by inhibiting reactive oxygen species accumulation, which may mitigate oxidative damage in the lungs.[17] Our findings are consistent with Christmann et al,[3] in which oral supplementation of DHA and EPA led to augmented levels of DHA- and EPA-containing GPCs in healthy equine plasma, synovial fluid, and surfactant. In that study, surfactant levels of GPCs containing DHA were significantly elevated following 30 days of oral supplementation. Our findings further suggest that orally administered DHA reaches the lower airways, where it is likely incorporated into cell membranes, thus changing membrane fluidity and flexibility and modulating cellular processes to potentially promote an anti-inflammatory environment.

Our study had several limitations. Only clinically healthy horses were used for this study to limit the number of variables affecting alveolar macrophage metabolism and the lower airway lipid and protein profile. There is evidence that disease state, including asthma, affects these parameters.[19] Although the study was performed within 1 season to minimize seasonal variability, seasonality and temperature can affect BAL cytology in individual horses,[35] and we did see changes in the percentage of mast cells on BAL cytology in horses in our study. Whole-blood fatty acid analysis was not performed at baseline; however, the placebo did not contain a source of DHA. Finally, alveolar macrophages are dynamic and can shift their phenotype based on the physiological demand.[13] For each condition (baseline, placebo, Aleira supplement), this study analyzed samples collected at a single time point within each condition, with 1 method of analysis for each parameter, and did not investigate other methods to analyze alveolar macrophage phenotype, such as flow cytometry. In future studies, we will combine analysis of macrophage metabolism with marker-based phenotyping.

In conclusion, this study demonstrates that 45 days of oral DHA supplementation (Aleira) increased alveolar macrophage aerobic metabolism as demonstrated by increased basal OCR and maximal respiratory capacity. This change could be consistent with a shift toward an M2 anti-inflammatory phenotype and should be confirmed in future studies. Docosahexaeonic acid supplementation was also associated with shifts in abundance of BAL supernatant proteins and lipids involved in metabolic and anti-inflammatory pathways. The contribution of these shifts to lower airway immune mechanisms, including alveolar macrophage phenotype, warrants further study. Given that the risk factors and triggers for the onset of equine asthma are incompletely understood, management practices and/or supplements that promote maintenance of lower airway health and decreased inflammatory response could benefit both healthy and asthmatic horses exposed to airway triggers in their environment.

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Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the composition of this manuscript.

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