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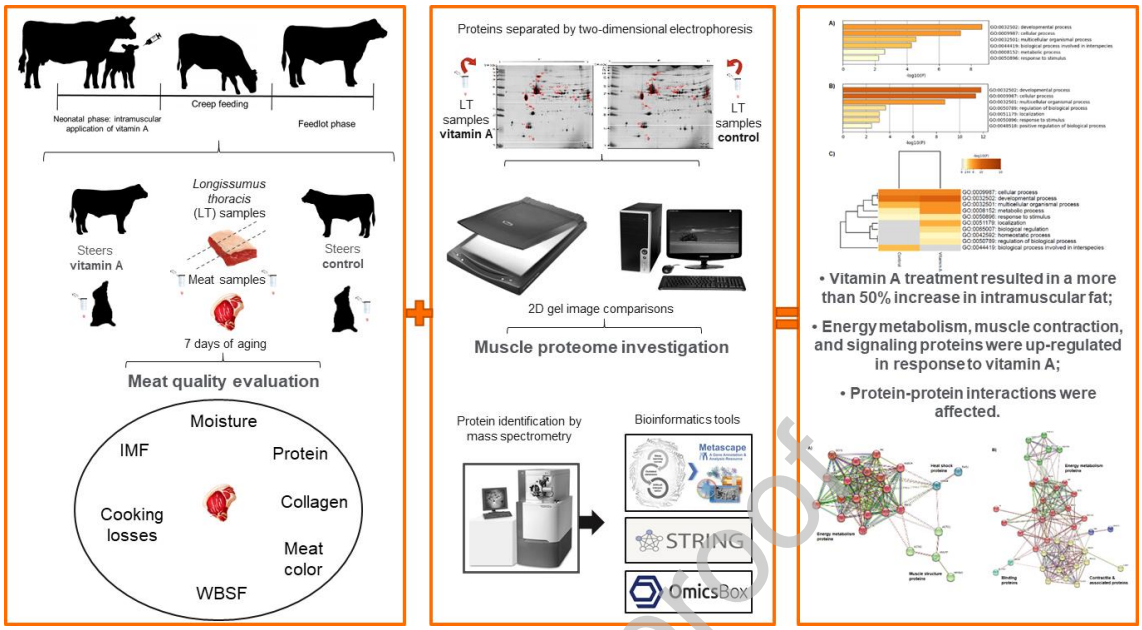
Graphical Abstract



Highlights

- Muscle proteome and meat quality of calves that received vitamin A at birth were described.
- Vitamin A treatment resulted in a more than 50% increase in intramuscular fat (IMF).
- Post-mortem muscle proteome and the protein-protein interactions were affected.
- Key biological processes related to energy metabolism, muscle contraction, and signaling were up-regulated in response to vitamin A.
- There was slight improvement in the tenderness of animals that received vitamin A at birth.

Abstract

This study aimed to evaluate the skeletal muscle proteome of calves that received an intramuscular application of vitamin A at birth and its impacts on meat quality. Forty male crossbred calves were used from birth to feedlot finishing. On the day of birth, 20 calves were injected intramuscularly with a single dose of 300,000 IU of vitamin A and the other 20 animals received placebo (control). After weaning at 210 days, the animals of the two treatments were feedlot finished for 180 days. *Longissimus* muscle samples were collected for proteome analysis and meat quality assessment. There was a trend ($P = 0.07$) towards more tender meat in animals that received vitamin A compared to control (WBSF = 3.92 ± 0.16 kg vs. 4.23 ± 0.15). Meat color, pH and cooking loss were not affected ($P > 0.05$). Vitamin A treatment resulted in a more than 50% increase in intramuscular fat (IMF) content ($P < 0.05$) compared to control (4.10 ± 0.35% vs. 2.57 ± 0.27%). The intramuscular injection of vitamin A affected the post-mortem muscle proteome and the protein-protein interactions. A greater abundance of proteins involved in three main pathways were observed these being energy metabolism (GAPDH, ENO3, TPI1, CKM, and COX5A), muscle contraction (ACTB, ACTC1, ACTG1, ACTG2, ACTA1, ACTA2, ACTN1, ACTN2, ACTN3, TPM1, TPM2, TPM3, MYH1, PDLIM3, and TNNT3), protein binding, transport, and signaling (TUBA4A, VIM, TBA1B, and EEF1A2). A greater abundance of oxidative stress and cellular defense proteins (HSPA8 and DNAJC18) was specifically observed in the control treatment. Vitamin A upregulated key biological processes related to energy metabolism, which favored IMF accumulation during the finishing phase.

Keywords: Adipogenesis, *Bos indicus*, feedlot, lipogenesis, retinoic acid; Muscle proteome; Biochemical processes

Introduction

The low intramuscular fat (IMF) content of beef is associated with the loss of sensory characteristics such as juiciness and tenderness, which affects consumer perception and approval (Hocquette et al., 2010). Within this context, the use of industrial crossing between two breeds with complementary characteristics (*Bos taurus* × *Bos indicus*), different nutritional strategies during the cow-calf phase, and feedlot finishing are some of the strategies allowing the improvement of animal performances, mainly by achieving better carcass and meat quality traits, particularly tenderness and marbling (Baldassini et al., 2023; Bonilha et al., 2008; Costa et al., 2015; Giusti et al., 2013).

In animals, adipose tissue hyperplasia, *i.e.*, an increase in the number of intramuscular cells, is more evident from the end of gestation to 250 days of age (Du et al., 2017). Within this context, vitamin A appears to be a key regulator of gene and protein expression, influencing adipogenesis and lipid metabolism in animal tissues. Vitamin A can cause changes in the recruitment of undifferentiated cells during the early stages of a calf's life through its metabolite, the retinoic acid. Consequently, more of these cells are directed towards the adipogenic lineage, especially intramuscular adipocytes (Pyatt and Berger, 2005). Injection of vitamin A after birth (adipogenic window for the formation of adipocytes) may thus increase IMF in cattle (Wang et al., 2016).

When applied during the postnatal period, this strategy seems to be more related

to an increase in the number of adipocytes (hyperplastic effect) than to their size (Kruk et al., 2018). Although vitamin A can boost the multiplication of adipocytes during this period, a fact that renders it an alternative for increasing the IMF content of meat, studies using crossbred beef cattle are still relatively few in number. Researchers evaluated the neonatal administration of vitamin A (control, 150,000 or 300,000 IU) to Angus (Harris et al., 2018) or crossbred calves (Maciel et al., 2022) with application of two doses of this vitamin, the first at birth and the second at 30 days of life.

Exploring muscle tissue proteome of beef cattle allow to better understanding rearing or nutritional strategies used in production systems, which is great importance for the meat industry in order to add value to the meat products. In literature, there are no studies that investigated the regulation of bovine muscle tissue proteins induced by vitamin A after birth. Within this context, the present study aimed to evaluate the muscle proteome of crossbred cattle that received an intramuscular application of vitamin A at birth and its impacts on carcass and meat quality traits.

Material and Methods

Animal production

All animal procedures followed the ethical guidelines established by the Animal Use Ethics Committee of São Paulo State University – UNESP (protocol number 0107, approved on June 21, 2018).

Forty male crossbred (F1 Montana × Nellore) calves were used from birth to finishing. On the day of birth, 20 calves were injected intramuscularly with a single dose of 300,000 IU of vitamin A (Monovin A, Bravet, Brazil), while the other 20 animals received a placebo. During the cow-calf phase, the animals of the two treatments were kept with their respective mothers in the same paddock. By

approximately 40 days of age, the animals were submitted to creep feeding until weaning, with concentrate supplementation (1% of their body weight [BW]). The creep feed supplement was composed of corn, soybean meal, salt, molasses, and a mineral premix (50, 25, 9, 1, and 5%, respectively).

All animals were immunocastrated by applying three doses of anti-gonadotropin-releasing factor (GnRF) vaccine (Bopriva®, Zoetis, Brazil). The first dose was applied at weaning and the second and third doses 30 and 90 days later, respectively. After weaning at 210 days of age, the animals were transferred to the feedlot and housed in individual covered pens with concrete floors that were equipped with individual feeders and drinkers. The two groups of animals were fed a diet formulated using RLM software v. 3.3.57 (Andrade et al., 2023; Lanna et al., 2011) – Table S1, The finishing diet consisted of 12.7% roughage (sugarcane bagasse) and 87.3% concentrates feeds (ground corn, soybean meal, cottonseed, potassium chloride and mineral-vitamin supplement). Mineral-vitamin mix had 1231.10 IU/kg of vitamin A. The diet was supplied *ad libitum*, twice a day at 8 am and 4 pm. For the determination of average daily weight gain (ADG), the animals were weighed at the beginning and end of the experimental period after fasting for 16 hours.

Slaughter, carcass and meat quality traits

In the current study, animals were produced according to the "super early-maturing" system, as described by (Ferraz and Felício, 2010). Thus, after 180 days in the feedlot and when weighing approximately 400 kg, all animals were slaughtered in a commercial slaughterhouse inspected and registered in SISP (Boituva, São Paulo, Brazil) by stunning and sectioning of the jugular vein, followed by hide removal and evisceration. The carcasses were identified, washed, and split into halves. Each half

carcass was weighed individually and cooled in a cold room at 1 ºC for approximately 24 h. Carcass measurements included hot carcass weight and carcass yield. Before deboning, backfat thickness and ribeye area were evaluated as described by USDA-AMS Standards for Grades of Carcass Beef (USDA, 1997).

Sample collection

In the slaughter line (hot carcass), individual *Longissimus thoracis* (LT) muscle samples from all animals (approximately 10 g) were collected from the 12th rib of the left half carcass and frozen in liquid nitrogen. These aliquots were then stored in an ultrafreezer (-80 ºC) until the time of proteome extraction and analysis. After cooling in the cold room (48 h), LT muscle samples were also collected between the 12th and 13th ribs of the left half carcass of all animals for meat quality evaluation. Briefly, the meat samples were cut into 2.54-cm-thick steaks, vacuum packed (plastic bags with low oxygen permeability) and kept at 2 ºC in a biological oxygen demand incubator (model TE-371, TECNAL, Brazil) for seven days. After this period, the steaks were frozen for analysis of meat quality.

Chemical composition of meat

To evaluate the proximate composition of meat, the samples were thawed in a refrigerator at 4 ºC for 24 h and the subcutaneous fat was removed with a scalpel. The steak was then ground in a multiprocessor for 5 minutes using approximately 180 g of sample (Anderson, 2007). The analyses were carried out by infrared spectroscopy in a FoodScan™ equipment (FOSS, Hillerød, Denmark), in which the average levels of moisture, protein, fat, and total collagen were determined. The average moisture, protein, fat, and ash contents were obtained through three readings per sample. At each

reading, the sample was removed from the plate, homogenized again, and returned to the plate for the next reading.

Cooking loss and shear force

The procedure proposed by Wheeler et al. (1994) was adopted to assess cooking loss and Warner-Bratzler shear force (WBSF), following the recommendations (AMSA, 2015). The samples were placed on a grid coupled to a glass refractory. A thermocouple connected to a digital thermometer (DT-612, ATP Instrumentation, Ashby-de-la-Zouch, England) was inserted in the center of each sample to monitor the internal end-point cooking temperature.

The samples were cooked in an industrial electric oven (Feri90 Venâncio, Venâncio Aires, Rio Grande do Sul, Brazil) preheated to 170 ºC and equipped with a thermostat to minimize temperature variations. Once the internal temperature of the steaks reached 40 ºC, they were turned over and remained in the oven until the final temperature reached 71 ºC. The samples were then kept at room temperature for 15 min, weighed, and refrigerated at 4 ºC for 24 h. Cooking loss was obtained by weighing the glass refractory before and after cooking the sample.

For the determination of WBSF, eight cores with a diameter of 1.27 cm were sectioned in a Brookfield CT-3 Texture Analyzer (AMETEK Brookfield, Middleborough, Massachusetts, United States). The device is equipped with a stainless-steel 3.07-mm-thick Warner-Bratzler blade with a vee-shaped (60° angle) cutting edge, with a load cell of 25 kilograms (kg) at a velocity of 20 cm/min. Results were reported as the average of six values per sample (in kg) by removing the two low and high extremes.

Instrumental meat color and pH

Meat color (L* - lightness/pale to dark; a* - redness/red to green; b* - yellowness/yellow to blue) was measured with the CIELAB system using a CR-400 spectrophotometer (Konica Minolta Sensing, Inc., Tokyo, Japan), equipped with a D65 standard illuminant (observation angle of 10º, opening of 5.0 cm, and Y display: 0.01 to 160% reflectance). The unit was calibrated using a plate for the black and white standard and color readings were taken at three locations of the LT muscle sample after a 30 min bloom time, as reported (AMSA, 2015; Gagaoua et al., 2018). An average of the three measurements was generated for L*, a* and b*. The chroma colorimetric index was calculated using the formula $\sqrt{(a*)^2 + (b*)^2}$, and hue angle was calculated using $tan(\frac{b*}{a*})^{-1}$.

The pH was determined with a Hanna digital pH meter (Model HI 99163, Hanna Instruments, Woonsocket, Rhode Island, United States) equipped with a penetration electrode. Standard buffers (pH 4.0 and 7.0) were used in calibration procedures.

Muscle proteome

For proteome analysis, frozen LT muscle samples were used. Six animals were chosen randomly from each experimental group (control *vs* vitamin A), totaling 12 samples in these analyses.

Protein extraction

Proteins were extracted following previously described procedures (Poleti et al., 2018). Briefly, around 0.5 g of LT muscle were homogenized in 5.0 mL lysis buffer (8 M urea, 2 M thiourea, 1% dithiothreitol, 2% CHAPS and 1% protease inhibitor Cocktails - Sigma-Aldrich) in Ultra-Turrax high shear mixer (Marconi – MA102/E,

Piracicaba, São Paulo, Brazil). Samples were vigorously shaken for 30 min on ice and centrifuged at 10,000 ×g for 30 min at 4 °C.

Total protein concentration was determined in a spectrophotometer Multiskan GO (Thermo Scientific, Massachusetts, United States) using PlusOne 2-D Quant Kit (GE Healthcare Product, Code 80-6483-56) according to manufacturer recommendations.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and image analysis

For 2D-PAGE, approximately 375 μg of protein was loaded onto 13-cm isoelectric linear strips containing pre-fabricated gel and ampholytes (pH gradient of 3 to 10). After separation of the proteins in the first dimension, the strips were transferred to a polyacrylamide gel (12.5%) for separation in the second dimension as previously described (Baldassini et al., 2015). The gels of the experimental groups were scanned and analyzed using the Image Master 2D Platinum software v.7.0 (GE Healthcare, Illinois, United States) to obtain the following parameters: number of spots, matching, isoelectric point (pI), and molecular weight (MW).

To ensure confidence in the detection and subsequent validation of the spot, the gels were run in triplicate. Thus, the mean of three values was calculated for each sample and spot. The amount of protein in each spot was expressed as volume, i.e., the sum of pixel intensity within the spot area. The relative volume (V%) was used to assess differences in the amount of each spot, which is a normalized value that remains relatively independent of variations. Matching, i.e., determination whether the spot present in one gel corresponds to that in another gel, was performed after detection of the spots.

Electrospray ionization tandem mass spectrometry (ESI-MS/MS)

After image analysis, the differentially abundant protein spots (between treatments) were extracted from the gels with a scalpel, cut into segments of approximately 1 mm, transferred to microtubes containing 1 mL of 5% acetic acid (v/v), and subjected to the following steps: dye removal, protein reduction and alkylation, and tryptic digestion with 10 ng mL^{-1} trypsin solution. Tryptic digestion of the spots was performed using a specific commercial kit (In-Gel DigestZP). The peptide sequences obtained by tryptic digestion were characterized by ESI-MS/MS as previously described (Shevchenko et al., 2007). The proteins were identified in the UniProt database (www.uniprot.org) based on homology.

Bioinformatics procedures

First, bioinformatics analyses were performed for the classification of differentially abundant spot proteins in muscle tissues from control versus vitamin A-treated animals in terms of biological processes (BP), molecular function (MF), and cellular components (CC). For this purpose, the proteins identified by ESI/MS/MS were analyzed using the OMICSBOX v.2.0 (https://www.biobam.com/omicsbox) and Blast2GO tools (Götz et al., 2008).

Next, the Metascape® open-source tool (https://metascape.org/) was used to identify the main enriched gene ontology (GO) terms among the protein lists following the procedures described (Gagaoua et al., 2021a). Since the *Bos taurus* taxonomy is not available in Metascape®, orthologs to *Homo sapiens* were retrieved using HCOP (www.genenames.org/tools/hcop/). Hierarchical heatmap clustering comparing the significantly enriched GO terms was performed. The overlap of the two proteomes was also investigated with a Circos plot, illustrating de the degree of overlap between

protein lists of control versus vitamin A. The cut-off criteria were the default values: degree cut-off = 2, node score cut-off = 0.2, Max depth = 100, and *K*-score = 2.

Further bioinformatics analysis between the proteins identified in the LT of animals was performed using the STRING database (Search Tool for Retrieval of Interacting Genes, v. 11.0 – https://string-db.org). A protein-protein interaction network relating the differential proteins according to the pathways to which they belong was then generated.

Statistical analysis

Data were analyzed using the SAS v. 9.4 statistical software (SAS Institute Inc., Cary, North Carolina, United States). The initial body weight was used as a covariate and analysis of variance (ANOVA) was applied to test the effects of treatment (control versus vitamin A) on the performance variables and carcass traits using the PROC GLM procedure according to the model:

$$Yij = m + ti + \varepsilon ij,$$

where *Y*ij is the value observed for the experimental unit of treatment *i* in repetition *j*; *m* is the overall effect of the mean; *t* is the effect of treatment (control or vitamin A), and $\mathcal{E}$ is the experimental error.

For the proteomics data, the mean and standard deviation of the selected spots were calculated. The images were compared between treatments (control and vitamin A). The spot data were tested for homogeneity of variances and normality using the Levene and Shapiro-Wilk tests, respectively. Next, differences in means between treatments were analyzed by the Student t-test. In addition, the Mann-Whitney test (Wilcoxon test) was used when the normality assumption was violated in either treatment. For all variables, a P value < 0.05 indicated significant differences. Trends

were considered when $0.05 \leq P < 0.10$.

Results

Performance and carcass and meat quality traits

The birth weight of control and vitamin A-treated animals was 28 ± 1.4 and 26 ± 1.2 kg, respectively. There was no significant effect of treatment control versus vitamin A on weaning weight or ADG during the cow-calf phase ($P > 0.05$). The productive performance (final body weight and ADG) were similar during the finishing phase ($P > 0.05$). Similarly, there was no effect of treatment on the carcass traits at slaughter ($P > 0.05$) (Table 1).

A trend ($P = 0.07$) towards more tender meat (lower WBSF) was observed for animals that received vitamin A compared to control (Table 2). There were no differences in cooking loss, meat color (L*, a*, b*, Chroma and Hue) or meat pH between treatments ($P > 0.05$). The meat of animals receiving vitamin A at birth had a higher IMF content ($P < 0.05$) than that of control animals. Vitamin A increased the IMF content of beef by 50%. On the other hand, moisture, protein, and collagen levels were similar ($P > 0.05$).

Muscle proteome

Comparison of the gels showed a mean number of protein spots per treatment of 273 ± 45 for the control treatment and of 266 ± 44 for the vitamin A treatment. Among these spots, image analysis identified 17 spots that were differentially abundant ($P < 0.05$) in the control treatment (Figure 1a) versus vitamin A treatment (Figure 1b). Forty-seven genes were found in proteins spots of control ($n = 10$) and vitamin A ($n = 37$).

Table 3 lists the proteins identified in the two treatments. In the control group,

there was greater abundance of oxidative stress and cell defense proteins (HSPA8 and DNAJC18), proteins involved in muscle structure/contraction (MYLPF, PDLIM3, TNNT3, and MYOZ1) and energy metabolism/oxidative pathway (LDHA, CA3, and MDH2), and transport and signaling proteins (H31, EEF1A1, SOCS2, H2BC12, and CNBP). On the other hand, animals supplemented with vitamin A during the cow-calf phase exhibited greater abundance of proteins involved in energy metabolism (GAPDH, ENO3, TPI1, CKM, and COX5A), muscle structure/contraction (ACTB, ACTC1, ACTG1, ACTG2, ACTA1, ACTA2, ACTN1, ACTN2, ACTN3, TPM1, TPM2, TPM3, MYH1, PDLIM3, and TNNT3), protein binding (STXBP6, LGALS1, H4, MYOZ1, H31, H2BC12, GUCA1A, and CNBP), and transport and cell signaling (TUBA4A, VIM, TBA1B, and EEF1A2).

Bioinformatics procedures and functional enrichment permitted to identify differences in BP, MF, and CC between the control versus vitamin A treatment (Figure 2). Positive and significant upregulation of BP such as generation of precursor metabolites and energy, phosphorylation, and ATP metabolic process was observed at the end of the finishing period in animals that received vitamin A at birth. Regarding MF, more catalytic processes were identified in control animals (Figure 2A), while binding processes were enriched in vitamin A-treated animals (Figure 2B). These differences in muscle tissue BP and MF between the control and vitamin A treatments help explain the IMF content of meat. Vitamin A influenced the number of key BP related to energy metabolism, concomitantly inhibiting catalytic processes. As a result, greater deposition of IMF occurred during the finishing phase.

Figures 3 and 4 summarize the main enriched terms and pathways identified in this study using the differentially abundant proteins for control versus vitamin A. Based on GO terms, several pathways were significantly upregulated in animals supplemented

with vitamin A: developmental, cellular/multicellular organismal and metabolic processes (Figure 3A-C). Thus, biological regulation pathways, generation of metabolites, and energy metabolism help explain the higher IMF content found in beef of vitamin A-treated animals.

Further bioinformatic analysis was used to compare the protein overlap between treatments (control vs. vitamin A) using Circos plots (Figure 3D) for the total number of proteins. This analysis displays the overlap and functional connections between proteins and allowed us to compare the enriched ontology terms between treatments in order to identify those that were shared by or specific for the treatments.

Next, we analyzed the protein-protein interactions between the control and vitamin A treatments (Figure 4). Additionally, enriched network analysis highlighting the degree of interconnectedness between proteins was performed (Figure S1).

Consistent interactions were found between the proteins identified in bovine muscle tissue samples. Three main clusters were observed in the control treatment: proteins related to energy metabolism, muscle tissue structure, and heat shock proteins (HSPA8). On the other hand, in animals supplemented with vitamin A, there were a larger number of proteins belonging to interconnected pathways involved in energy metabolism, muscle contraction, and interactions with binding proteins such as TUBA4A and VIM. Some of these proteins are considered key molecules in energy metabolism, such as GAPDH, CKM, and ENO3.

Discussion

Vitamin A and meat quality: molecular mechanisms

This is the first study that demonstrated that the intramuscular administration of vitamin A to calves shortly after birth (cow-calf phase) affects the muscle proteome,

improving IMF deposition by more than 50% during the finishing phase. Although the animals in the current study were slaughtered with light BW, if animals reach greater final BW in the feedlot system, the differences in meat quality traits should be the same or even greater. However, additional studies are need to confirm this hypothesis. To supply their own butcher shops, some Brazilian meat-packing companies pay better for carcasses derived from the biological type known as "super early-maturing" (low *Bos indicus* content; steers feedlot-finished and slaughtered up to 15 months of age).

Previous studies have suggested the ability of vitamin A to stimulate postnatal angiogenesis (Pyatt and Berger, 2005; Taniguchi and Mizoguchi, 2015). Vitamin A, through its metabolite retinoic acid, can cause changes in the recruitment of undifferentiated cells, with more of these cells being directed towards the adipogenic lineage (Wang et al., 2016), particularly intramuscular adipocytes. A study using a transcriptome approach in Aberdeen Angus calves injected with two doses of vitamin A, one at birth and another one month later at concentrations of 150,000 or 300,000 IU, found that two doses of 150,000 IU improved IMF deposition and upregulated adipogenic genes (Harris et al., 2018).

Similarly, a previous study revealed that intramuscular injection of vitamin A after birth (dose of 300,000 IU) increased the IMF content in Montana × Nellore calves after the finishing phase (Maciel et al., 2022). In that study, treatment with vitamin A at birth upregulated the *ZNF423*, *FABP4* and *SREBF1* genes during the early phases of life of calves. These genes are crucial for pre-adipocyte recruitment and the regulation of lipogenesis (Ladeira et al., 2018). When adipocytes mature, several genes with specific lipogenic functions can also affect the muscle proteome (Campos et al., 2020), with consequences on energy metabolism that lead to an increase in IMF, as observed in the present study.

Energy metabolism proteins and vitamin A

The most abundant proteins identified in LT muscle of F1 Montana × Nellore steers (GAPDH, CA3, MDH2, CKM, TPM2, HSPA8, ENO3, TPI1, TUBA4A, and TNNT3) have been associated with meat quality traits such as tenderness (Bjarnadóttir et al., 2012; Poleti et al., 2018; Zhu et al., 2021) and IMF (Baldassini et al., 2022; Picard et al., 2019; Santiago et al., 2023; Silva et al., 2019a). Particularly interesting among the most abundant proteins in the vitamin A treatment are those related to energy metabolism (GAPDH, ENO3, TPI1, and CKM), which could explain the higher IMF content in these animals.

The genes encoding these molecules have been described in other studies on beef cattle as regulators of carcass and meat quality traits, including muscle growth (Silva-Vignato et al., 2017), IMF (Gagaoua et al., 2020), and meat tenderness (Gagaoua et al., 2021a) and color (Gagaoua et al., 2021b). The GAPDH enzyme, for example, regulates glycolysis and gluconeogenesis by converting glyceraldehyde 3-phosphate into 1,3-bisphosphoglycerate. Within this context, studies [30,35] have shown higher expression of GAPDH in the proteome of heifers and steers, respectively, which was accompanied by higher IMF content, in agreement with the present results.

Another important protein is carbonic anhydrase (CA3), which catalyzes the reversible hydration of carbon dioxide (CO_2) and is closely related to increased oxidative metabolism, acting as a regulator of IMF in cattle (Severino et al., 2022). In proteome studies of Qinchuan (Zhang et al., 2010) and Hanwoo (Shen et al., 2012) steers, CA3 was also less abundant in animals with greater IMF. These results agree with the present study in which CA3 was more abundant in muscle tissue of control animals (less IMF).

Vitamin A, muscle tissue proteome and meat tenderness

There was slight improvement in the tenderness of beef from animals that received an intramuscular injection of vitamin A at birth, with a reduction of approximately 0.3 kg in WBSF. Studying Limousin × Luxi crossbred steers, Wang et al. (Wang et al., 2007) reported lower WBSF of meat from animals receiving diets enriched with vitamin A (1,100 to 2,200 IU). These results suggest that the intramuscular injection of vitamin A in the early phases of life of beef cattle can lead to long-term changes in metabolism and in the contractile structure of muscle fibers (Wang et al., 2018), influencing meat tenderness, as observed in the present study.

Vitamin A regulated the abundance of heat shock proteins, which have been negatively associated with meat tenderness in other studies (Balan et al., 2014; Vermeulen et al., 2014). Additionally, there was greater abundance of HSPA8 and BAG1 in the control treatment. These proteins have been described in the literature to delay meat tenderization during the onset of rigor mortis (Carvalho et al., 2014; Souza Rodrigues et al., 2017). This fact would explain the trend towards higher WBSF in the control group since these proteins may have interfered with postmortem proteolysis, delaying meat tenderization. According to literature (Picard and Gagaoua, 2017), greater abundance of HSP60 and HSPA8 in bovine muscle tissue compromises meat tenderness, as reported for bulls (Santiago et al., 2023).

The myofibrillar proteins α-actin (ACTA1), β-actin (ACTB), γ-actin (ACTG1), and MYH1 were significantly more abundant in the vitamin A treatment. These proteins have been associated with improved tenderness of beef (Gagaoua et al., 2021a). For example, α-actin has been proposed as a biomarker for meat tenderness in Norwegian Red cattle (Bjarnadóttir et al., 2012) and has also been associated with tenderness of

Nellore beef (Rosa et al., 2018).

Conclusion

The administration of a dose of 300,000 IU of vitamin A during the neonatal phase of beef calves is a strategy that exerts a long-term effect on the regulation of key biological processes related to energy metabolism, muscle contraction, and signaling, which strongly control the deposition of IMF and moderately regulate meat tenderness. The trend towards more tender meat in animals that received an intramuscular injection of vitamin A at birth may be related to higher IMF content, as well as to changes in the muscle proteome, notably molecular mechanisms involved in the conversion of muscle to meat. The use of this strategy under favorable nutritional conditions (e.g., feedlot finishing) contributed to improve economically important traits such as marbling and meat tenderness.

Acknowledgment

This research was funded by São Paulo Research Foundation (FAPESP), grant numbers 2019/ 14572-2 and 2020/11300-9. This study was also financed in part by CAPES - Finance Code 001.

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Figure captions

Figure 1. Protein spots selected for characterization by mass spectrometry (ESI-MS/MS) after image analysis. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): 12.5% (w/v) and pH gradient of 3-10. Muscle samples (*Longissimus thoracis*) obtained from F1 Montana × Nellore steers of the control (**A**) and vitamin A (**B**) group.

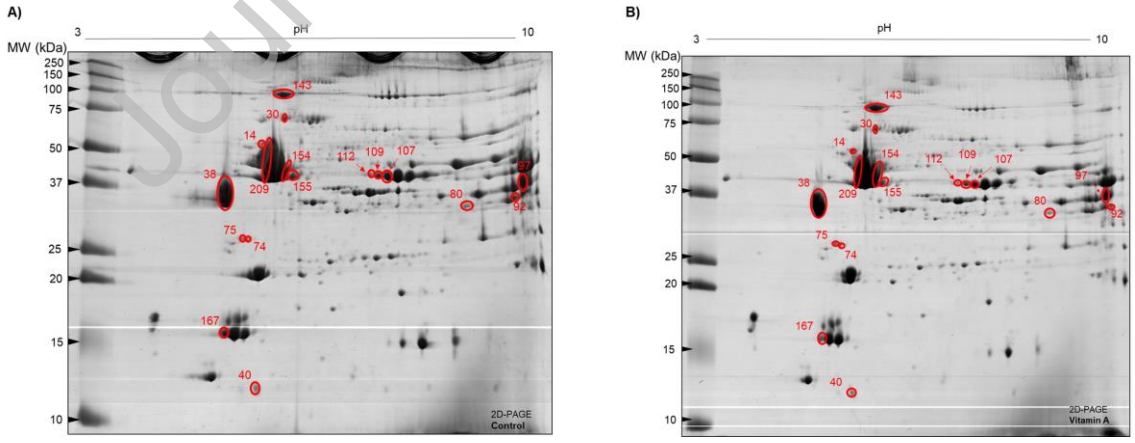

Figure 2. Classification of proteins identified in tissue samples (*Longissimus thoracis*) of feedlot-finished F1 Montana × Nellore steers. **A)** control; **B)** vitamin A. The

OMICSBOX software was used to classify the proteins according to biological process (BP), molecular function (MF), and cellular component (CC).

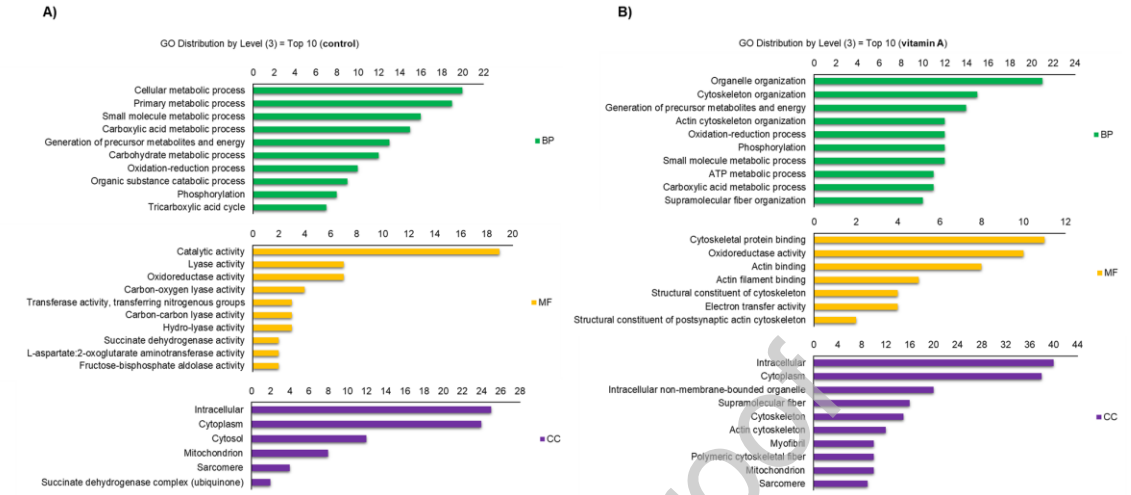

Figure 3. Bioinformatics analyses based on the differentially abundant proteins identified in *Longissimus thoracis* muscle tissue of feedlot-finished F1 Montana × Nellore steers in response to treatments. **A)** Enriched ontology clusters based on the significantly enriched gene ontology (GO) terms obtained using the protein lists of the control treatment. **B**) Enriched ontology clusters based on the significantly enriched GO terms obtained using the protein lists of the vitamin A treatment. **C**) Hierarchical heatmap clustering comparing the enriched GO terms within steers and heifers, as well as the main biological process in each condition. The heatmaps with *P*-values are indicated by color, where grey cells indicate the lack of significant enrichment, paler brown indicates a low *P*-value, and darker brown indicates a high *P*-value. **D**) Protein overlap analysis using a Circos plot that illustrates the degree of overlap between experimental treatments (control vs. vitamin A) based on the list of proteins identified in muscle tissue.

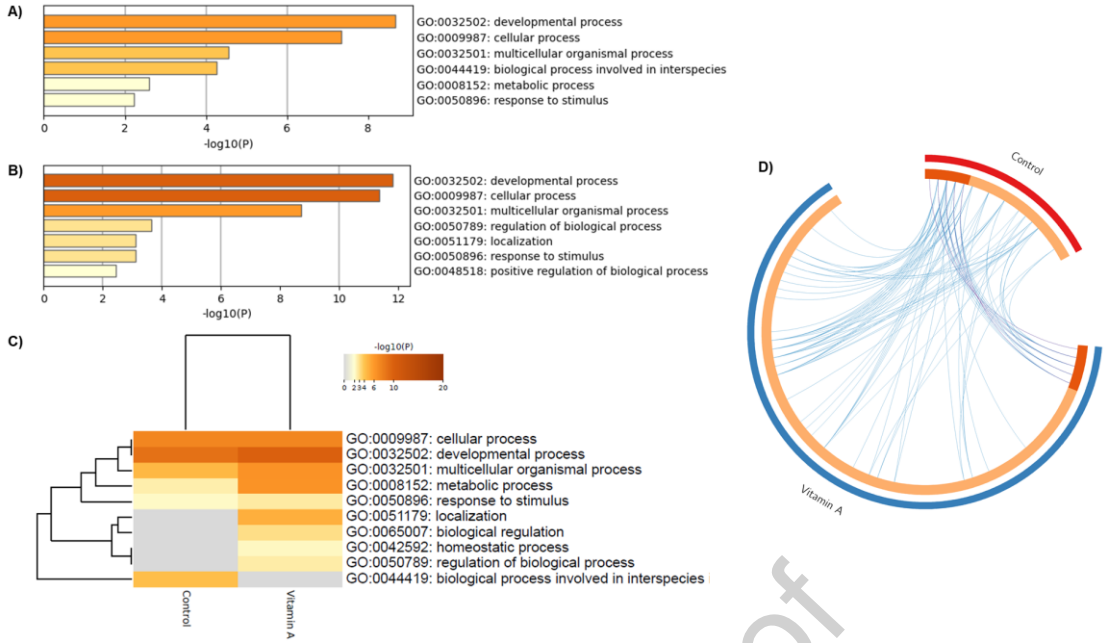

Figure 4. Analysis of protein-protein interactions using the differentially abundant proteins in muscle tissue (*Longissimus thoracis*) of feedlot-finished F1 Montana × Nellore steers. Treatments: **A**) control group; **B**) vitamin A.

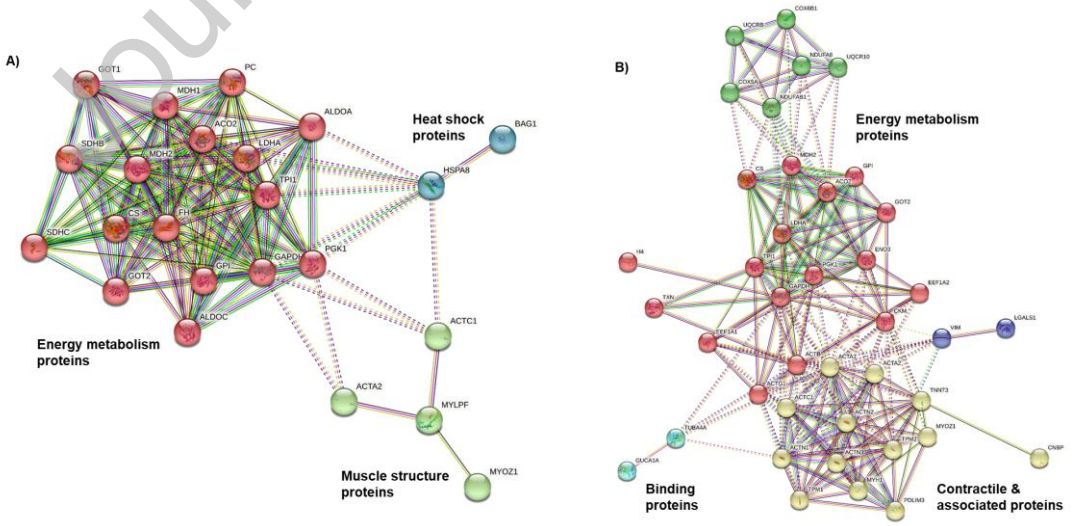

Figure S1. Enriched network related to the previous terms of control versus vitamin A treatment, highlighting the degree of interconnectedness. **A**) Each term is represented by a circle node, where its size is proportional to the number of input genes fall under that term, and its color represent its cluster identity (i.e., nodes of the same color belong to the same cluster). Terms with a similarity score > 0.3 are linked by an edge (the thickness of the edge represents the similarity score). **B**) The same enrichment network has its nodes colored by p-value, as shown in the legend. The dark the color, the more statistically significant the node is (see legend for p-value ranges).

Table 1. Productive performance and carcass traits of feedlot-finished F1 Montana × Nellore steers supplemented or not with injectable vitamin A at birth.

Variables	Control	Vitamin A	SEM	*P-value*
Cow-calf phase				
Weight at birth (kg)	28	26	1.2	0.281
Weaning weight (kg)	237	236	11	0.580
Average daily gain (kg/d)	0.79	0.77	0.14	0.705
Finishing phase				
Final body weight (kg)	398	415	5.5	0.707
Average daily gain (kg/d)	0.96	0.95	0.19	0.650
Carcass traits				
Hot carcass weight (kg)	222.8	231.2	5.98	0.298
Carcass yield (%)	55.9	55.7	1.22	0.794
Ribeye area (cm^2)	60.5	64.3	7.33	0.263
Backfat thickness (mm)	10.6	10.9	1.58	0.901

Table 2. Effect of vitamin A supplementation on the quality and proximate chemical composition of meat from F1 Montana × Nellore steers.

Variables[1]	Control	Vitamin A	SEM[1]	*P*-value
Lightness (L*)	34.70	35.10	0.40	0.880
Redness (a*)	16.40	16.70	0.21	0.143

Yellowness (b*)	6.20	6.40	0.13	0.151
Chroma	17.74	18.19	0.29	0.172
Hue	20.47	21.04	0.31	0.175
Meat pH (7 days of aging)	5.68	5.70	0.02	0.650
WBSF (kg)	4.23	3.92	0.16	0.070
Cooking loss (%)	26.70	26.10	0.67	0.770
Moisture (%)	74.04	73.57	0.18	0.418
Protein (%)	22.54	22.60	0.11	0.506
Intramuscular fat (%)	2.57	4.10	0.28	0.002
Collagen (%)	1.21	1.20	0.01	0.915

[1]WBSF = Warner-Bratzler shear force (7 days of aging)

Table 3. Muscle proteins (*Longissimus thoracis*) of F1 Montana × Nellore steers separated by two-dimensional electrophoresis and characterized by mass spectrometry.

Spot ID	Uniprot ID	Gene symbol	Full protein name	Score	Coverage (%)	pI/MW experimental[2]	pI/MW theoretical	Fold-change (control/vitamin A)[1]
167	Q0P571	MYLPF	Myosin regulatory light chain 2_skeletal muscle isoform	489,3139	54.76	4.71/ 15,800	4.68/ 19,000	1.094/ -0.143 (UP in control)*
80	Q8MKI3	TNNT3	Troponin T_ fast skeletal muscle	473,3224	38.37	8.60/ 32,615.4	5.93/ 32,106	0.606/ -0.707 (UP in control)*
97	Q8MKI3	TNNT3	Troponin T_ fast skeletal muscle	2636, 316	49.07	9.60/ 36,230.8	5.93/ 32,106	0.596/ -0.695 (UP in control)*
107	Q3SYZ8	PDLIM3	PDZ and LIM domain protein 3	848,494	55.06	7.30/ 39,692.3	7.86/ 34,331	0.535/ -0.624 (UP in control)*
112	Q3SYZ8	PDLIM3	PDZ and LIM domain protein 3	1169, 761	58.54	7.01/ 40,615.4	7.86/ 34,331	0.603/ -0.704 (UP in control)*
30	P19120	HSPA8	Heat shock cognate 71 kDa protein	8712, 229	59.84	5.54/ 71,363.6	5.20/ 71,196	0.768/ 0.014 (UP in control)*
92	Q861R0	SOCS2	Suppressor of cytokine signaling 2	148,0309	25.75	9.65/ 34,555.6	8.38/ 22,200	0.921/ -0.230 (UP in control)*
92	P19858	LDHA	L-lactate dehydrogenase A chain	3946, 7	65.97	8.60/ 32,615.4	8.01/ 36,574	0.606/ -0.707 (UP in control)*
92	Q3SZX4	CA3	Carbonic anhydrase 3	238,4126	56.15	9.65/ 34,555.6	7.78/ 29,351	0.606/ -0.707 (UP in

								control)*
80	Q5EA26	DNAJC18	DnaJ homolog subfamily C member 18	247,768	18.15	8.60/ 32,615.4	8.04/ 41,576	0.596/ -0.695 (UP in control)*
97	Q32LG3	MDH2	Malate dehydrogenase_ mitochondrial	1835,295	62.42	9.60/ 36,230.8	8.64/ 35,645	0.596/ -0.695 (UP in control)*
80	Q8SQ24	MYOZ1	Myozenin-1	755,83	60.60	9.60/ 36,230.8	9.53/ 31,653	0.596/ -0.695 (UP in control)*
112	P68432	H31	Histone H3.1	1328,632	33.09	7.01/ 40,615.4	11.54/ 15,394	0.603/ -0.704 (UP in control)*
112	P68103	EEF1A1	Elongation factor 1-alpha 1	3367,631	16.45	7.01/ 40,615.4	9.34/ 50,109	0.603/ -0.704 (UP in control)*
112	Q2M2T1	H2BC12	Histone H2B type 1-K	548,1394	11.90	7.01/ 40,615.4	10.71/ 13,866	0.603/ -0.704 (UP in control)*
209	Q3T0Q6	CNBP	CCHC-type zinc finger nucleic acid binding protein	364,5128	67.65	5.30/ 42,384.6	7.56/ 18,729	0.483/ -0.563 (UP in control)*
14	Q3ZC07	ACTC1	Actin_ alpha cardiac muscle 1	2528,468	48.54	5.23/ 55,818.2	5.07/ 41,991	0.069/ 0.719 (UP in vit. A)*
38	Q5KR48	TPM2	Tropomyosin beta chain	7021,083	88.38	4.68/ 34,846.1	4.45/ 32,816	-0.495/ 0.578 (UP in vit. A)*
38	Q5KR49	TPM1	Tropomyosin alpha-1 chain	4352,101	56.34	4.68/ 34,846.1	4.49/ 32,674	-0.495/ 0.578 (UP in vit. A)*
38	Q5KR47	TPM3	Tropomyosin alpha-3 chain	1905,464	49.65	4.68/ 34,846.1	4.49/ 32,798	-0.495/ 0.578 (UP in vit. A)*
74	Q3ZC07	ACTC1	Actin_ alpha cardiac muscle 1	4033,7	37.13	5.03/ 27,461,5	5.07/ 41,991	-0.575/ 0.670 (UP in vit. A)*
74	Q5KR49	TPM1	Tropomyosin alpha-1 chain	414,3358	59.86	5.03/ 27,461.5	4.49/ 32,674	-0.575/ 0.670 (UP in vit. A)*
75	Q5E9B5	ACTG2	Actin_ gamma-enteric smooth muscle	944,8882	37.76	4.95/ 27,923.1	5.16/ 41,849	-0.471/ 0.549 (UP in vit. A)*
143	Q0III9	ACTN3	Alpha-actinin-3	21469,2	86.79	5.58/ 99,384.6	5.15/ 103,086	-0.768/ 0.895 (UP in vit. A)*
143	Q3ZC55	ACTN2	Alpha-actinin-2	11696,69	67.45	5.58/ 99,384.6	5.15/ 103,713	-0.768/ 0.895 (UP in vit. A)*
143	P62739	ACTA2	Actin_ aortic smooth muscle	485,5888	46.68	5.58/ 99,384.6	5.07/ 41,981	-0.768/ 0.895 (UP in vit. A)*
143	Q3B7N2	ACTN1	Alpha-actinin-1	398,6213	17.94	5.58/ 99,384.6	5.09/ 102,915	-0.768/ 0.895 (UP in vit. A)*
143	Q9BE40	MYH1	Myosin-1	320,7703	33.44	5.58/ 99,384.6	5.43/ 222,850	-0.768/ 0.895 (UP in vit. A)*
154	Q3ZC07	ACTC1	Actin_ alpha cardiac muscle 1	21499,1	79.84	5.62/ 40,846.1	5.07/ 41,991	-0.492/ 0.573 (UP in vit. A)*
154	P60712	ACTB	Actin_ cytoplasmic 1	251,4295	63.20	5.62/ 40,846.1	5.14/ 41,709	-0.492/ 0.573 (UP in vit. A)*
155	P68138	ACTA1	Actin_ alpha skeletal muscle	23305,23	75.33	5.74/ 41,076.9	5.07/ 42,023	-0.495/ 0.577 (UP in vit. A)*
155	P63258	ACTG1	Actin_cytoplasmic 2	147,8195	26.67	5.74/ 41,076.9	5.16/ 41,765	-0.495/ 0.577 (UP in vit. A)*
14	Q9XSC6	CKM	Creatine kinase M-type	724,686	61.15	5.23/ 55,818.2	6.67/ 42,961	0.070/ 0.719 (UP in vit. A)*

40	Q5E956	TPI1	Triosephosphate isomerase	475,6502	49.39	5.22/ 11,923.1	6.51/ 26,672	-0.434/ 0.752 (UP in vit. A)*
40	P00426	COX5A	Cytochrome c oxidase subunit 5A_ mitochondrial	255,1726	27.63	5.22/ 11,923,1	6.49/ 16,724	-0.434/ 0.752 (UP in vit. A)*
74	Q9XSC6	CKM	Creatine kinase M-type	141,5909	53.54	5.03/ 27,461.5	6.68/ 42,961	-0.575/ 0.670 (UP in vit. A)*
75	Q9XSC6	CKM	Creatine kinase M-type	215,1218	46.19	4.95/ 27,923.1	6.68/ 42,961	-0.470/ 0.549 (UP in vit. A)*
154	Q3ZC09	ENO3	Beta-enolase	3075,726	60.37	5.62/ 40,846.1	7.66/ 47,066	-0.491/ 0.573 (UP in vit. A)*
38	Q2YDL1	STXBP6	Syntaxin-binding protein 6	500,6852	26.67	4.68/ 34,846.1	9.69/ 23,652	-0.495/ 0.577 (UP in vit. A)*
40	P11116	LGALS1	Galectin-1	14888,66	71.11	5.22/ 11,923,1	5.16/ 14,734	-0.645/ 0.752 (UP in vit. A)*
40	P62803	H4	Histone H4	460,3494	49.56	5.22/ 11,923.1	11.77/ 11,360	-0.645/ 0.752 (UP in vit. A)*
40	O97680	TXN	Thioredoxin	203,4148	36.19	5.22/ 11,923.1	4.78/ 11,804	-0.645/ 0.752 (UP in vit. A)*
155	P46065	GUCA1A	Guanylyl cyclase-activating protein 1	155,5674	50.73	5.74/ 41,076.9	4.19/ 23,495	-0.495/ 0.577 (UP in vit. A)*
14	P81948	TUBA4A	Tubulin alpha-4A chain	10080,27	76.34	5.23/ 55,818.2	4.75/ 49,892	0.070/ 0.719 (UP in vit. A)*
14	P48616	VIMN	Vimentin	8105,088	82.62	5.23/ 55,818.2	4.87/ 53,695	0.070/ 0.719 (UP in vit. A)*
14	P81947	TBA1B	Tubulin alpha-1B chain	1203,786	28.82	5.23/ 55,818.2	4.76/ 50,119	0.070/ 0.719 (UP in vit. A)*
14	Q32PH8	EEF1A2	Elongation factor 1-alpha 2	289,2056	86.93	5.22/ 11,923.1	9.34/ 50,438	0.070/ 0.719 (UP in vit. A)*

[1]The number of spots in the reference gels was 230 ± 45 for the control treatment and 251 ± 44 for the vitamin A treatment. Protein spot expression in experimental groups (control versus vitamin A). For the comparisons of 2D gel images, one reference gel per treatment is listed, which contained the largest number and best definition of spots. The reference gel of one treatment was contrasted with each gel of the other treatment, totaling 10 comparisons.

[2]Isoelectric point (pI) and molecular weight (MW).

Conflict of Interest

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial interest or non financial interest in the subject matter or materials discussed in this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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M. Gagaoua: Writing - Review & Editing.

G.D. Ramírez-Zamudio: Investigation and Writing - Review & Editing.

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R.N.S. Torres: Writing - Review & Editing.

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R.A. Curi: Writing - Review & Editing.

L. A. Chardulo: Project administration, Funding acquisition, Conceptualization, and Investigation.