Feedlot diets containing increasing starch levels and different feed additives changes cecal proteome profile involved on energy metabolism and inflammatory response of Nellore cattle

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Abstract

Background: Diets for feedlot cattle require higher energy density, thus contributing to the high rate of fermentable carbohydrate. The use of feed additives is necessary to reduce possible metabolic disorders. The objective of this study was to analyze the post-rumen effects of different levels of starch (25, 35, and 45%) and additives (Monensin, Blend of essential oil + exogenous α-Amylase) in diets for Nellore cattle feedlot. The cecum tissue proteome was separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and then, with the verification of differentially expressed protein SPOTS, these were characterized by electrospray ionization mass spectrometry (ESI-MS/MS).

Results: The expression of nine enzymes participating in the Steps of the glycolysis pathway was verified, such as: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Glyceraldehyde-3-phosphate dehydrogenase like-17 protein, Triosephosphate isomerase (Step 1); Phosphoglycerate mutase and Phosphoglycerate mutase 2 (Step 2); Alpha-enolase (ENO1), Beta-enolase (ENO3) and Fructose-bisphosphate aldolase (ALDOB) (Step 4); and Pyruvate Kinase (PKM) (Step 5). There was expression of three enzymes linked to catalytic activities participating in the synthesis of lactate from...
pyruvate: L-lactate dehydrogenase B, L-lactate dehydrogenase A chain and L-lactate dehydrogenase. The ATP synthase subunit beta and ATP synthase subunit beta mitochondrial participate in the electron transport chain, producing ATP from ADP in the presence of a proton gradient across the membrane. Due to the manipulation of diets, the expression of the Leukocyte elastase inhibitor protein, associated with the inflammatory response.

**Conclusions:** The use of blends of essential oil associated with α-amylase as a feed additive promoted the greater expression of enzymes in the pathway of glycolysis and gluconeogenesis (and the absence of proteins linked to inflammation (Leukocyte elastase inhibitor) in cecum tissues. On the other hand, the increase in starch in the diets promoted a reduction in enzymes linked to carbohydrate degradation with increased responses linked to inflammatory injuries.

**Keywords:** Blend Essential Oils, Exogenous α-Amylase, Glucose, Monensin, Proteomic, Spectrometry Mass

**Background**

Among the limitations to enhance meat production is the large energy requirement, which means that feedlot diets have a higher net energy demand (EL) [1]. Thus, increased starch in the diet, tests and physiological limits the digestive animals for the large amount of fermentable carbohydrates in the rumen and ruminal escape increased starch.

In the rumen, the fermentation of glucose from starch occurs, being converted mainly into volatile fatty acids (AGV) and lactate [2] which are metabolized in the liver and provide the greatest source of energy for ruminants [3,4]. However, the use of large amounts of starch can lead to disorders and metabolic diseases due to the accumulation of organic acids in ruminal fluid, especially acidosis and bloat [5]. Thus, feed additives that eliminate harmful processes of ruminal fermentation are employed, such as sodium monensin, which is a polyester carboxylic ionophore used in growth and
finishing diets [5]. In addition to acting bacteriostatically on gram positive ruminal bacteria, but with
the possible impact of residues on products of animal origin and microbial resistance [6]. Alternative
additives have shown the potential to replace monensin, such as blends of essential oil associated with
the exogenous enzyme $\alpha$-amylase, which has demonstrated gains in performance and carcass weight,
in addition to reducing hepatic abscesses and fecal starch in diets with high levels of starch [7–9].

With high levels of starch in the diet, the rate of passage and post-ruminal digestion increases
[5]. The rumen microbiota can digest around 70–80% of the starch consumed [5,10–13], however, the
digestion and absorption of post rumen starch are partially impaired, as enzymatic digestion by
pancreatic $\alpha$-amylase in the duodenum is limited in the small intestine [14,15]. Another important
factor postulated by others is that glucose cannot be absorbed and transported in large quantities from
the lumen into the bloodstream due to insufficient levels of the glucose transporters SLGT1 and
GLUT2 [5,16,17], which favors the escape of part of the starch to the large intestine and increases the
potential for digestion and use of this starch in the cecum. This favors the escape of some of the starch
to the large intestine and increases the potential for digestion and use of this starch in the cecum.

Therefore, feedlot diets that usually contain increased amounts of energy due to high levels of
concentrate inclusion [18] can cause excessive fermentation in the cecum, thereby contributing to the
metabolizable energy of ruminants [19,20], however, it may generate inflammatory reactions in the
cecal epithelium. Feed additives that are able to increase the use of starch in the rumen, reducing the
escape of starch to the intestines, as well as lower starch levels in the diet of feedlot cattle, can avoid
the risk of cecal acidification.

Therefore, it is important to understand how the digestion and absorption sites act in the use of
starch in beef cattle. Due to the levels of starch in diets for cattle and their respective effects on the
extension of the gastrointestinal tract, associated with different feed additives, the objective of this
study is to map the proteome of the cecum of feedlot cattle, and to elucidate how protein expression
acts on metabolism in different nutritional strategies.
Material and Methods

Animals, facilities, feeding and animal care

The field trial was conducted in at the feedlot facilities of the Innovation and Applied Science Center of DSM Nutritional Products (I & AS Beef Center) (Rio Brilhante, Mato Grosso do Sul, Brazil). Nellore bulls (n = 210) (Bos taurus indicus), with an average body weight of ± 380 kg, from the grazing system were used. The animals were randomly allocated to pens (7 animals/pen), with 12 m² of area/animal, drinking fountains and collective troughs (50 cm linear/animal). The program for receiving the animals consisted of weighing, deworming and vaccinating according to the annual prophylactic calendar. The animals were submitted to a pre-adaptation period of 10 days in order to standardize their rumen population and adapt to the facilities and management. The diets were formulated using the LRNS system (Large Ruminant Nutrition System, [21]), level 2, meeting the nutritional requirements for daily weight gain between 1.5 and 1.7 kg.day.animal⁻¹. Feeding was done twice a day at 8:00 am (40% of the total) and 3:00 pm (60% of the total), with constant water in the automatic drinkers. The experimental diets were composed of bagasse sugarcane in nature, ground corn, soybean hulls, cottonseed, soybean, mineral-vitamin core, urea and additives. The management of the animals' adaptation period to the finishing diet was as follows: duration of 14 days, two diets with 65% and 75% concentrate being provided for seven days each. From the 15th day of the experiment until slaughter of the animals, the finishing diet containing 85% concentrate was provided (Table1).
Table 1. Experimental diets containing increasing starch levels (25, 35, and 45%) and additives (Monensin, Blend of essential oil + exogenous α-Amylase) in diets for Nellore cattle feedlot

| Diets | Starch level (%) | 25 | 35 | 45 |
|-------|-----------------|----|----|----|
|       | Adap. 1         | Adap. 2 | Finishin | Adap. 1 | Adap. 2 | Finishin | Adap. 1 | Adap. 2 | Finishin |
|       | 1               | 2    | g    | 1    | 2    | g    | 1    | 2    | g    |
| Sugarcane bagasse | 350 | 250 | 150 | 350 | 250 | 150 | 350 | 250 | 150 |
| Corn grain grind | 300 | 330 | 360 | 300 | 400 | 500 | 300 | 470 | 640 |
| Soybean meal | 90 | 55 | 20 | 90 | 65 | 40 | 90 | 75 | 60 |
| Whole cottonseed | 60 | 80 | 100 | 60 | 80 | 100 | 60 | 80 | 100 |
| Soybean hulls | 150 | 235 | 320 | 150 | 155 | 160 | 150 | 75 | 0 |
| Mineral and Vitamin supplement | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |

| Nutrient Content (Dry matter, g kg⁻¹) |
|-------------------------------------|
| CP³ | 146 | 147 | 146 | 146 | 147 | 146 | 146 | 145 | 145 |
| TDN⁴ | 660 | 680 | 690 | 660 | 690 | 730 | 660 | 720 | 770 |
| DPI⁵ | 510 | 510 | 500 | 510 | 510 | 520 | 510 | 520 | 530 |
| NDF⁶ | 437 | 424 | 412 | 437 | 382 | 330 | 437 | 316 | 252 |
| peNFD⁷ | 360 | 300 | 250 | 360 | 290 | 230 | 360 | 280 | 220 |
| Ca⁸ | 7.7 | 7.5 | 7.3 | 7.7 | 7.5 | 7.3 | 7.7 | 7.6 | 7.5 |
| P⁹ | 3.1 | 2.8 | 2.5 | 3.1 | 3.1 | 3.1 | 3.1 | 3.6 | 3.7 |
| Starch | 209.5 | 230.8 | 254.6 | 209.5 | 284.0 | 355 | 209.5 | 372.8 | 458.0 |
| NE | 2.40 | 2.44 | 2.48 | 2.40 | 2.51 | 2.63 | 2.40 | 2.62 | 2.77 |

1Adap 1 = Adaptation 1, 0-7 days; 2Adap 2 = Adaptation 2, 7-14days, 14-89 days; ³Crude protein (CP); ⁴Total digestible nutrients (TDN); ⁵Digestive protein intake (DPI); ⁶Neutral detergent fiber (NDF); ⁷Physically effective neutral detergent fiber (peNFD); ⁸Calcium (Ca); ⁹Phosphor (P); ¹⁰Net energy (NE).

Experimental design

The factorial arrangement 3 x 2 was used, being the factors: STARCH LEVEL (25 × 35 × 45%) and ADDITIVES (Monensin × Essential Oil Blend: CRINA® + Exogenous α-Amylase: Rumistar®).

Sodium monensin (Rumensin, Elanco Animal Health, Indianapolis, IN) used was included in the diet at a dose of 26 mg kg⁻¹ of dry matter. The blend of functional oils (CRINA RUMINANTS®; DSM...
Nutritional products, Basel, Switzerland) containing thymol, eugenol, limonene and vanillin [22], and
the exogenous enzyme α-amylase (RONOZYME RUMISTAR™; DSM Nutritional products, Basel,
Switzerland) were added to the diet at a dose of 90 mg kg⁻¹ of dry matter, 560 mg kg⁻¹ of dry matter,
respectively. The pens were distributed in a completely randomized block design, totaling 6 treatments
with 5 repetitions, totaling 30 experimental units. Thus, the distribution of treatments within the blocks
was as follows: T1 (25MON), T2 (25BEOα), T3 (35MON), T4 (35BEOα), T5 (45MON), T6
(45BEOα). According to the statistical model:

\[ Y_{ijk} = \mu + B_k + C_i + (C \times A)_j + \varepsilon_{ijk} \]

Where: \( Y_{ijk} \) = Dependent variable; \( \mu \) = Overall mean; \( B_k \) = Block effect; \( C_i \) = Concentrate;
\( A_j \) = Additive; \( (C \times A)_j \) = Interaction between concentrate and additive effects; \( \varepsilon_{ijk} \) = Residual error.

Sample Collection and preparation

During the humane slaughter of animals, cecum samples were collected with an area of about
4 × 4 cm, which were then washed with phosphate buffered saline (PBS), transferred to 15 ml
polypropylene bottles and placed in liquid nitrogen (-196°C) for later protein extraction. The pen was
considered the experimental unit, so a pool of samples was made from the homogenization of cecal
tissue of the same treatment, wherein three animals per experimental unit were considered (totaling 15
animals/pool).

Extraction, precipitation and quantification of proteins

During the protocol, different extraction solutions were tested (buffer solution Tris-HCl pH
8.50 with protease inhibitors Leupepetin and Phenyl Methyl Sulfonyl Fluoride - PMSF, Tris-
HCl/Sucrose buffer also with protease inhibitors Leupepetin and Phenyl Methyl Fluoride Sulfonyl -
PMSF, with ultrapure water in the presence of protease inhibitors). The buffer which showed the best results for protein extraction was ultrapure water.

To extract the protein fraction, the tissue was macerated with a mortar and pestle in the presence of liquid nitrogen. The extracting solution was added in a proportion of 1g/1 mL (tissue/ultrapure water), and then homogenized with an OMMI-BEAD RUPTOR4 cell disruptor (Kennesaw, Georgia, United States) with 3 cycles of 30 seconds. They were subsequently separated into protein extracts and supernatant after refrigerated centrifugation (-4°C) with an UNIVERSAL 320R HETTICH (Tuttlingen, Baden-Württemberg, Germany). Thus, the proteins were precipitated in 80% (v/v) acetone (J.T. Baker, Phillipsburg, New Jersey, United States), using 300 μL of supernatant and 600 μL of 80% acetone. The samples were stored at 2°C for 1.5 hours and then centrifuged at 14,000 rpm for 30 minutes; the supernatant was discarded and the protein pellet was solubilized in 1 mL of 0.50 mol L⁻¹ NAOH (Merck, Darmstadt, Germany). Protein concentrations were determined by the Biuret method [23] using an analytical curve with a concentration range from 0–100 g L⁻¹ of standard bovine albumin solution (Acros Organics, NJ, United States) at the concentration 100 g L⁻¹.

**Electrophoretic separations of protein fractions using 2D-PAGE**

For isoelectric focusing, about 375 μg of proteins was applied to the strips; the sample was resolubilized with a solution containing 7 mol L⁻¹ urea, 2 mol L⁻¹ thiourea, 2% CHAPS (m/v) (GE Healthcare, Uppsala, Sweden), ampholytes pH 3 to 10 at 0.5% (v/v) (GE Healthcare, Uppsala, Sweden) and 0.002% bromophenol blue (GE Healthcare, Uppsala, Sweden), in addition to 2.8 mg DTT (USB, Cleveland, Ohio, USA). Approximately 900 μL of mineral oil was added at room temperature for 12 hours to rehydrate the strips. After this period, the strips were added to the EttanTMIPGphorTM3 isoelectric focusing system (IEF) (GE Healthcare, Uppsala, Sweden). The electrical voltage used was established by the protocol described by Braga et al. (2015). At the end of focusing, the strip was balanced in two stages lasting 15 minutes each. At first, using 10 ml of solution
containing 6 mol L\(^{-1}\) urea, 2% SDS (w/v), 30% glycerol (v/v), 50 mmol L\(^{-1}\) Tris-HCl (pH 8.8), 0.002% bromophenol blue (w/v) and 2% DTT (w/v), to keep the proteins in their reduced forms [24,25]. In the second stage, a solution with a similar composition was used; however, DTT was replaced with 2.5% (w/v) iodoacetamide, to obtain alkylation of the thiol groups of the proteins and thereby prevent possible reoxidation. After the strip balance steps, the second dimension of the electrophoretic process (SDS-PAGE) was submitted. The strip was applied to a 12.5% (w/v) polyacrylamide gel previously prepared on a glass plate (180 x 160 x 1.5 mm). The gel was placed next to the strip, with a piece of filter paper containing 6 µL of a molar mass standard (GE Healthcare, Uppsala, Sweden), with proteins of different molar masses (β-phosphorylase (97.0 kDa), albumin (66.0 kDa), ovoalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). The strip and filter paper were sealed with 0.5% agarose solution (w/v), to ensure contact with the polyacrylamide gel. The race program was then applied at 100 volts for 30 minutes, and a further 250 volts for 2 hours. After the run period, the gels were immersed in a fixative for 30 minutes containing 10% acetic acid (v/v) and 40% ethanol (v/v); soon after, the proteins were revealed using the colloidal Coomassie dye (USB, Cleveland, Ohio, USA) for 72 h and then removed by washing with deionized water [24–27].

The gels obtained were scanned and their images analyzed using the image processing program ImageMaster 2D Platinum 7.0 (GeneBio, Geneva, Switzerland), which allows the isoelectric points and the molecular masses of the separated proteins to be estimated, and the number of SPOTS obtained in gel electrophoresis to be calculated. Three replicates of each gel run were used to evaluate the reproducibility of each protein SPOT obtained in the replicates of the gels, by overlaying the image from one gel over the other, using the image treatment program [24–27].

**Protein identification by mass spectrometry (ESI MS)**

The protein spots were characterized by ESI-MS after being subjected to tryptic digestion and the elution of peptides according to the methodology described by [28]. The aliquots of the solutions
containing the peptides were analyzed to obtain the mass spectra through the nanoAcquity UPLC system coupled to the Xevo G2 QTof mass spectrometer (Waters, Milford, MA, USA). The identification of proteins was performed by searching in database UniProt (2020) with the *Bos taurus* species. After the identification of the proteins, their sequences were analyzed by the FASTA software OMICSBOX (BLAST2GO) and thus categorized by their molecular function, biological processes and biochemical activities with Gene Ontology (GO). The same sequences were used to analyze metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes function (KEGG pathways), making it possible to map the expressions of proteins encoding enzymes found in the database.

**Statistical analysis**

The fixed effects analyzed were STARCH LEVEL AND ADDITIVE in a factorial design; thus, the comparison between groups was by means of contrasts in order to verify differentially expressed protein SPOTS. The images were analyzed using ImageMaster Platinum software version 7.0, which establishes correlation (matching) between groups. For this correlation, the 3 replicates of gels were used comparing volume, distribution, relative intensity, isoelectric point and molecular mass for analysis of variance (ANOVA) considering significance to determine the differentially expressed protein SPOTS. Figure 1 describes the chronology and execution of the activities carried out.
Results and Discussion

Image analysis and SPOTS expression

In the “Workspace”, Classes (Groups) were created to analyze differences in protein expression; for that, the analysis of variance (ANOVA) tests the hypothesis \( H_0 \) that the expressed SPOTS are identical (as shown in supplementary material). When testing all classes, protein SPOTS were differentially expressed, as described in Table 2.
Table 2. Differentially expressed SPOTS in Nellore beef cattle cecum fed with diets containing increasing starch levels (25, 35, and 45%) and additives (Monensin, Blend of essential oil + exogenous α-Amylase)

| SPOT (n) | MON × BEO*  |  | MON*  |  | BEO*  |  |
|----------|-------------|---|-------|---|-------|---|
|          | 25×25 | 35×35 | 45×45 | 25×35 | 35×45 | 25×45 | 25×35 | 35×45 | 25×45 |
| Up       | 9     | 3     | 7     | 14    | 3     | 8     | 5     | 0     | 1     |
| Down     | 11    | 16    | 5     | 6     | 28    | 4     | 10    | 6     | 13    |
| +        | 10    | 59    | 14    | 22    | 65    | 35    | 34    | 16    | 27    |
| Ø        | 37    | 11    | 14    | 81    | 19    | 42    | 18    | 8     | 16    |
| Total    | 67    | 89    | 40    | 125   | 115   | 89    | 67    | 30    | 57    |

*P ≤ 0.05; UP: Up-regulated SPOT; Down: Down-regulated SPOT; +: SPOT present in the first group in relation to the second; Ø: SPOT absent in the first group in relation to the second

Figure 2 describes the distribution of proteins and their biological processes, molecular functions and cellular component.

Figure 2. Classification of the proteins sequences found in beef cattle cecum proteome using OMICSBOX software analysis (Blast2GO).
Proteins characterization by ESI-MS/MS

The differentially expressed SPOTS were characterized from mass spectrometry, after the identification was standardized considering the highest Score Protein, pI and molecular mass (MM) closest to the theoretical and experimental results. Among the proteins identified, 15 were addressed as functional for the purpose of the study, which involve energy metabolism and inflammatory response. Table 3 shows the differentially expressed protein profile in the cecum of Nellore beef cattle under different levels of starch and feed additives.

Table 3. Protein profile differentially expressed in Nellore beef cattle cecum fed with diets containing increasing starch levels (25, 35, and 45%) and additives (Monensin, Blend of essential oil + exogenous α-Amylase) using ESI-MS/MS

| Protein                                         | Access     | Score    | pI/MM theoretical (Da) | pI/MM experimental (Da) |
|-------------------------------------------------|------------|----------|------------------------|-------------------------|
| **Glucose and energy metabolism**                |            |          |                        |                         |
| Alpha-enolase                                   | Q9XSJ4     | 1783.3310| 6.37/47326.13          | 6.70/56906              |
| Beta-enolase                                    | Q3ZC09     | 440.2993 | 7.60/47096.01          | 6.43/48539              |
| Triosephosphate isomerase                       | Q5E956     | 193.3130 | 6.45/26689.51          | 7.24/25458              |
| L-lactate dehydrogenase B                       | Q5E9B1     | 4599.0320| 6.02/36723.64          | 6.37/39211              |
| L-lactate dehydrogenase A chain                 | P19858     | 1327.3960| 8.12/36597.64          | 6.37/39211              |
| Pyruvate Kinase                                 | A5D984     | 98.4805  | 7.96/57948.91          | 5.9/57613               |
| Fructose-bisphosphate aldolase                  | A6QLL8     | 1850.8330| 8.45/39436.12          | 6.37/39211              |
| Phosphoglycerate mutase                         | F1N2F2     | 427.2343 | 9.01/28699.04          | 6.37/39211              |
| Phosphoglycerate mutase 2                       | Q32KV0     | 413.5597 | 8.99/28685.05          | 6.37/39211              |
| L-lactate dehydrogenase                         | F1MK19     | 70.7983  | 5.72/36724.58          | 6.37/39211              |
| Glyceraldehyde-3-Phosphate dehydrogenase        | P10096     | 11907.1000| 8.51/35868.09         | 8.12/29321              |
| Glyceraldehyde-3-phosphate dehydrogenase like-17| Q9XSN4     | 1934.1340| 9.22/11514.31          | 9.70/26577              |
| protein                                         |            |          |                        |                         |
| ATP synthase subunit beta mitochondrial          | P00829     | 533.0471 | 5.15/56283.53          | 5.49/47920              |
| ATP synthase subunit beta                        | A0A452DI18 | 533.0471 | 5.47/62225.55          | 5.70/38338              |

**Inflammatory response**

| Leukocyte elastase inhibitor                    | Q1JPB0     | 300.0084 | 5.70/42235.75          | 5.70/38338              |
Proteins associated with glucose metabolism and energy synthesis (Table 3) and macromolecules involved in the degradation of carbohydrates linked to the glycolytic pathway, gluconeogenesis and oxidative phosphorylation were detected in cecal tissue. The expression of nine enzymes participating in the Steps of the glycolysis pathway was verified, such as: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Glyceraldehyde-3-phosphate dehydrogenase like-17 protein, Triosephosphate isomerase (Step 1); Phosphoglycerate mutase and Phosphoglycerate mutase 2 (Step 2); Alpha-enolase (ENO1), Beta-enolase (ENO3) and Fructose-bisphosphate aldolase (ALDOB) (Step 4); and Pyruvate Kinase (PKM) (Step 5). There was expression of three enzymes linked to catalytic activities participating in the synthesis of lactate from pyruvate: L-lactate dehydrogenase B, L-lactate dehydrogenase A chain and L-lactate dehydrogenase. The ATP synthase subunit beta and ATP synthase subunit beta mitochondrial participate in the electron transport chain, producing ATP from ADP in the presence of a proton gradient across the membrane.

Due to the manipulation of diets, the expression of the Leukocyte elastase inhibitor protein, associated with the inflammatory response, was verified (Table 3); this plays an essential role in regulation of the innate immune response, inflammation and cellular homeostasis, and mainly acts to protect cell proteases released into the cytoplasm during stress or infection.

Table 4. Expression values (ANOVA, \( P \leq 0.05 \)) in beef cattle cecum protein profile fed starch levels (25, 35 and 45%) and additives (Monensin and Blend Essential Oil + \( \alpha \)-Amylase)

| Protein                           | MON x BEO\( \alpha \) | MON | BEO\( \alpha \) |
|-----------------------------------|------------------------|-----|----------------|
|                                   | 25x2 35x3 45x4         | 25x3 35x4 45x4 | 25x3 35x4 45x4 |
|                                   | 5 5 5                   | 5 5 5 | 5 5 5          |
| Glucose and energy metabolism     |                        |      |                |
| Alpha-enolase                     | +/-/\( \emptyset \) NS  | 1,55 | +/-/-1,48 +/-/\( \emptyset \) | 1,65 NS +/-/\( \emptyset \) |
| Beta-enolase                      | \( \emptyset \)/+ NS  | 1,55 | \( \emptyset \)/+/-1,48 \( \emptyset \)/+ | NS NS NS |
| Triosephosphate isomerase         | -3,55 -2,55 NS         | +/-/-2,39 NS | NS NS -1,84 NS |
| L-lactate dehydrogenase           | \( \emptyset \)/+ NS  | -1,47 | \( \emptyset \)/+ NS NS | NS NS NS |
| L-lactate dehydrogenase B        | \( \emptyset \)/+ NS  | NS | NS NS NS | NS NS NS |
|                                | NS | NS | NS | NS | NS | -2,54 | NS | NS |
|--------------------------------|----|----|----|----|----|--------|----|----|
| L-lactate dehydrogenase A chain| Ø/+| NS | NS | NS | Ø/+| NS     | NS | NS |
| Pyruvate Kinase                | NS | NS | -1,47 | NS | NS | NS     | NS | NS |
| Fructose-bisphosphate aldolase | NS | NS | Ø/+ | +/Ø | Ø/+ | NS     | NS | NS |
| Phosphoglycerate mutase        | NS | NS | -1,49 | NS | NS | Ø/+     | +/Ø | NS |
| Phosphoglycerate mutase 2      | NS | NS | +/Ø | NS | NS | Ø/+     | +/Ø | NS |
| Glyceraldehyde-3-phosphate dehydrogenase | NS | NS | NS | NS | Ø/+ | +/Ø | NS | NS |
| Glyceraldehyde-3-phosphate dehydrogenase like-17 protein | NS | NS | NS | NS | Ø/+ | +/Ø | NS | NS |
| ATP synthase subunit beta      | NS | NS | NS | NS | Ø/+ | +/Ø | NS | NS |
| ATP synthase subunit beta      | NS | NS | NS | NS | Ø/+ | +/Ø | NS | NS |
| Inflammatory response          |    |    |    |    |    |        |    |    |
| Leukocyte elastase inhibitor   | +/Ø| NS | +/Ø| NS | NS | -1,22 | NS | NS |
|                                |    |    |    |    |    |        |    |    |

**Inflammatory response**

|                                | NS | NS | NS | NS | NS | Ø/+ | +/Ø | NS | NS |
|--------------------------------|----|----|----|----|----|-----|-----|----|----|
| Leukocyte elastase inhibitor   | +/Ø| NS | +/Ø| NS | NS | -1,22| NS | NS | -1,29 |
Figure 3. Heatmap of the differentially expressed proteins (ANOVA, \( P \leq 0.05 \)) among the diets contending different starch levels and additives. Color-coded matrix showed the correlation coefficient of the SPOTS expression values. Each row and column represent one group and protein, respectively.

Effects of feed additives and starch level on glucose and energy metabolism

The expression values (\( P \leq 0.05 \)) (Table 4) were grouped from the hierarchical cluster analysis, and ordered by the homogeneity between the treatments tested. Animals fed with identical levels of starch, but submitted to different feed additives, showed differentiation for proteins that exercise functions in energy metabolism.

The animals fed with low starch in their diet (25%) associated with BEO\(\alpha\) increased expression of the proteins pyruvate kinase (EC 2.7.1.40), beta-enolase (EC 4.2.1.11), triosephosphate isomerase (EC 5.3.1.1) and L-lactate dehydrogenase (EC 1.1.1.27) compared to those treated with monensin; both proteins are enzymes catalyzing the synthesis of pyruvate, which is responsible for the degradation of carbohydrates. The highest level of starch tested (45%) promoted the greater synthesis of L-lactate dehydrogenase (EC 1.1.1.27), fructose-bisphosphate aldolase (EC 4.1.2.13) and
phosphoglycerate mutase (EC 5.4.2.4); it is noted that the intermediate starch level showed a higher expression of triosephosphate isomerase (EC 5.3.1.1) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12). Thus, the tested range shows a greater expression of glycolysis intermediates when using BEOα (Figure 4). As demonstrated by Thomas, Webb, Ghimire, Blair, Olson, Fenske & Scaria (2017), the effect of monensin is more evident in the rumen, mainly in the diversity of microorganisms, but a proportion below 10% results in post-ruminal action, corroborating the hypothesis that antibiotic additives have a limited effect on the microbiota and intestinal fermentation of ruminants.

Protein expression differs depending on the starch levels in the diet ($P \leq 0.05$); the cluster analysis shows differentiation in the profile of the identified proteins involved in energy metabolism (Figure 3), but the effect is greater when contrasting starch levels of 25% vs. 35% and 35% vs. 45%, mainly when using monensin as a feed additive. It is important to note that this was not observed when assessing the range of levels (25% vs. 45%), but there was a greater expression of proteins involved in inflammatory responses (Figure 3), a fact that is attributed to the greater increase in carbohydrate in the diets, which may have contributed to the lower expression of proteins associated with energy metabolism.
Figure 4. Expression protein profile encoding enzymes in glycolysis and gluconeogenesis pathway.

KEGG key: EC 4.1.2.13: Fructose-bisphosphate aldolase; EC 5.3.1.1: Triosephosphate isomerase; EC 1.2.1.12: Glyceraldehyde-3-phosphate dehydrogenase; EC 1.2.1.59: Glyceraldehyde-3-phosphate dehydrogenase like-17 protein; EC 5.4.2.4: Phosphoglycerate mutase; EC 4.2.1.11 Beta-enolase; EC 2.7.1.40 Pyruvate Kinase; EC 1.1.1.27 L-lactate dehydrogenase.

In view of the different feeding strategies, key enzymes were identified in the degradation of carbohydrates in the large intestine of cattle. Fructose-bisphosphate aldolase (ALDOB), an enzyme
that converts fructose-1,6-bisphosphate to fructose 6-phosphate catalyzed by Triosephosphate isomerase (TPI), is a precursor of glyceraldehyde-3-phosphate (GA3P), which is acted upon by the glyceral enzyme 3-phosphate dehydrogenase (GAPDH) during glycolysis. Alpha-enolase (ENO1) and beta-enolase (ENO3) are isoforms of enolase that are involved in Step 4 of glycolytic metabolism. Phosphoglycerate mutase (PGM) is a catalytic enzyme that converts 3-phosphoglycerate to 2-phosphoglycerate, and finally pyruvate kinase (PKM), which synthesizes pyruvate in the last step of glycolysis. In ruminants, a high concentration of starch enables the fermentation of carbohydrates in the cecum with lactate production, which increases glucose metabolism in the intestine observed expression of the enzyme L-lactate dehydrogenase and its isoforms L-lactate dehydrogenase B and L-lactate dehydrogenase A, which are responsible for the synthesis of lactate from pyruvate.

Inflammatory response

In the protein SPOTS of groups 25BEOα and 45BEOα, in relation to those fed MON, there was an absence in the expression of leukocyte elastase inhibitor, which is a serine protease inhibitor that is essential in the regulation of inflammation responses, and which limits the activity of inflammatory caspases [29]. When comparing 25% vs. 45% of starch in the diet, regardless of the type of additive used, there was a greater expression of this protein, corroborating with previous studies, which demonstrate that inflammatory injuries are caused by the increased use of concentrate in diets [30,31].

Conclusions

To verify the differential expression of the cecal proteome in cattle, our results show that the blend of essential oils associated with α-amylase, incorporated as a feed additive for beef cattle, increased the expression of enzymes related to carbohydrate degradation, participated in glycolysis and gluconeogenesis and reduced the inflammatory response when compared to monensin as a feed
additive. Conversely, higher concentrations of starch reduced the expression of proteins involved in energy metabolism, and increased the expression of inflammatory responses.

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**Author’s contributions**

LR designed and performed the experiment, analyzed samples and datas and wrote the manuscript. VC and AP designed and performed the experiment. MB, JA, AA and RM analyzed samples and datas. DM, JV and PP designed, supervised and analyzed samples and datas. All authors helped to revise, read and approved the final version of the manuscript.

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**Availability of data and materials**

The datasets used can be made available by the corresponding author on reasonable request.

**Ethics approval and consent to participate**

The experiment was carried out according to the standards issued by the National Council for Animal Experimentation Control - CONCEA, and approved by the Ethics and Use of Animals
Committee of the Universidade Estadual Paulista – UNESP, Botucatu -SP, under protocol n° 0107/2019.

Consent for publication

All authors provide their consent to this publication.

Competing interests

The authors declare that they have no conflicts of interest.

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References

1. Brake DW, Swanson KC. RUMINANT NUTRITION SYMPOSIUM: Effects of postruminal flows of protein and amino acids on small intestinal starch digestion in beef cattle. J Anim Sci. 2018;96:739–50. Available from: https://academic.oup.com/jas/advance-article/doi/10.1093/jas/skx058/4827744

2. Allen MS, Bradford BJ, Oba M. BOARD-INVITED REVIEW: The hepatic oxidation theory of the control of feed intake and its application to ruminants. J Anim Sci. 2009;87:3317–34. Available from: https://academic.oup.com/jas/article/87/10/3317-3334/4563378

3. Reynolds CK, Maltby SA. Regulation of Nutrient Partitioning by Visceral Tissues in Ruminants. J
4. Lozano O, Theurer CB, Alio A, Huber JT, Delgado-Elorduy A, Cuneo P, et al. Net absorption and hepatic metabolism of glucose, L-lactate, and volatile fatty acids by steers fed diets containing sorghum grain processed as dry-rolled or steam-flaked at different densities. J Anim Sci. 2000;78:1364. Available from: https://academic.oup.com/jas/article/78/5/1364-1371/4668495

5. National Academies of Sciences, Engineering, and Medicine - NASCEM. Nutrient Requirements of Beef Cattle - NRBC. 8th ed. Washington, D.C.: National Academies Press; 2016. Available from: http://www.nap.edu/catalog/19014

6. Silva FRN, Pereira AD, Baptista DP, Pereira MU, Spisso BF, Gigante ML, et al. Monensin residues in the production of Minas Frescal cheese: Stability, effects on fermentation, fate and physicochemical characteristics of the cheese. Food Res Int. 2020;137:109440. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0963996920304658

7. Meyer NF, Erickson GE, Klopfenstein TJ, Greenquist MA, Luebbe MK, Williams P, et al. Effect of essential oils, tylosin, and monensin on finishing steer performance, carcass characteristics, liver abscesses, ruminal fermentation, and digestibility1. J Anim Sci. 2009;87:2346–54. Available from: https://academic.oup.com/jas/article/87/7/2346-2354/4731227

8. Meschiatti MAP, Gouvêa VN, Pellarin LA, Batalha CDA, Biehl M V, Acedo TS, et al. Feeding the combination of essential oils and exogenous α-amylase increases performance and carcass production of finishing beef cattle1. J Anim Sci. 2019;97:456–71. Available from: https://academic.oup.com/jas/article/97/1/456/5142563

9. Toseti LB, Goulart RS, Gouvêa VN, Acedo TS, Vasconcellos GSFM, Pires A V., et al. Effects of a blend of essential oils and exogenous α-amylase in diets containing different roughage sources for
finishing beef cattle. Anim Feed Sci Technol. 2020;269:114643. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0377840120305472

10. Harmon DL, Yamka RM, Elam NA. Factors affecting intestinal starch digestion in ruminants: A review. Can J Anim Sci. 2004;84:309–18. Available from: http://www.nrcresearchpress.com/doi/10.4141/A03-077

11. Huntington GB, Harmon DL, Richards CJ. Sites, rates, and limits of starch digestion and glucose metabolism in growing cattle1. J Anim Sci. 2006;84:E14–24. Available from: https://academic.oup.com/jas/article/84/suppl_13/E14/4776389

12. Moharrery A, Larsen M, Weisbjerg MR. Starch digestion in the rumen, small intestine, and hind gut of dairy cows - a meta-analysis. Anim Feed Sci Technol. Elsevier B.V.; 2014;192:1–14. Available from: http://dx.doi.org/10.1016/j.anifeedsci.2014.03.001

13. Westreicher-Kristen E, Robbers K, Blank R, Tröscher A, Dickhoefer U, Wolffram S, et al. Postruminal digestion of starch infused into the abomasum of heifers with or without exogenous amylase administration. J Anim Sci. 2018;96:1939–51. Available from: https://academic.oup.com/jas/article/96/5/1939/4958201

14. Owens FN, Zinn RA, Kim YK. Limits to Starch Digestion in the Ruminant Small Intestine1,2. J Anim Sci. 1986;63:1634–48. Available from: https://academic.oup.com/jas/article/63/5/1634-1648/4662251

15. Huntington GB. Starch utilization by ruminants: from basics to the bunk. J Anim Sci. 1997;75:852. Available from: https://academic.oup.com/jas/article/75/3/852-867/4637334

16. Lohrenz A-K, Duske K, Schönhusen U, Losand B, Seyfert HM, Metges CC, et al. Glucose transporters and enzymes related to glucose synthesis in small intestinal mucosa of mid-lactation dairy cows fed 2 levels of starch. J Dairy Sci. 2011;94:4546–55. Available from:
17. Mills JAN, France J, Ellis JL, Crompton LA, Bannink A, Hanigan MD, et al. A mechanistic model of small intestinal starch digestion and glucose uptake in the cow. J Dairy Sci. American Dairy Science Association; 2017;100:4650–70. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030217302758

18. Pinto ACJ, Millen DD. Nutritional Recommendations and Management Practices Adopted By Feedlot Cattle Nutritionists: the 2016 Brazilian Survey. Can J Anim Sci. 2018;1–46.

19. Hoover WH. Digestion and Absorption in the Hindgut of Ruminants. J Anim Sci. 1978;46:1789–99. Available from: https://academic.oup.com/jas/article/46/6/1789-1799/4699332

20. Siciliano-Jones J, Murphy MR. Production of Volatile Fatty Acids in the Rumen and Cecum-Colon of Steers as Affected by Forage: Concentrate and Forage Physical Form. J Dairy Sci. 1989;72:485–92. Available from: https://linkinghub.elsevier.com/retrieve/pii/S002203028979130X

21. Fox D., Tedeschi L., Tylutki T., Russell J., Van Amburgh M., Chase L., et al. The Cornell Net Carbohydrate and Protein System model for evaluating herd nutrition and nutrient excretion. Anim Feed Sci Technol. 2004;112:29–78. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0377840103002979

22. McIntosh FM, Williams P, Losa R, Wallace RJ, Beever DA, Newbold CJ. Effects of Essential Oils on Ruminal Microorganisms and Their Protein Metabolism. Appl Environ Microbiol. 2003;69:5011–4. Available from: https://aem.asm.org/content/69/8/5011

23. Doumas BT, Bayse DD, Carter RJ, Peters T, Schaffer R. A candidate reference method for determination of total protein in serum. I. Development and validation. Clin Chem. 1981;27:1642–50. Available from: http://www.ncbi.nlm.nih.gov/pubmed/6169466
24. Santos FA, Lima PM, Neves RCF, Moraes PM, Pérez CA, Silva MOA, et al. Metallomic study on plasma samples from Nile tilapia using SR-XRF and GFAAS after separation by 2D PAGE: Initial results. Microchim Acta. 2011;173:43–9.

25. Neves RCF, Lima PM, Baldassini WA, Santos FA, Moraes PM, Castro GR, et al. Fracionamento de cobre em proteínas do plasma, músculo e fígado de tilápia do Nilo. Quim Nova. 2012;35:493–8.

26. Moraes PM, Santos FA, Padilha CCF, Vieira JCS, Zara LF, De M. Padilha P. A preliminary and qualitative metallomics study of mercury in the muscle of fish from amazonas, Brazil. Biol Trace Elem Res. 2012;150:195–9.

27. Silva FA, Cavecci B, Baldassini WA, Lima PM, Moraes PM, Roldan PS, et al. Selenium fractionation from plasma, muscle and liver of Nile tilapia (Oreochromis niloticus). J Food Meas Charact. 2013;7:158–65.

28. Shevchenko A, Tomas H, Havli J, Olsen J V, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc. 2006;1:2856–60. Available from: http://www.nature.com/articles/nprot.2006.468

29. Choi YJ, Kim S, Choi Y, Nielsen TB, Yan J, Lu A, et al. SERPINB1-mediated checkpoint of inflammatory caspase activation. Nat Immunol. 2019;20:276–87. Available from: http://www.nature.com/articles/s41590-018-0303-z

30. Liu J, Xu T, Zhu W, Mao S. High-grain feeding alters caecal bacterial microbiota composition and fermentation and results in caecal mucosal injury in goats. Br J Nutr. 2014;112:416–27. Available from: https://www.cambridge.org/core/product/identifier/S0007114514000993/type/journal_article

31. Chang G, Ma N, Zhang H, Wang Y, Huang J, Liu J, et al. Sodium Butyrate Modulates Mucosal Inflammation Injury Mediated by GPR41/43 in the Cecum of Goats Fed a High Concentration Diet. Front Physiol. 2019;10. Available from:
