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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Research

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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

52 finishing diets [5]. in addition to acting bacteriostatically on gram positive ruminal bacteria, but with
53 the possible impact of residues on products of animal origin and microbial resistance [6]. Alternative
54 additives have shown the potential to replace monensin, such as blends of essential oil associated with
55 the exogenous enzyme α-amylase, which has demonstrated gains in performance and carcass weight,
56 in addition to reducing hepatic abscesses and fecal starch in diets with high levels of starch [7–9].
57 With high levels of starch in the diet, the rate of passage and post-ruminal digestion increases
58 [5]. The rumen microbiota can digest around 70–80% of the starch consumed [5,10–13], however, the
59 digestion and absorption of post rumen starch are partially impaired, as enzymatic digestion by
60 pancreatic α-amylase in the duodenum is limited in the small intestine [14,15]. Another important
61 factor postulated by others is that glucose cannot be absorbed and transported in large quantities from
62 the lumen into the bloodstream due to insufficient levels of the glucose transporters SLGT1 and
63 GLUT2 [5,16,17], which favors the escape of part of the starch to the large intestine and increases the
64 potential for digestion and use of this starch in the cecum. this favors the escape of some of the starch
65 to the large intestine and increases the potential for digestion and use of this starch in the cecum.
66 Therefore, feedlot diets that usually contain increased amounts of energy due to high levels of
67 concentrate inclusion [18] can cause excessive fermentation in the cecum, thereby contributing to the
68 metabolizable energy of ruminants [19,20], however, it may generate inflammatory reactions in the
69 cecal epithelium. Feed additives that are able to increase the use of starch in the rumen, reducing the
70 escape of starch to the intestines, as well as lower starch levels in the diet of feedlot cattle, can avoid
71 the risk of cecal acidification.
72 Therefore, it is important to understand how the digestion and absorption sites act in the use of
73 starch in beef cattle. Due to the levels of starch in diets for cattle and their respective effects on the
74 extension of the gastrointestinal tract, associated with different feed additives, the objective of this
75 study is to map the proteome of the cecum of feedlot cattle, and to elucidate how protein expression
76 acts on metabolism in different nutritional strategies.

77

78 **Material and Methods**

79 ***Animals, facilities, feeding and animal care***

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

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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[1]	Adap. 2[2]	Finishing	Adap. 1[1]	Adap. 2[2]	Finishing	Adap. 1[1]	Adap. 2[2]	Finishing
	Ingredients (g kg^{-1})								
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})								
CP[3]	146	147	146	146	147	146	146	145	145
TDN[4]	660	680	690	660	690	730	660	720	770
DPI[5]	510	510	500	510	510	520	510	520	530
NDF[6]	437	424	412	437	382	330	437	316	252
peNFD[7]	360	300	250	360	290	230	360	280	220
Ca[8]	7.7	7.5	7.3	7.7	7.5	7.3	7.7	7.6	7.5
P[9]	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 Mcal/kg DM[10]	2.40	2.44	2.48	2.40	2.51	2.63	2.40	2.62	2.77

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

$$Y_{ijk} = \mu + B_{k} + C_i + A_J + (C \times A)_{ij} + \varepsilon_{ijk} \quad (1)$$

Where: Y_{ijk} = Dependent variable; μ = Overall mean; B_k = Block effect; C_i = Concentrate; A_J = Additive; $(C \times A)_{ij}$ = Interaction between concentrate and additive effects; ε_{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 beset 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 parthways), 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.

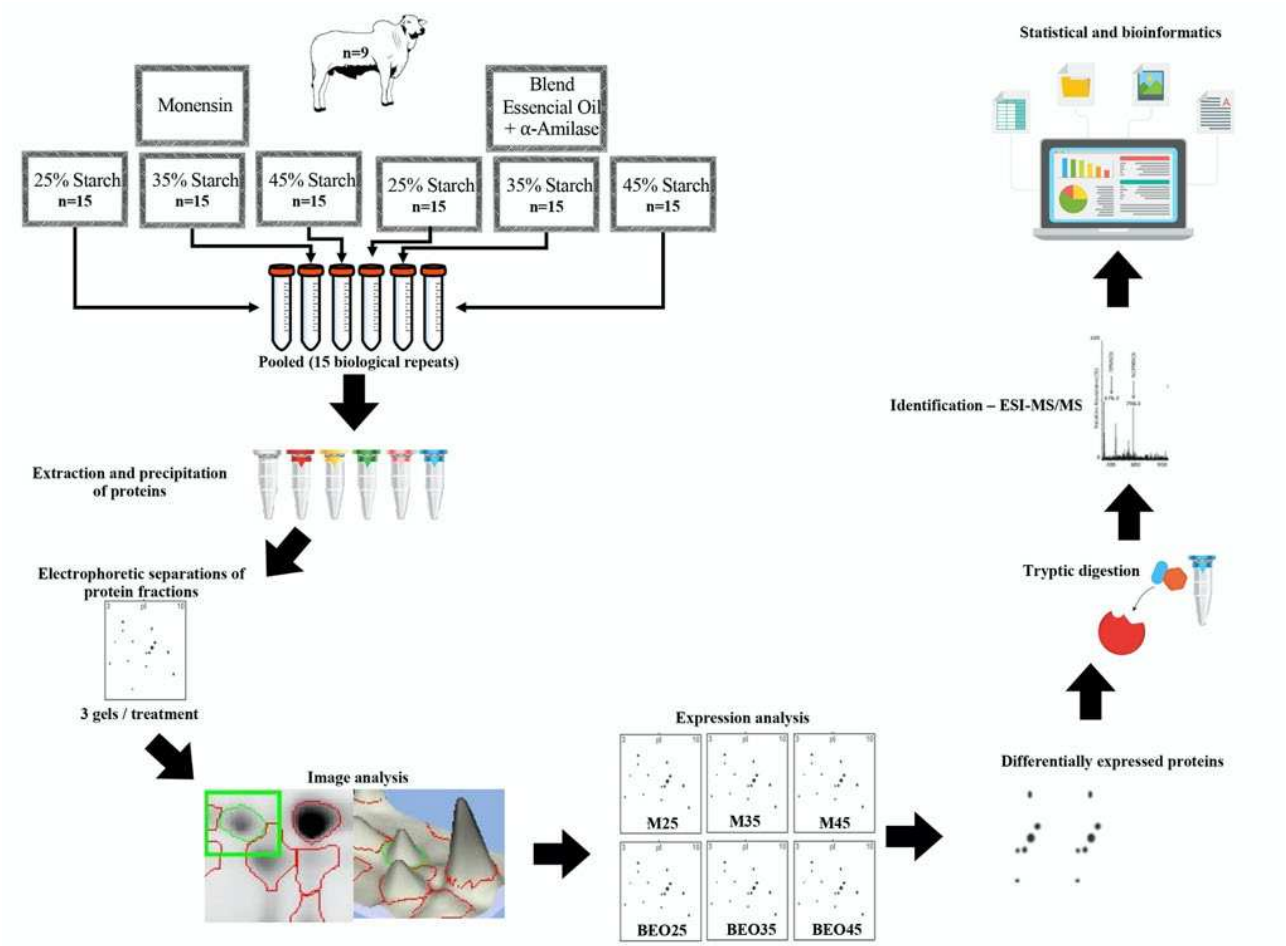

Figure 1. Graphical abstract

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
∅	37	11	14	81	19	42	18	8	16
Total	67	89	40	125	115	89	67	30	57

* $P \leq 0.05$;UP: Up-regulated SPOT; Down: Down-regulated SPOT; +: SPOT present in the first group in relation to the second;∅: 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.

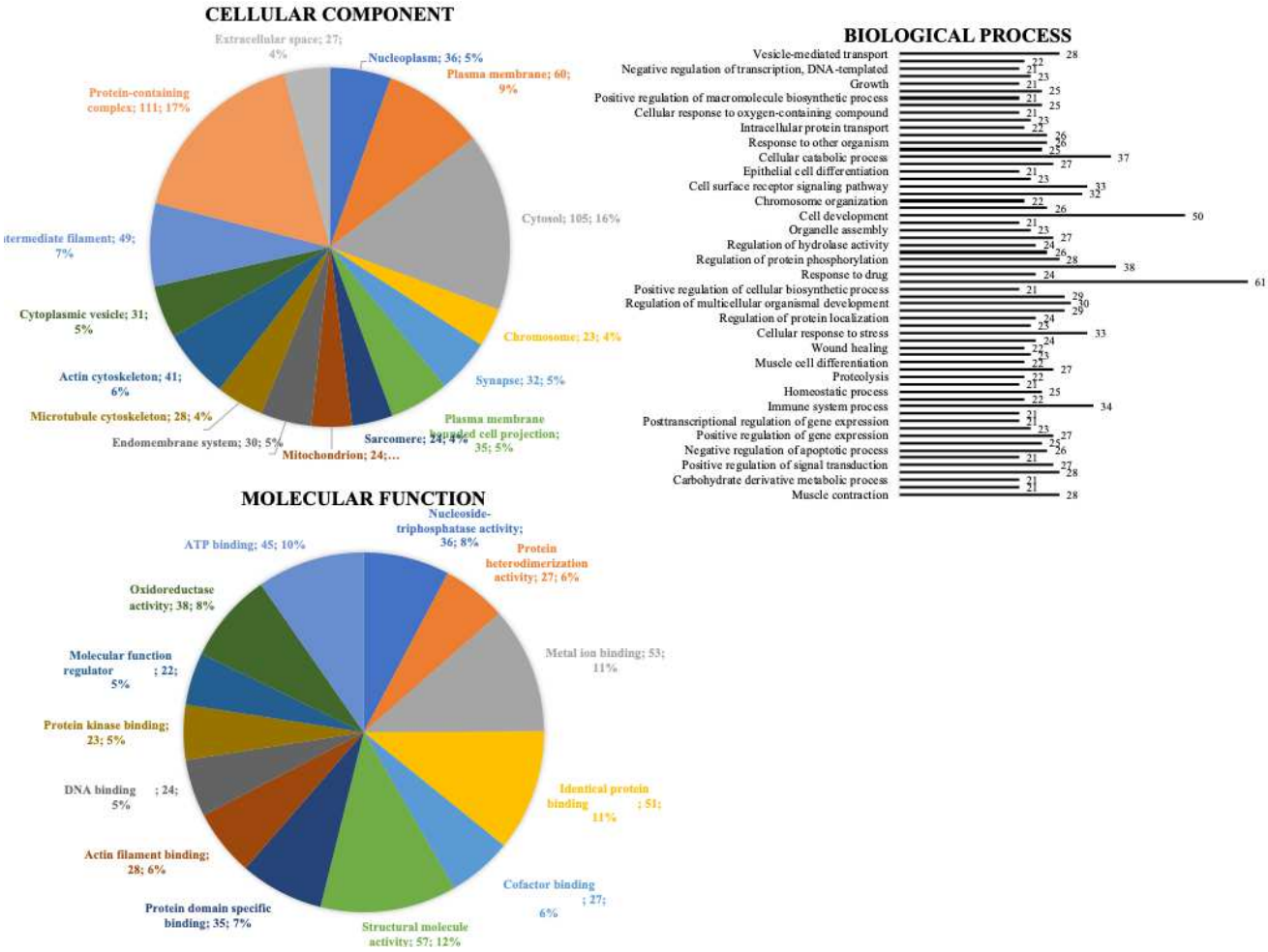

Figure 2. Classification of the proteins sequences found in beef cattle cecum proteome using OMICSBOX software analysis (Blast2GO).

230 ***Proteins characterization by ESI- MS/MS***

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

237

238 **Table 3.** Protein profile differentially expressed in Nellore beef cattle cecum fed with diets containing
239 increasing starch levels (25, 35, and 45%) and additives (Monensin, Blend of essential oil + exogenous
240 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 protein	Q9XSN4	1934.1340	9.22/11514.31	9.70/26577
ATP synthase subunit beta_ mitochondrial	P00829	533.0471	5.15/56283.53	5.49/47920
ATP synthase subunit beta	A0A452DII8	533.0471	5.47/62225.55	
Inflammatory response				
Leukocyte elastase inhibitor	Q1JPB0	300.0084	5.70/42235.75	5.70/38338

241

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 + α-Amylase)

Protein	MON × BEOα			MON			BEOα		
	25×25	35×35	45×45	25×35	35×45	25×45	25×35	35×45	25×45
Glucose and energy metabolism									
Alpha-enolase	+/∅	NS	1,55	+/∅	-1,48	+/∅	1,65	NS	+/∅
Beta-enolase	∅/+	NS	1,55	∅/+	-1,48	∅/+	NS	NS	NS
Triosephosphate isomerase	-3,55	-2,55	NS	+/∅	-2.39	NS	NS	NS	-1,84
L-lactate dehydrogenase	∅/+	NS	-1,47	∅/+	NS	NS	NS	NS	NS
L-lactate dehydrogenase B	∅/+	NS	NS	NS	NS	NS	NS	NS	NS

L-lactate dehydrogenase A chain									
Pyruvate Kinase	∅/+	NS	NS	NS	∅/+	NS	-2,54	NS	NS
Fructose-bisphosphate aldolase									
Phosphoglycerate mutase	NS	NS	-1,47	NS	NS	NS	NS	NS	NS
Phosphoglycerate mutase 2									
Glyceraldehyde-3-phosphate dehydrogenase	NS	-1,49	NS	∅/+	+/∅	∅/+	NS	NS	NS
Glyceraldehyde-3-phosphate dehydrogenase like-17 protein	NS	+/∅	NS	∅/+	+/∅	∅/+	NS	NS	NS
ATP synthase subunit beta_ mitochondrial	NS	NS	NS	NS	NS	NS	∅/+	+/∅	NS
ATP synthase subunit beta									
Inflammatory response									
Leukocyte elastase inhibitor	+/∅	NS	+/∅	NS	NS	-1,22	NS	NS	-1,29

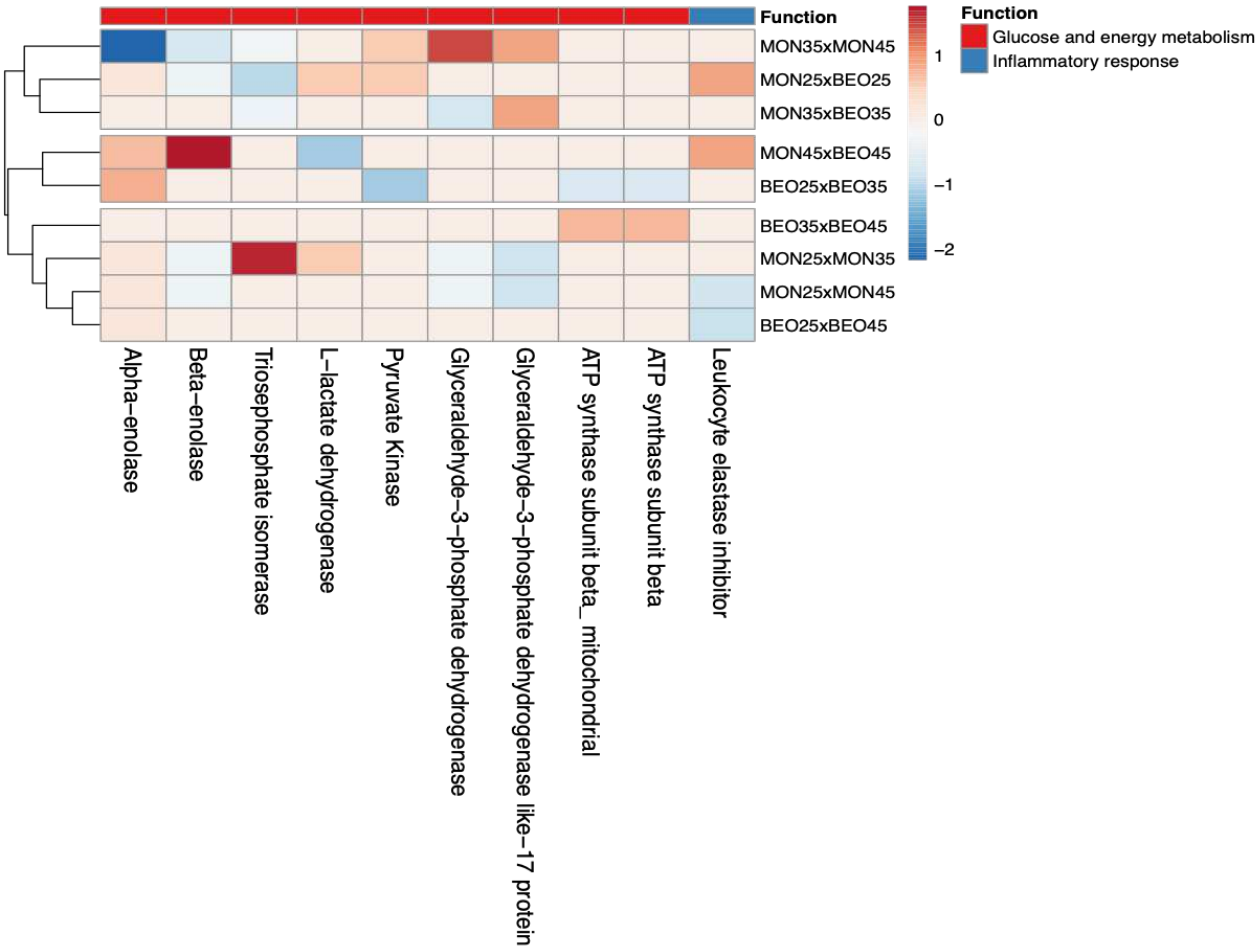

Figure 3. Heatmap of the differentially expressed proteins (ANOVA, $P \leq 0.05$) among the diets contending different starch levels and additives. Color-coded matrix showed the correlation coefficient of the SPOTS expression values. Each row and column represent one group and protein, respectively.

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 BEOα 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

277 phosphoglycerate mutase (EC 5.4.2.4); it is noted that the intermediate starch level showed a higher
278 expression of triosephosphate isomerase (EC 5.3.1.1) and glyceraldehyde-3-phosphate dehydrogenase
279 (EC 1.2.1.12). Thus, the tested range shows a greater expression of glycolysis intermediates when
280 using BEOα (Figure 4). As demonstrated by Thomas, Webb, Ghimire, Blair, Olson, Fenske & Scaria
281 (2017), the effect of monensin is more evident in the rumen, mainly in the diversity of microorganisms,
282 but a proportion below 10% results in post-ruminal action, corroborating the hypothesis that antibiotic
283 additives have a limited effect on the microbiota and intestinal fermentation of ruminants.

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

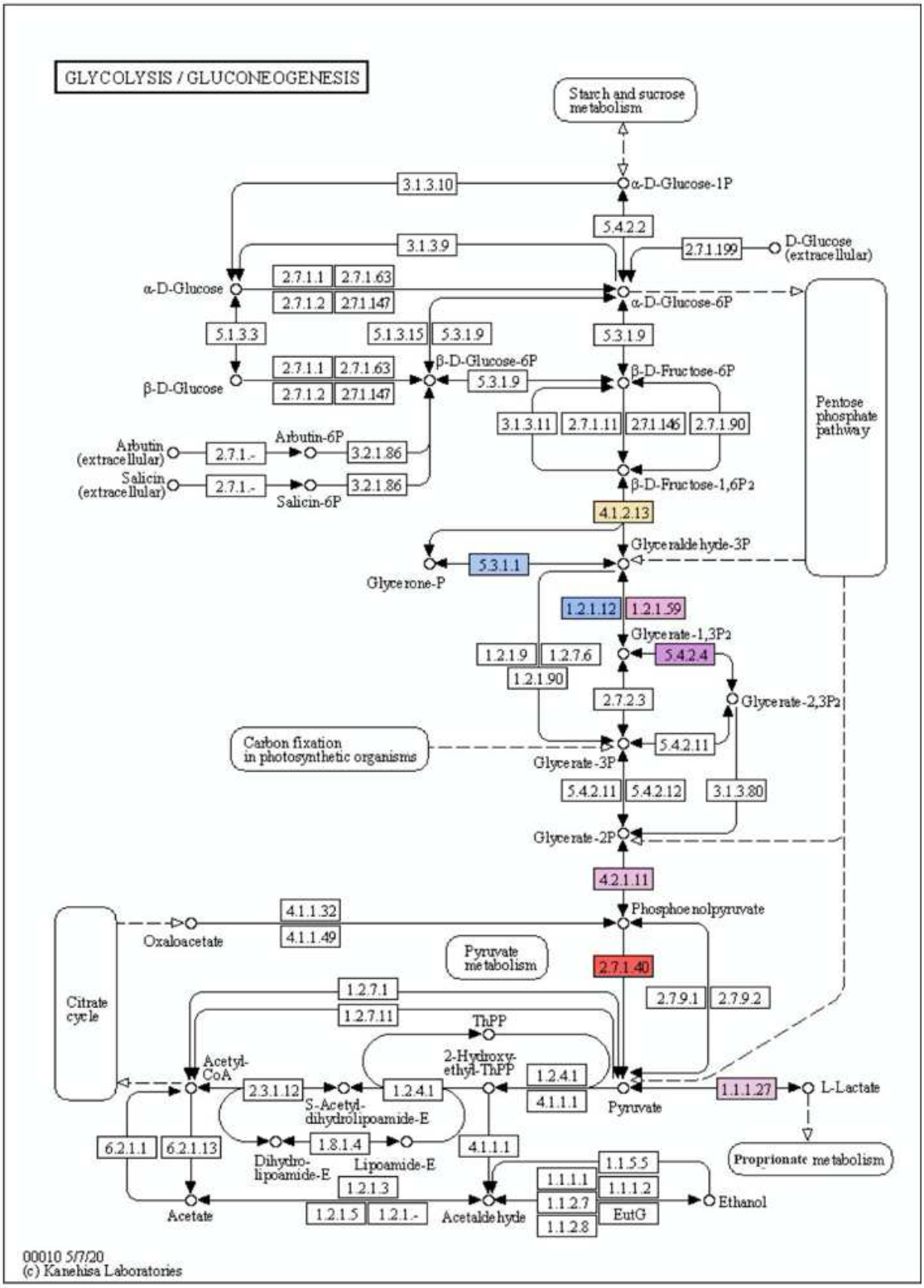

Figure 4. Expression protein profile encoding enzymes in glycolysis and gluconeogenesis pathway.
KEGG key: EC 4.1.2.13: Fructose-bisphosphate aldolase; EC 5.3.1.1: Triosephosphate isomerase; EC 1.2.1.12: Glyceraldehyde-3-phosphate dehydrogenase; EC 1.2.1.59: Glyceraldehyde-3-phosphate dehydrogenase like-17 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

301 that converts fructose-1,6-bisphosphate to fructose 6-phosphate catalyzed by Triosephosphate
302 isomerase (TPI), is a precursor of glyceraldehyde-3-phosphate (GA3P), which is acted upon by the
303 glyceral enzyme 3-phosphate dehydrogenase (GAPDH) during glycolysis. Alpha-enolase (ENO1) and
304 beta-enolase (ENO3) are isoforms of enolase that are involved in Step 4 of glycolytic metabolism.
305 Phosphoglycerate mutase (PGM) is a catalytic enzyme that converts 3-phosphoglycerate to 2-
306 phosphoglycerate, and finally pyruvate kinase (PKM), which synthesizes pyruvate in the last step of
307 glycolysis. In ruminants, a high concentration of starch enables the fermentation of carbohydrates in
308 the cecum with lactate production, which increases glucose metabolism in the intestine observed
309 expression of the enzyme L-lactate dehydrogenase and its isoforms L-lactate dehydrogenase B and L-
310 lactate dehydrogenase A, which are responsible for the synthesis of lactate from pyruvate.
311
312 ***Inflammatory response***
313 In the protein SPOTS of groups 25BEOα and 45BEOα, in relation to those fed MON, there
314 was an absence in the expression of leukocyte elastase inhibitor, which is a serine protease inhibitor
315 that is essential in the regulation of inflammation responses, and which limits the activity of
316 inflammatory caspases [29]. When comparing 25% vs. 45% of starch in the diet, regardless of the type
317 of additive used, there was a greater expression of this protein, corroborating with previous studies,
318 which demonstrate that inflammatory injuries are caused by the increased use of concentrate in diets
319 [30,31].
320
321 **Conclusions**
322 To verify the differential expression of the cecal proteome in cattle, our results show that the
323 blend of essential oils associated with α-amylase, incorporated as a feed additive for beef cattle,
324 increased the expression of enzymes related to carbohydrate degradation, participated in glycolysis
325 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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Figures

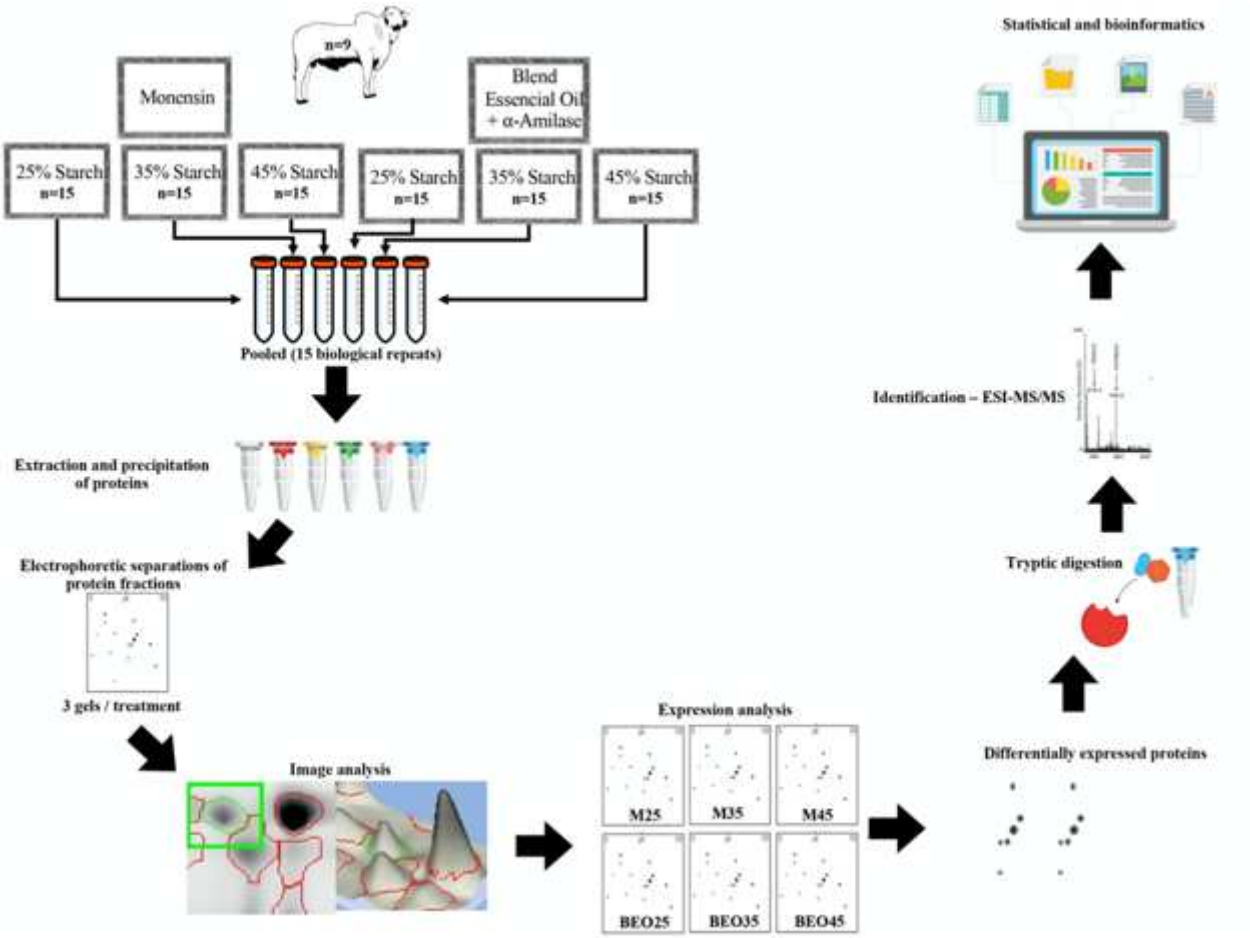

Figure 1

Graphical abstract

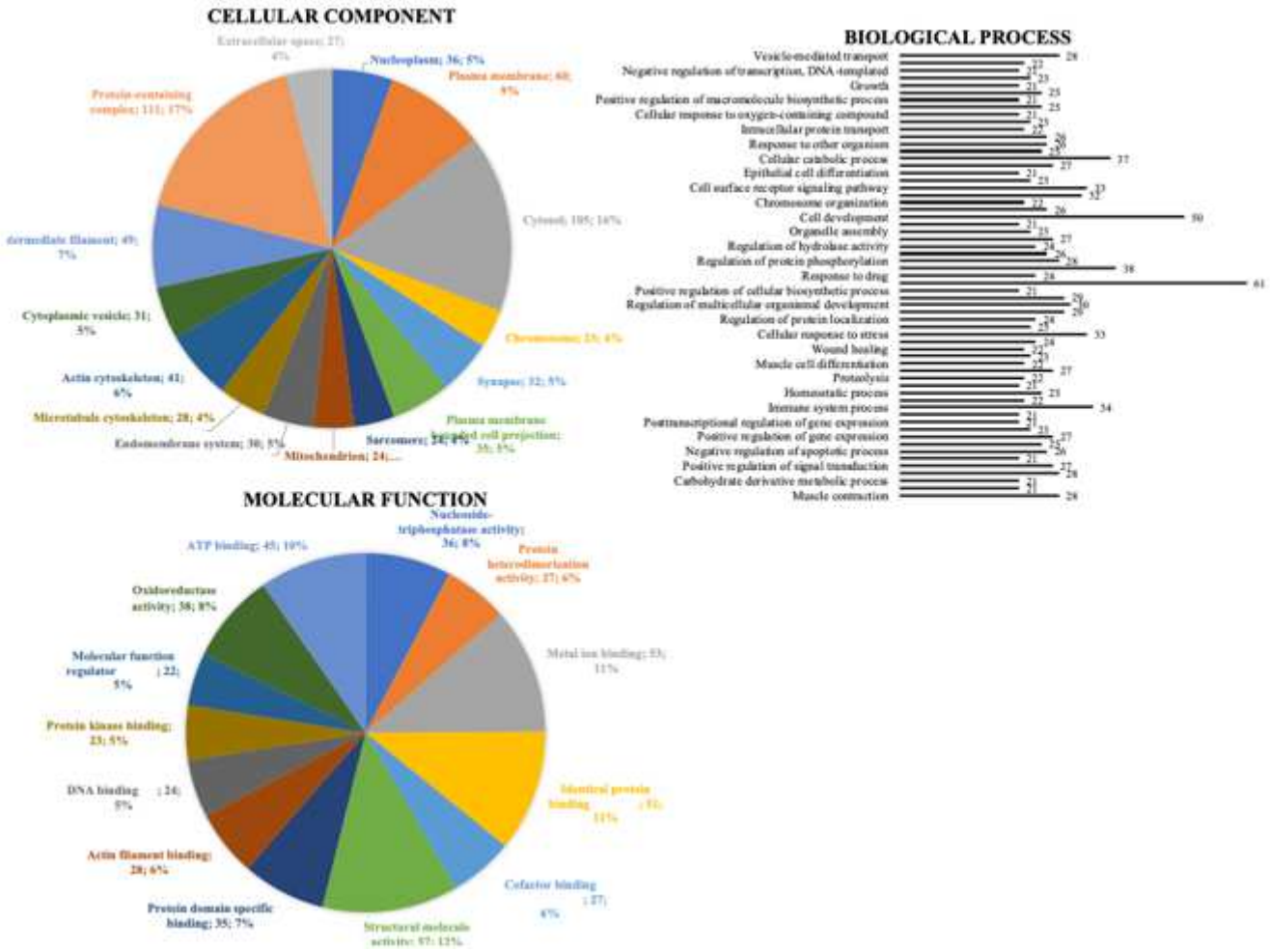

Figure 2

Classification of the proteins sequences found in beef cattle cecum proteome using OMICSBOX software analysis (Blast2GO).

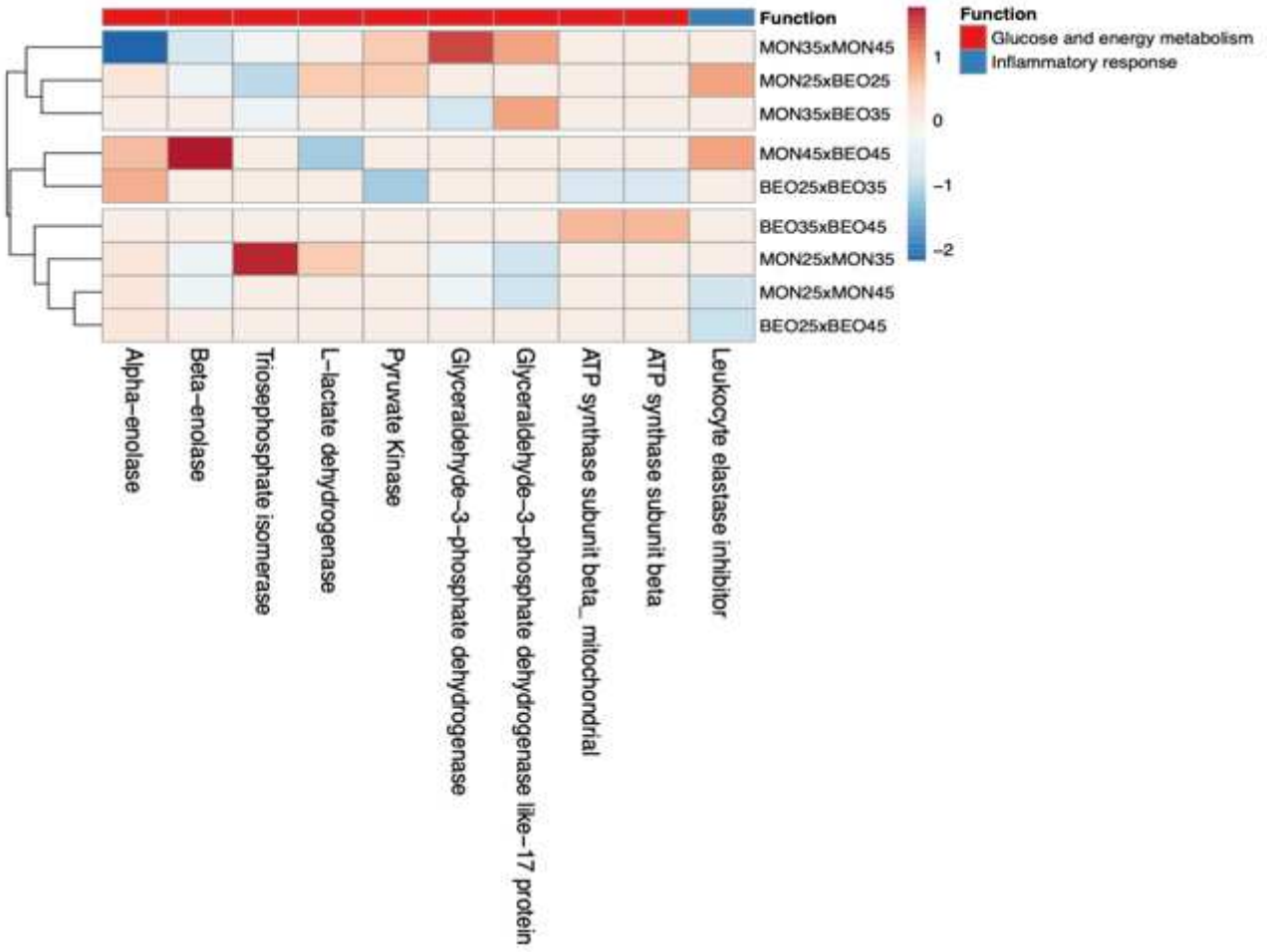

Figure 3

Heatmap of the differentially expressed proteins (ANOVA,) 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.

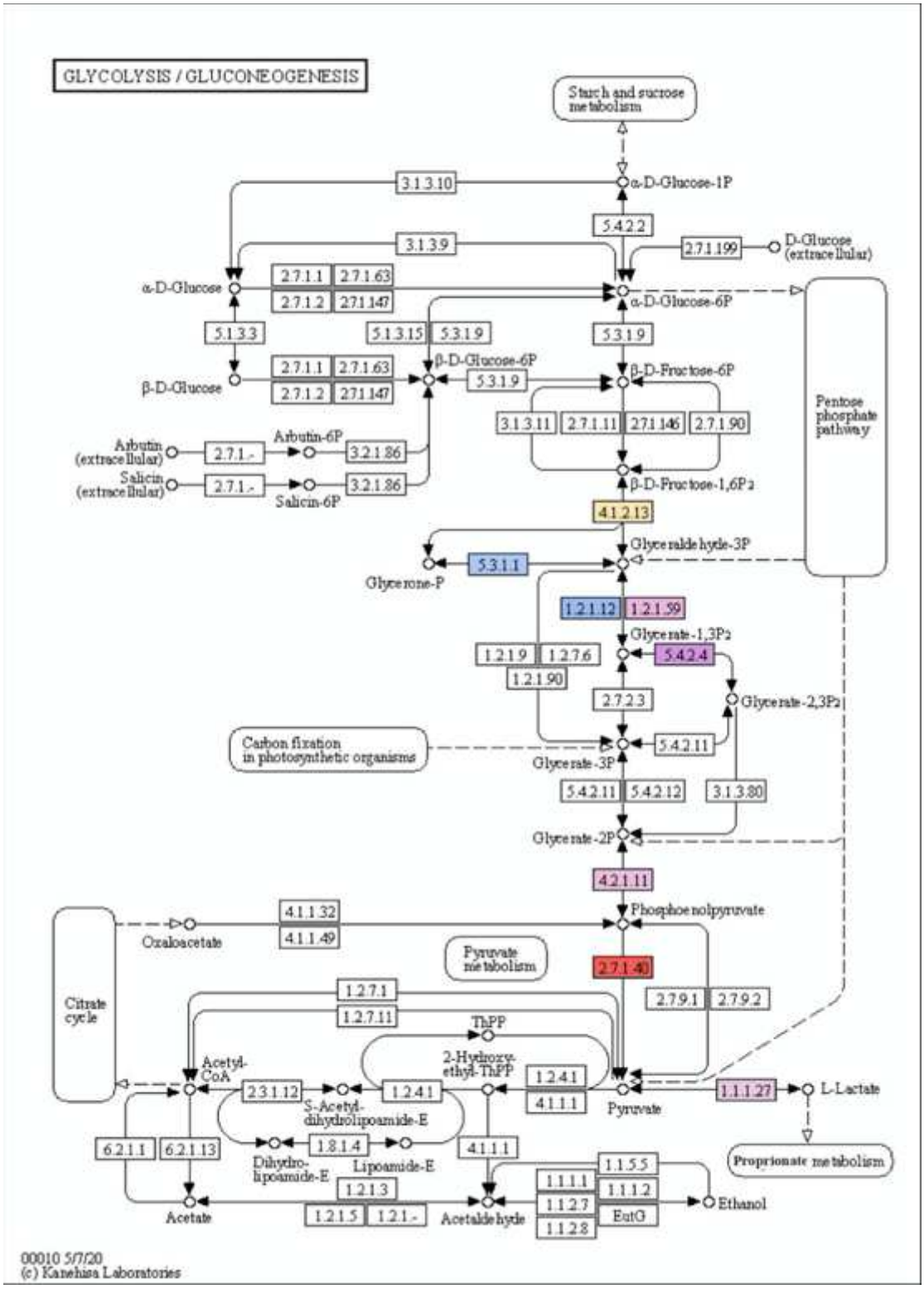

Figure 4

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.