UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
CÂMPUS DE BOTUCATU

PROTEÔMICA DE TECIDO RUMINAL E CECAL DE BOVINOS NELORE CONFINADOS COM DIFERENTES ESTRATÉGIAS NUTRICIONAIS

LEONE CAMPOS ROCHA

Tese apresentada ao Programa de Pós-graduação em Zootecnia como parte das exigências para obtenção do título de Doutor em Zootecnia

BOTUCATU - SP
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Leone Campos Rocha, nascido em 04 de abril de 1994, na cidade de Macarani/BA, filho de José Rocha Filho e Joana Maria Campos Rocha, ingressou no curso de Zootecnia da Universidade Estadual do Sudoeste da Bahia – Campus de Itapetinga em 14 de fevereiro de 2011 e graduou-se em 31 de outubro de 2015. Durante a graduação foi bolsista de iniciação científica no período de 3 anos (2012-2013, 2013-2014 e 2014-2015) financiado por bolsa da Universidade Estadual do Sudoeste da Bahia e Conselho Nacional de Desenvolvimento e Tecnológico (CNPq). Trabalhou como Zootecnista Trainee na Agropecuária Jacarezinho S/A (Jan-abril de 2016), atuando no gerenciamento de confinamento, nutrição e manejo de bovinos confinados e a pasto. Iniciou o curso de Mestrado em 04 de abril de 2016 pelo Programa de Pós-Graduação em Zootecnia – PPZ junto à Universidade Estadual do Sudoeste da Bahia com ênfase em Nutrição de Ruminantes e Forragicultura e Pastagens, sendo concluído em 26 de fevereiro de 2018. Iniciou o curso de Doutorado em Zootecnia em 01 de março de 2018 na Unesp - Faculdade de Medicina Veterinária e Zootecnia - Câmpus de Botucatu, onde foi bolsista pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), e atuou nas áreas de nutrição de bovinos confinados e análise proteômica aplicada à nutrição e metabolismo animal.

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"Eis que estarei com vocês todos os dias, até o fim do mundo."

(Mt 28, 20)

RESUMO GERAL

A literatura apresenta poucos estudos que investigaram o proteoma de ceco e rúmen de bovinos submetidos a estratégias alimentares com uso de aditivos. Essa proposta é pioneira na identificação, expressão e compreensão do perfil proteico ruminal e cecal de bovinos nelore confinados. **Artigo 1:** Objetivou-se avaliar a expressão proteica, a fim identificar possíveis moléculas candidatas a biomarcadores e suas relações com funções metabólicas e nutricionais aplicados a animais confinados com diferentes níveis de amido (25, 35 e 45%) e aditivos (Monensina × blend de óleos essências + amilase exógena), as dosagens de monensina sódica, blend de óleos essenciais e amilase exógena foram 26, 90 e 560 mg/kg MS, respectivamente. Foram utilizados machos Nelore (n = 210) (*Bos taurus indicus*), não castrados (Peso Inicial = ± 380 kg). Após o abate foram coletadas amostras de ceco e acondicionadas imediatamente em nitrogênio líquido. Foi feito um *pool* de amostras utilizando 90 animais (15 animais/tratamento) no processo de precipitação do pellet proteico. A separação e caracterização do proteoma das amostras biológicas foram feitas por eletroforese bidimensional (2D-PAGE) e identificadas por cromatografia líquida acoplada à espectrometria de massas (LC-MS/MS). Os géis de poliacrilamida foram analisados utilizando ImageMaster 2D Platinum 7.0 para verificar as diferenças na expressão de proteínas, para as comparações do % volume normalizado dos *spots*. Após a identificação das proteínas foram analisadas as vias metabólicas usando a função da Enciclopédia de Genes e Genomas de Kyoto (KEGG), análise de enriquecimento da via Reactome para mapear as expressões das proteínas que codificam enzimas e suas respectivas funções nas vias afetadas. O uso de *Blend* de Óleo essenciais associado com α-Amilase como aditivo alimentar promoveu maior expressão de enzimas na via da glicólise e gliconeogênese e ausência de proteína ligada a inflamação (Leukocyte elastase inhibitor). Por outro lado, o incremento de amido nas dietas promoveu redução de enzimas ligadas a degradação de carboidrato com aumento de respostas atribuídas à injúrias inflamatórias no rúmen de bovinos Nelore confinados. **Artigo 2:** Objetivou-se mapear o proteoma do epitélio ruminal de bovinos nelore confinados (n=60) com diferentes níveis de amido (Baixo: 25 e Alto: 45%) e aditivos (Monensina × blend de óleos essências + amilase exógena). As separações e fracionamento das proteínas foram através da eletroforese bidimensional em gel de poliacrilamida (2D-PAGE), e posteriormente a identificação por espectrometria de massas acoplada a cromatografia líquida (LC-MS/MS). Após a identificação das proteínas foram utilizados os acessos das proteínas para a classificação em suas funções moleculares, processos biológicos e componentes celulares utilizando Blast2GO, posteriormente a análise de enriquecimento via String para mapear as

redes e interação entres proteínas caracterizadas suas respectivas funções no metabolismo de glicose e ácidos graxos. Dietas contendo blend de óleos essenciais associados à amilase exógena promoveram maior expressão de macromoléculas que participam da degradação de carboidratos pela via glicolítica e cetogênica. Foram identificadas 14 proteínas com maior expressão e presentes no tecido epitelial do rúmen envolvidas na oxidação da glicose, a proteína hidroximetilglutaril-CoA liase que catalisa parte do metabolismo metabólico intermediário, uma etapa fundamental na cetogênese. Nossos resultados sugerem que houve aumento da glicólise a partir da oxidação do gliceraldeído-3-fosfato, que participa da primeira etapa da produção de acetato e butirato e da descarboxilação oxidativa no epitélio ruminal de bovinos nelore confinados. A monensina melhora os precursores de propionato, a maior expressão de metilmalonil-CoA mutase sugere síntese de propionato via propionil-CoA que participa do ciclo do ácido cítrico através do succinil-CoA, que pode aumentar a energia metabolizável e reduzir a ingestão de alimentos.

Palavras-chave: 2D-PAGE, amido, aditivos, espectrometria de massas, proteômica

ABSTRACT

Few studies have investigated the proteome of cecum and rumen of cattle feeding different strategies with the use of additives. This proposal is a pioneer in the identification, expression and understanding of the ruminal and cecal protein profile of feedlot Nellore cattle. **Manuscript 1:** The objective was to evaluate protein expression in order to identify possible candidate molecules for biomarkers and their relationships with metabolic and nutritional functions applied to animals fed with different levels of starch (25, 35 and 45%) and additives (Monensin × blend of essential oils + exogenous amylase), the dosages of sodium monensin, blend of essential oils and exogenous amylase were 26, 90 and 560 mg/kg DM, respectively. Nellore bulls (n = 210) (*Bos taurus indicus*) (initial weight = ± 380 kg) were used. After slaughter, cecum samples were collected and immediately placed in liquid nitrogen. Samples were pooled using 90 animals (15 animals/treatment) in the protein pellet precipitation process. The separation and characterization of the proteome of biological samples were performed by two-dimensional electrophoresis (2D-PAGE) and identified by liquid chromatography coupled to mass spectrometry (LC-MS/MS). Polyacrylamide gels were analyzed using ImageMaster 2D Platinum 7.0 to verify differences in protein expression for the % volume normalized spot comparisons. After identifying the proteins, the metabolic pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) function, enrichment analysis of the Reactome pathway to map the expressions of proteins encoding enzymes and their respective functions in the affected pathways. The use of blend essential oil associated with α-Amylase as a feedd additive promoted greater expression of enzymes in the glycolysis and gluconeogenesis pathway and absence of protein linked to inflammation (Leukocyte elastase inhibitor). On the other hand, the increase in starch in the diets reduced enzymes linked to carbohydrate degradation with increased responses attributed to inflammatory injuries in the rumen of feedlot Nellore cattle. **Manuscript 2:** The objective was to map the proteome of the ruminal epithelium of feedlot Nellore cattle (n=60) with different levels of starch (Low: 25 and High: 45%) and additives (Monensin × blend of essential oils + exogenous amylase) the dosages of sodium monensin, blend of essential oils and exogenous amylase were 26, 90 and 560 mg/kg DM, respectively. Protein separations were performed using two-dimensional polyacrylamide gel electrophores (2D-PAGE), followed by identification by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). After protein identification, protein accessions were used to classify their molecular functions, biological processes and cellular component using Blast2GO, then enrichment analysis pathways String to map the networks and interaction

between proteins characterized and their respective functions in glucose and fatty acids metabolic. Diets containing blend of essential oils associated with exogenous amylase promoted greater expression of macromolecules that participate in carbohydrate degradation via glycolytic and ketogenic pathways. We identified 14 proteins with greater expression and present in the epithelial tissue of the rumen involved in glucose oxidation, the protein hydroxymethylglutaryl-CoA lyase that catalyzes part of intermediary metabolic metabolism, a fundamental step in ketogenesis. Our results suggest that there was an increase in glycolysis from the oxidation of glyceraldehyde-3-phosphate, which participates in the first stage of acetate and butyrate production and oxidative decarboxylation in the ruminal epithelium of feedlot Nellore cattle. Monensin improves propionate precursors, the increased expression of methylmalonyl-CoA mutase suggests propionate synthesis via propionyl-CoA that participates in the citric acid cycle through succinyl-CoA, which can increase metabolizable energy and reduce feed intake.

Keywords: 2D-PAGE, additives, starch, mass spectrometry, proteomic

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LISTA DE ABREVIATURAS

2D-PAGE	Eletroforese bidimensional em gel de poliacrilamida
3-PGDH	D-3-phosphoglycerate dehydrogenase
6PGL	6-phosphogluconolactonase
ADP	Adenosina difosfato
ALDOB	Fructose-bisphosphate aldolase
Alpha-ETF	Electron transfer flavoprotein subunit alpha
AGV	Ácido graxos voláteis
ANOVA	Análise de Variância
ATP	Adenosina Trifosfato
BEO	Blend Essential Oil
Ca	Cálcio
CAC	Citric Acid Cycle
CAPES	Coordenação de Aperfeiçoamento Pessoal de Nível Superior
CONCEA	Conselho Nacional de Controle de Experimentação Animal
CP	Crude Protein
DHAP	Dihydroxyacetone phosphate
DM	Dry Matter
DMI	Dry Matter Intake
DTT	1,4-dithiothreitol
ENO1	Alpha-enolase
ENO3	Beta-enolase
ESI-MS	Electrospray Ionization Mass Spectrometry
IEF	Focalização isoelétrica
IMS	Ingestão de Matéria Seca
IVD	Isovaleryl-CoA dehydrogenase
FDN	Fibra em Detergente Neutro
GA3P	Glyceraldehyde-3-phosphate (GA3P)
GAPD4	Glyceraldehyde-3-phosphate dehydrogenase
GO	Gene Ontology
GPI	Glucose-6-phosphate isomerase
HCl	Ácido Clorídrico
IBGE	Instituto Brasileiro de Geografia e Estatística
KDa	Quilodalton
LC – MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LDH-A	L-lactate dehydrogenase A chain
LRNS	Large Ruminant Nutritional System
MALDI	Matrix-Assisted Laser Desporption Ionization
MM	Massa Molecular
MON	Monensin
MS	Matéria Seca
NAOH	Cloreto de Sódio
NAD	Nicotinamida Adenina Dinucleotídeo
NADH	Nicotinamida Adenina Dinucleotídeo reduzido
NASCEM	National Academies of Sciences, Engineering and Medicine
NE	Net Energy
NDF	Neutral Detergent Fiber
NRBC	Nutrient Requirements of Beef Cattle
P	Phosphorus
PBS	Phosphate Buffered Saline

peNDF	Physically Effective Neutral Detergent Fiber
pH	Potencial Hidrogeniônico
PKM	Pyruvate Kinase
PMSF	Phenyl Methyl Sulfonyl Fluoride
SDS-PAGE	Second Dimension of the Electrophoretic Process
TIM	Triosephosphate isomerase
TPI	Triosephosphate isomerase
VFA	Volatile Fatty Acid
XR	L-xylulose reductase

SUMÁRIO

CAPÍTULO 1

CONSIDERAÇÕES INICIAIS

As atualidades e perspectivas da cadeia produtiva da carne nacional têm expressado enorme potencial econômico. O Brasil é determinante na produção de carne mundial, em 2021 as exportações totais do Brasil devem aumentar em 5% quando comparado ao ano anterior, sendo o décimo ano consecutivo de alta, o que expressa competitividade e relevância no mercado global (USDA, 2021) Nota-se que em 10 anos, o crescimento ponderal foi de 39% na série histórica das exportações (ABIEC, 2021). Em contrapartida ao crescimento da demanda, temos a necessidade de aperfeiçoamento do sistema de produção, logo, o uso de estratégias que busquem o encurtamento do período necessário para abate é eficaz em um mercado promissor como descrito.

A manipulação das dietas tem movido produtores e nutricionistas para busca de ferramentas que maximizem, encurtem o ciclo e tragam eficiência à cadeia produtiva. Preconiza-se então, abate de animais jovens, os quais são submetidos a dietas com alto concentrado. Ao passo que a nutrição caminha em busca de maximização de respostas produtivas, o limite fisiológico é testado, distúrbios metabólicos e limitações fisiológicas surgem como principais impedimentos aos altos níveis de amido em dietas para bovinos de corte.

Estratégias alimentares são adotadas para aumentar o metabolismo energético, principalmente em ruminantes com alta demanda energética, que confere maior sensibilidade a desordens metabólicas pelo excesso de fermentação de carboidratos. Majoritariamente, suprimento de energia dos ruminantes é obtido pela gliconeogênese hepática através de derivados da fermentação ruminal (propionato) e aminoácidos, porém há questionamentos quanto ao entendimento do aproveitamento a nível de intestino. O maior aporte de carboidratos altamente fermentescíveis é caracterizado por desordens digestivas em função do acúmulo de ácidos orgânicos, provocado pela assimetria entre a capacidade de absorção via lúmen ruminal e taxa de síntese microbiana (NAGARAJA; TITGEMEYER, 2010; NASCEM, 2016). Posteriormente, no intestino há limitações fisiológicas que devem ser ressaltadas principalmente em dietas com maiores níveis de amido, que aumenta o fluxo de glicose na forma de amido para sofrer digestão intestinal. Assim, falta clareza quanto à capacidade de digestão de enzimas pancreáticas, disponibilidade de transportadores lúmen-sistema aporta e possíveis alterações nos tecidos trazidos pelo aumento de carboidratos nas dietas.

Assim, a partir de estudos proteômicos é possível verificar a expressão de centenas de proteínas através do mapeamento do proteoma correspondente ao fenótipo do organismo analisado, em que esse fenótipo pode ser em função de diferentes estratégias alimentares ao

qual os animais foram submetidos. Em estudo constatado por Loor et al. (2015) observaram notável tendência no uso de ferramentas proteômicos integradas a biologia de sistemas, nutrição, fisiologia e metabolismo animal com o objetivo de elucidar como proteínas podem regular vias metabólicas, e como a síntese e degradação dessas macromoléculas afetam fenótipos complexos (nutrientes e/ou fatores dietéticos). Ao aprimorar mecanismos complexos por meio da proteômica, com possibilidade de identificar biomarcadores vinculados ao estado nutricional e doenças digestivas que a posteriori podem ser prevenidas e/ou evitadas, aumentando a eficiência de sistemas produtivos.

No estudo proposto por Campos et al. (2020) utilizaram a proteômica pela técnica 2D-PAGE (Gel de poliacrilamida) para caracterizar o perfil proteico de animais suplementados com vitamina A e os possíveis efeitos na supressão da deposição de gordura intramuscular. Assim nesse estudo foi possível identificar a expressão de proteínas do grupo HSP70 para os animais que não receberam a suplementação de vit. A, corroborando com a hipótese que o uso de vitaminas prejudica a expressão dessas proteínas que estão associadas ao metabolismo energético e melhora a captação de glicose pelo tecido para uso do carbono na síntese de gordura intramuscular. Foi identificada a proteína GAPDH para o grupo suplementado, essa é uma enzima glicolítica que demonstra que houve glicólise ao invés de metabolismo oxidativo e assim menor teor de gordura intramuscular.

Ferramentas bioquímicas tal como a proteômica são potencialmente aplicáveis à ciência animal, com o intuito de compreender processos biológicos, identificar perfil proteico e suas respectivas funções, permitem maior assertividade e inferência na nutrição e metabolismo animal. Assim, objetiva-se nessa proposta caracterizar proteínas do ceco e rúmen de bovinos nelore confinados submetidos a modificações dietéticas e possíveis vias metabólicas afetadas

1. REVISÃO BIBLIOGRÁFICA

1.1 Proteômica

Os organismos desempenham diversas atividades celulares continuamente, dado ao estímulo exógeno aplicado ao indivíduo. Logo, conhecer a expressão de um determinado proteoma permite considerar proteínas sub ou superexpressas e assim, inferir suas funções nos processos celulares e possíveis respostas ao ambiente (FRANÇOIS, 2010).

Estudos proteômicos permitem a identificação e caracterização do perfil proteico (quali e quantitativamente) e possíveis mudanças pós traducionais, sendo possível compreender como mecanismos moleculares alteram fenótipos complexos. A síntese proteica confere ampla possibilidade de proteínas sintetizadas por um mesmo genoma, logo, há complexidade na

síntese de transcritos até expressão proteica (NAABY-HANSEN; WATERFIELD; CRAMER, 2001; PRASAD et al., 2017). O genoma possui bases de DNA pré-definidas e representam o conjunto de genes de um organismo, já a proteômica possui representação funcional do genoma em função do ambiente ou fase de desenvolvimento do organismo.

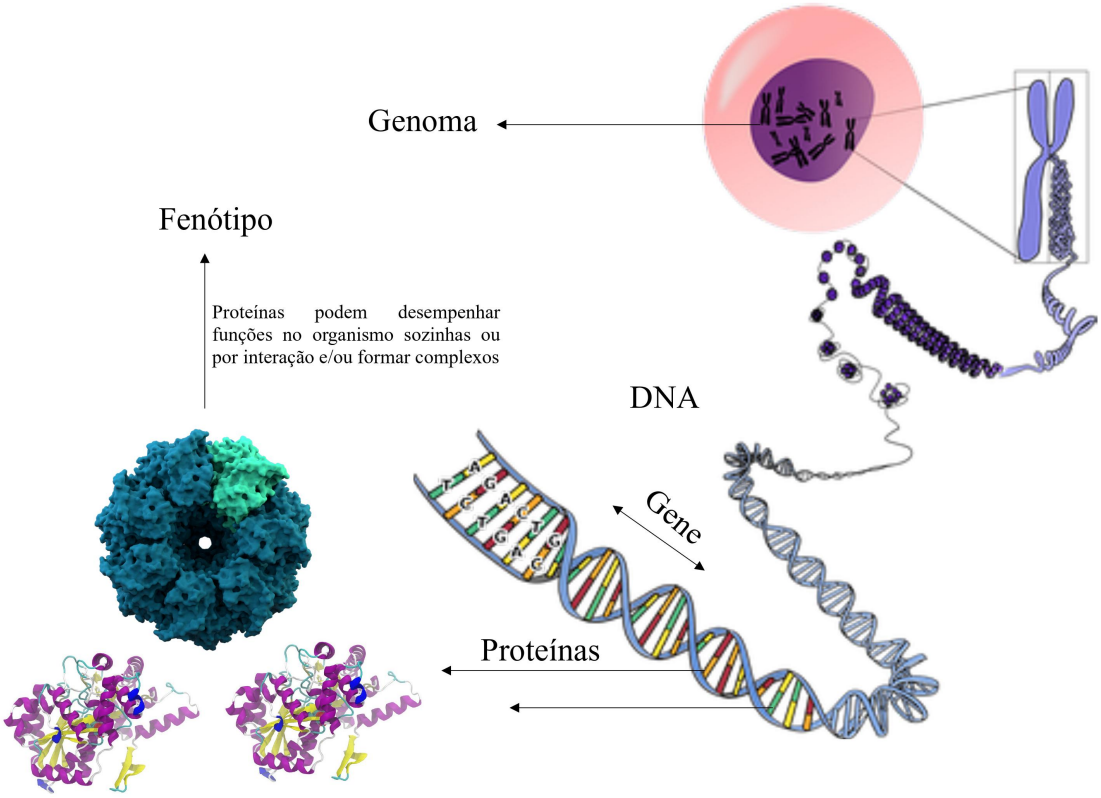

Figura 1. Representação esquemática da síntese proteica. Adaptado: www.pixabay.com.br/Google Imagens

O proteoma pode ser caracterizado como produto da expressão gênica, capaz de complementar as informações geradas pela síntese de nucleotídeos, que por si, é insuficiente na compreensão dos mecanismos de regulação proteicos, composição de organelas, interação proteína-proteína, proteólise e funções proteicas (PANDEY; MANN, 2000). Ao traçar esse perfil é provável identificar biomarcadores e o potencial entendimento de vias metabólicas e funções exercidas por um determinado grupo de proteínas dentro de um sistema biológico.

1.2 Eletroforese Bidimensional em gel de poliacrilamida (2D-PAGE)

As abordagens padrões que envolvem estudos proteômicos utilizam ferramentas como eletroforese, cromatografia líquida seguida de espectrometria de massas. A técnica eletroforese bidimensional é uma ferramenta analítica capaz de separar e quantificar em nível de proteínas

intactas, isoformas e modificações pós traducionais (ex.: fosforilação), baseada na focalização isoelétrica (IEF), que promove determinação simultânea de proteínas por ponto isoelétrico (pI) e/ou massa molecular (MM) em géis de poliacrilamida (O'FARRELL, 1975; SHEN et al., 2006).

O processo de separação e fracionamento das proteínas compreende duas etapas analíticas, primeira e segunda dimensão. Na primeira ou focalização isoelétrica (IEF) ocorre a separação de misturas de proteínas totais por pI, ou migração das proteínas até seus respectivos pH, isso devido a aplicação de um campo elétrico na fita contendo a solução proteica. As proteínas migram na faixa de pH da fita até que tenham cargas nulas, ou seja, quando o somatório de cargas parciais é igual a 0, assim as proteínas interrompem a migração no gel e determina-se o pI (O'FARRELL, 1975). Na etapa seguinte (SDS-PAGE), utiliza-se o surfactante aniônico (SDS) (carga negativa) capaz de romper possíveis ligações não covalentes, desdobrar e impossibilitar a reestruturação para formas superiores (terciárias e quaternárias) tornando-se estruturas lineares. SDS tem função de ligar-se a cargas positivas das proteínas e revestir efetivamente para carga negativa, fazendo com que as proteínas migrem na malha do gel de poliacrilamida até o polo positivo. Posteriormente ocorre o fracionamento das proteínas de acordo com a massa molecular, através de uma corrida eletroforética em gel de poliacrilamida.

O gel bidimensional é analisado conforme sua expressão proteica, para isso necessita-se de fases como digitalização, detecção e análise de imagem dos *spots*, esses são alinhados e para análise estatística utiliza-se % volume normalizado, intensidade e os respectivos pI e MM. Assim, utilizam-se ferramentas bioinformáticas (Software) aptos a verificar variações na expressão proteica de *spots* sob o gel de poliacrilamida. Considerando a análise proteômica por meio da eletroforese bidimensional, após a identificação dos *spots* e digestão tríptica (clivagem das extremidades de Lisina e Arginina), eluição dos peptídeos em misturas complexas, as soluções contendo os peptídeos podem ser caracterizados por espectrometria de massas.

1.3 Espectrometria de massas (MS)

Espectrometria de massas é uma técnica analítica capaz de determinar massa de moléculas presentes na amostra que são convertidas em íons em fase gasosa, pela fragmentação de íons (DE HOFFMANN, 2005), em seguida são separados no espectrômetro de massas de acordo sua relação massa carga sobre a carga (*m/z*). A cromatografia pode ser combinada para obter as vantagens da cromatografia (alta seletividade e eficiência de separação) com as vantagens da espectrometria de massas (obtenção de informação estrutural, massa molar e

aumento adicional da seletividade) (CHIARADIA et al., 2008). As técnicas cromatográficas mais comumente acopladas à espectrometria de massas são a cromatografia gasosa (GC) e a cromatografia líquida de alta eficiência (LC).

Dados obtidos pela espectrometria são buscados em banco de dados de sequência de aminoácidos por logaritmos de pesquisa, os peptídeos identificados recebem score, e com a combinação desses montam-se as listas de proteínas identificadas. Utilizam-se diferentes fontes de ionização aplicadas a proteômica, seja ESI (*Electrospray*) ou MALDI (*Matrix-Assisted Laser Desporption Ionization*), ambas, com função de ionizar e transferir espécies para fase gasosa (CANTÚ et al., 2008). Na técnica ESI, as amostras são dissolvidas em solução tampão ou solvente que, por conseguinte são dispensadas em gotas micrométricas com alta voltagem, assim, formam uma nuvem gasosa que transmitem íons carregados positivamente ou negativamente ao analito, logo a intensidade do espectro é proporcional a massa (GASKELL, 1997). Tal ferramenta gera um conjunto de dados, que necessitam de avaliações conclusivas, que devido a grandeza e complexidade é necessário extrair informações biológicas relevantes (HAOUDI & BENSMAIL, 2006).

1.4 Bioinformática e análise de dados proteômicos

Bioinformática engloba pesquisa, desenvolvimento ou aplicação de ferramentas computacionais em abordagens biológicas para incluir, analisar e facilitar a visualização de dados. Assim, essa análise baseia-se no uso de programas computacionais capaz de explorar informações a partir das sequências de nucleotídeos e/ou aminoácidos.

A partir de um formato FASTA contendo as sequencias supracitadas, é possível criar uma lista de proteínas resultante do experimento realizado por meio de uma biblioteca de dados. Utiliza-se então software de análise funcional a partir das mesmas sequências, para assim categorizar os dados genômicos em classes funcionais (CONESA et al., 2005). Para isso, o software compara as sequências fornecidas com uma biblioteca funcional padrão, conhecida como Gene Ontology, e posteriormente é gerado anotações que podem ser apresentados na forma de gráfico, com distribuição das proteínas em seus respectivos processos biológicos, funções moleculares e componentes celulares (HEMANDEZL et al., 2006), complementar a isso, analises de enriquecimento de dados são incorporadas, como redes de interação molecular, análises hierárquicas, vias metabólicas e etc.

1.5 Digestão e absorção de carboidratos

Os polissacarídeos presentes nas plantas são responsáveis por fornecer a maioria do aporte de energia requerida pelos ruminantes, seja parede celular ou conteúdo celular vegetal. Assim, o conteúdo celular é composto de amido, açucares solúveis e frutosanas, sendo esses, solúveis em água, já a parede celular é composta por porções solúveis e insolúveis em água; a fração insolúvel, chamada de FDN é composta por hemicelulose, celulose e lignina e pequenas fragmentos de nitrogênio e amido que podem ser encontrados caso não seja aplicado nenhum tratamento com amilase. O FDN não inclui a pectina, que está presente na parede celular, porém compõe a fração solúvel (Hall, 2003), como demonstrado na Figura 1.

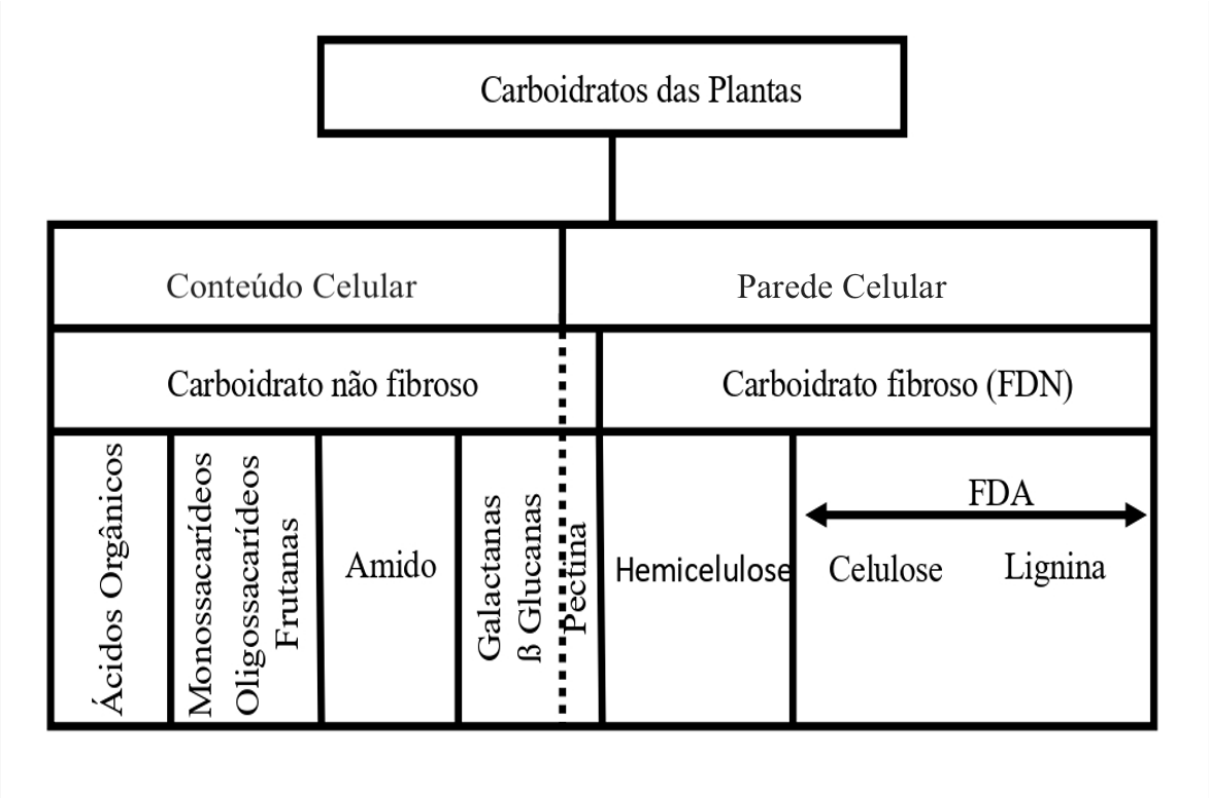

Figura 2. Fração dos carboidratos para ruminantes (Adaptado de Hall (2003) e NASCEM (2016).

No rúmen, essas macromoléculas são degradadas por enzimas associadas a membrana celular dos microorganismos e no citoplasma degradados e metabolizados em piruvato e por conseguinte, em ácidos graxos voláteis (AGV) para formar a maior fonte de energia dos ruminantes (KOSLOSKI, 2011). A fermentação anaeróbica ruminal dispõe formação de ácidos fracos (ácidos graxos voláteis) e ácido lático, que em condições normais de pH (6,8) apresentam relação de 100:1 para duas formas, ionizada:protonada. A alteração do potencial hidrogeniônico promove seletividade no ecossistema microbiano, que por sua vez, regula produtos (ácidos orgânicos) da fermentação de hexoses (glicose), ao observar aumento das proporções molares

para propionato e butirato (HUNTINGTON, 1997; PENNER et al., 2009, 2011; ALLEN, 2010; KOZLOSKI, 2011). Assim, a fração do carboidrato (solúvel ou insolúvel) é determinante para atender as exigências energéticas dos bovinos, pois para cada ácido orgânico sintetizado pelos microorganismos ruminais é observada diferente estequiometria da conversão de um mol de glicose.

As rações de bovinos confinados são compostas principalmente por amido como a maior fração dos carboidratos, podendo exceder 50% da matéria seca nas dietas. A saliva dos ruminantes não contém amilase, logo, o amido é degradado inicialmente pelo efeito enzimático resultante de enzimas bacterianas e posteriormente colonização dos grânulos por bactérias amilolíticas e protozoários (NOZIÈRE et al., 2010).

A digestibilidade total do amido tende ser uniforme devido à digestão compensatória no intestino, embora o local de digestão do amido possa ter um efeito profundo no perfil dos substratos absorvidos do trato digestivo dos ruminantes (OWENS; ZINN; KIM, 1986; HUHTANEN; SVEINBJÖRNSSON, 2006). A eficiência de utilização de amido para produção de energia absorvida se dá pela hidrólise no rúmen que fornece energia para síntese de proteína microbiana e produção de ácidos graxos voláteis (AGVs), do qual, apenas o propionato gera biossíntese de glicose no fígado, diferentemente da digestão intestinal que fornece glicose ao sistema aporta (OWENS; ZINN; KIM, 1986; REYNOLDS et al., 2001; REYNOLDS, 2006).

A digestão do amido no intestino delgado pode ter potenciais benefícios, principalmente pelo fornecimento eficiente de energia, e diminuição de incidências de timpanismo, acidose, e laminite (RÉMOND et al., 2004). Nesse caso, ocorre hidrólise de moléculas percursoras da glicose que podem ser diretamente absorvidas pelo sistema porta hepática. Enzimas que atuam na digestão intestinal sofrem efeito da capacidade de tamponamento da digesta abomasal, em que, na porção anterior do duodeno ocorre secreção de bile e secreções pancreáticas que contém bicarbonato, com efeito de neutralização do ácido clorídrico. Logo, o aumento de pH a níveis neutros ou levemente alcalinos favorecem atuação das enzimas amilolíticas secretadas pela mucosa intestinal e pâncreas (HARMON; YAMKA; ELAM, 2004; NASCEM, 2016; MILLS et al., 2017).

A digestão duodenal contribui com cerca de 88% da digestão pós rúmen (SUTTON; REYNOLDS, 2011), assim, a capacidade de secreção de enzimas amilolíticas maximizam consideravelmente o aproveitamento de amido. Considerando a maior digestão de amido intestinal, preconiza-se o aumento glicose hepática e aproveitamento através da glicogênese. Porém o efeito da secreção pancreática foi postulado como modelo inversamente linear (HARMON, 1993), ou seja, aumento do fluxo de amido ou glicose duodenal tende reduzir

secreção pancreática e, por conseguinte decréscimo na hidrólise do amido. Verifica-se grande acúmulo de maltase, maltotrioses, e açucares simples no íleo, corroborando com inexistência de maltase e maltotriases as dietas ricas em amido (NASCEM, 2016). A infusão de glicose ou aumento do fluxo duodenal de amido promove declínio da absorção de glicose via veia porta hepática, tal efeito é atribuído à redução da digestibilidade do amido pelo decréscimo do tempo de retenção do amido junto a menor atividade enzimática (WEISS; STEINBERG; ENGSTROM, 2011; MILLS et al., 2017; WESTREICHER-KRISTEN et al., 2018). Outro fator importante é a baixa produção de transportadores dependentes de sódio *SLGT1,* que conduzem glicose do lúmen ao sistema aporta, esses, não mostram adaptação a dietas ricas em amido e necessitam de gasto energético (NASCEM, 2016).

1.6 Aditivos alimentares

A incorporação de aditivos em dietas para ruminantes preconiza alteração de perfil fermentativo e aproveitamento de alimentos no trato gastrintestinal com efeitos no metabolismo pós-absortivo (NAGARAJA et al., 1997; NASCEM, 2016), seja com intuito de prevenção de desordens digestivas e/ou aumento da eficiência alimentar. Parte da energia consumida pelos ruminantes pode ser perdida na forma de metano, o uso de aditivos além de atuar em bactérias fermentadoras de metano, modula a estequiometria de AGVs, favorecendo a produção de propionato, que por sua vez, reduz a formação de metano ao utilizar hidrogênio do meio ruminal. Além de atuar como efeito bacteriostático sobre bactérias ruminais gram positivas, mas com possível impacto de resíduos em produtos de origem animal e resistência microbiana. Sendo assim, recentemente alguns estudos demonstram que a combinação de *blend* de óleos essenciais com amilases exógenas promovem respostas produtivas semelhantes ao uso de monensina (MESCHIATTI et al., 2019) sem oferecer possíveis riscos a saúde humana, pois há preocupação com a resistência antimicrobiana e resíduos em produtos de origem animal (KHIAOSA-ARD & ZEBELI., 2013).

1.6.1 Monensina

Aditivos como ionóforos são poliésteres carboxílicos com efeito tóxico para os microrganismos ruminais, esse mecanismo de ação se dá pela ligação aos cátions e facilidade de transporte através da membrana celular (NASCEM, 2016). Os principais efeitos disso, é a alteração do potencial elétrico e gradiente de pH intracelular, característico por importação de solutos. Ionóforos foram implementados em dietas para animais confinados pela indústria

americana, em sua maioria, 77% dos nutricionistas americanos optam pela utilização de monensina em relação a outros ionóforos em dietas com nível de grãos (SAMUELSON et al., 2016), e no Brasil o percentual é cerca de 86% que utilizam ionóforo na dietas de confinamento (incluindo monensina, lasalocida, salinomicina, e virginiamicina) , como descrito por Pinto & Millen (2018). Os efeitos do uso de monensina em animais alimentados com alto grão é redução de bactérias fermentadoras de ácido lático (principal causador de acidose ruminal), captura de energia que seria desprendida para formação de metano e aumento da proporção molar de propionato, por conseguinte, aumento de energia metabolizável (NASCEM, 2016). A recomendação de monensina preconizada pelo Nutrient Requirements of Beef Cattle, é de 28 mg/kg MS, com aumento de 2,3% de energia metabolizável, redução de 4% na IMS de bovinos confinados (NASCEM, 2016).

1.6.2 Blend de Óleos Essenciais e α-Amilase

Óleos essenciais são metabolitos oriundos das plantas, os quais são obtidos por destilações a vapor e representam a fração volátil dos compostos de plantas (NASCEM, 2016), a mistura de vários componentes confere ao Blend, capacidade de manipulação da fermentação ruminal, por ter efeito antibacteriano e reduzir a digestibilidade de proteína no rúmen (MCINSTOSH et al., 2003), que aumenta a proteína metabolizável por garantir que parte da proteína dietética não sofra ação ruminal. Embora o efeito antimicrobiano seja conhecido, os óleos essenciais tem diversos compostos que podem ser extraídos de plantas, isso faz com que o mecanismo exato pelo qual esses compostos afetam a fermentação ruminal não seja totalmente conhecido (MEYER et al., 2009, NASCEM, 2016).

Enzimas exógenas possibilitam maior hidrólise de frações dietéticas com incremento na eficiência alimentar, *α*-amilase pode atuar na hidrólise do amido em oligossacarídeos que podem ser aproveitados pelo metabolismo de bactérias amilolíticas (TOSETI et al., 2020). Assim, a combinação desses aditivos alimentares visa otimizar o aproveitamento do amido, com o potencial de aumentar a síntese de proteína microbiana, ganhos no desempenho e na carcaça, além de redução de abscessos hepáticos e amido fecal em dietas com altos teores de amido quando comparado ao uso de monensina (GOUVEA et al., 2019; MESCHIATTI et al., 2019; TOSELI et al., 2020).

1.7 Utilização de amido em bovinos de corte

As dietas brasileiras destinadas a bovinos confinados comprovam a grande utilização de grãos, ao verificar o cenário atual sobre recomendações utilizadas por consultores brasileiros

(responsáveis por 4.228.254 animais), (PINTO; MILLEN, 2018) constataram que 51,5% e 33,3% destes adicionam grãos de 510-650 e acima de 660 g/kg MS da dieta, respectivamente. No mesmo estudo verifica-se que, o milho é a fonte energética predominante utilizada, do qual, o amido representa cerca de 75% dos valores energéticos (FERRARETTO; CRUMP; SHAVER, 2013). Entretanto, verifica-se variação na digestibilidade e absorção do amido a partir de fatores fisiológicas e dietéticos.

No trato gastrintestinal dos bovinos aplica-se efeito digestivo complementar, ou seja, o amido que escapa da degradação ruminal é pretendido a digestão e absorção intestinal. Alguns fatores como tempo de exposição para hidrólise, acesso de enzimas aos grânulos de amido, capacidade de absorção de glicose e atividade de enzimas hidrolíticas (OWENS; ZINN; KIM, 1986; HUNTINGTON, 1997), e fatores dietéticos como tipo de amido, processamento e possíveis interações entre componentes da dieta (MILLS et al., 2017) são fatores que manipulam digestão e aproveitamento do amido ao longo do trato gastrointestinal.

2. JUSTIFICATIVA E OBJETIVO

Essa proposta compõe a identificação, expressão e compreensão do perfil proteico ruminal e cecal de bovinos confinados com diferentes níveis de amido e aditivos, e suas possíveis associações à nutrição e metabolismo animal. Afim de responder os seguintes questionamentos. Quais alterações as dietas convencionais de confinamento podem trazer ao proteoma bovino? O que muda na síntese proteica nos tecidos de rúmen e ceco?

O Capítulo II, intitulado: **“Feedlot diets containing increasing starch levels and different feed additives changes cecal proteome profile involved on energy metabolism and inflammatory response of Nellore cattle”,** foi submetido a revisão por pares de acordo com as normas editoriais do periódico **Scientific Reports**, sob responsabilidade editorial do grupo Nature. O objetivo desse estudo é mapear o proteoma do ceco de bovinos confinados alimentados com diferentes níveis de amido (25, 35, 45 %) e aditivos (Monensina × *blend* de óleos essências + enzima exógena)**,** e elucidar como a expressão proteica atua no metabolismo energético e respostas inflamatórias no trato digestivo em função das diferentes estratégias nutricionais.

O Capítulo III intitulado de “**Protein profiles identified by LC MS / MS demonstrate change in beta oxidation, ketogenesis and propionate metabolism in rumen epithelium with different additives”,** o manuscrito está de acordo com as normas editoriais do periódico **Scientific Reports**, sob responsabilidade editorial do grupo Nature. O Objetivo desse estudo é caracterizar o perfil de proteínas expressas no epitélio ruminal de bovinos Nelore confinados

submetidos a níveis de amido (25 x 45 %) aditivos (Monensina × blend de óleos essências + enzima exógena) e traçar funcionalidade do perfil encontrado com o metabolismo animal.

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CAPÍTULO 2

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

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

Abstract

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 analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and then differentially expressed protein spots were identified by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). The use of blends of essential oil associated with α-amylase as a feed additive promoted upregulation of enzymes in pathways such as glycolysis and gluconeogenesis and the absence of proteins related to inflammation process (Leukocyte elastase inhibitor) in cecum tissues. However, the increase in starch in the diets promoted a downregulation in enzymes linked to carbohydrate degradation, probably caused by damage to the cecum epithelium due to increased responses linked to inflammatory injuries.

Keywords: blend essential oils, exogenous α-Amylase, glucose, mass spectrometry, monensin, proteomics

1. Introduction

Among the limitations to enhance meat production is the large energy requirement, which means that feedlot diets have a higher net energy demand (NE) (Brake and Swanson,

2018). Thus, increased starch in the diet, 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 (VFA) and lactate (Allen et al., 2009) which are metabolized in the liver and provide the greatest source of energy for ruminants (Lozano et al., 2000; Reynolds and Maltby, 1994). 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 (NASCEM, 2016). Thus, feed additives that decrease harmful processes of ruminal fermentation are employed, such as sodium monensin, which is a polyester carboxylic ionophore used in growth and finishing diets (NASCEM, 2016). 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 (Silva et al., 2020). Alternative additives have shown the potential to replace monensin, such as blends of essential oil associated with the exogenous enzyme α-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 (Meschiatti et al., 2019; Meyer et al., 2009; Toseti et al., 2020).

With high levels of starch in the diet, the rate of passage and post-ruminal digestion increases (NASCEM, 2016). The rumen microbiota can digest around 70–80% of the starch consumed (Harmon et al., 2004; Huntington et al., 2006; Moharrery et al., 2014; NASCEM, 2016; Westreicher-Kristen et al., 2018), however, the digestion and absorption of post rumen starch are partially impaired, as enzymatic digestion by pancreatic α-amylase in the duodenum is limited in the small intestine (Huntington, 1997; Owens et al., 1986). 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 (Lohrenz et al., 2011; Mills et al., 2017; NASCEM, 2016), 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 (Pinto and Millen, 2018) can cause excessive fermentation in the cecum, thereby contributing to the metabolizable energy of ruminants (Hoover, 1978; Siciliano-Jones and Murphy, 1989), however, high grain diets can result in hindgut acidosis it may generate inflammatory reactions in the cecal epithelium. Large amounts of starch in the cecum may contribute to the fermentation of VFA, NH_3 and lactic acid combined with a decreased pH (NASCEM, 2016), besides that, this compartment has limited buffering capacity as compared to rumen, where saliva and protozoa modulate pH fluctuations (Sanz-Fernandez et al., 2020). 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. In most studies with feed additives, it focuses only on the rumen effects, however it is necessary to understand what the effect of feed additives on post rumen.

Therefore, it is important to understand how the digestion and absorption sites act when high proportions of starch are included in feedlot diets. 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 feedlot diets.

2. Material and Methods

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 São Paulo State University – UNESP, Botucatu – SP, under protocol n° 0107/2019.

2.1 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 m^2 of area/animal 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, (Fox et al., 2004)), level 2, meeting the nutritional requirements for daily weight gain between 1.5 and 1.7 kg.day.animal^{-1}. Animals were fed for 92 days and diets were offered *ad libitum* twice a day (8 a. m. and 3 p.m.). 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[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	**355.0**	209.5	372.8	**458.0**
NE Mcal/kg DM[10]	2.4	2.4	2.4	2.4	2.5	2.6	2.4	2.6	2.7

[1]Adap 1 = Adaptation 1, 0-7 days; [2]Adap 2 = Adaptation 2, 7 -14days, 14-92 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]Calcium (Ca); [9]Phosphor (P); [10]Net energy (NE). Monensin: 26 ppm. Blend Essential Oil: 90 mg kg^{-1} of dry matter. α-Amylase: 560 mg kg^{-1} of dry matter.

2.2 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 essential oils (CRINA RUMINANTS®; DSM Nutritional products, Basel, Switzerland) containing thymol, eugenol, limonene and vanillin (McIntosh et al., 2003), 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 and 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:

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

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.

2.3 Sample Collection and preparation

The animals were transported to a commercial slaughterhouse where they were stunned by brain concussion using a captive dart gun, followed by bleeding hide removal and evisceration 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 (pen = 5) were considered, i.e., 15 animals/group or 90 animals totals (15 animals x 6 groups).

2.4 Extraction, precipitation and quantification of proteins

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 was collected and 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 proportion 1:2 of supernatant: acetone. The samples were stored at 2°C for 1.5 hours and then centrifuged at 14,000 rpm for 30 min; the supernatant was discarded and the protein pellet was solubilized in 1 mL of 0.50 mol L^{-1} NaOH (Merck, Darmstadt, Germany) only for the quantification of proteins was used. Protein concentrations were determined by the Biuret method (Doumas et al., 1981) 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}.

2.5 Electrophoretic separations of protein fractions using 2D-PAGE

For isoelectric focusing, about 375 µg of proteins from each group was applied to their respective 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 of Dithiothreitol (USB, Cleveland, Ohio, United States). Approximately 900 µL of mineral oil was added at room temperature for 12 hours to rehydrate the strips, prevent evaporation and urea crystals. After this period, the strips were added to the Ettan™ IPGphor™ 3 isoelectric focusing system (IEF) (GE Healthcare, Uppsala, Sweden). The electrical voltage used was established by the protocol described by Braga et al. (2015) to separate the proteins according to their isoeletric point. At the end of focusing, the strip was balanced in two stages of 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 (Neves et al., 2012; Santos et al., 2011). 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 proceed.

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 molecular mass standard (GE Healthcare, Uppsala, Sweden), with proteins of different molecular 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 run 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 fix solution for 30 minutes containing 10% acetic acid (v/v) and 40% ethanol (v/v); and then the proteins were revealed using the colloidal Coomassie G-250 (USB, Cleveland, Ohio,

United States) for 72 h and then removed by washing with ultrapure water (Moraes et al., 2012; Neves et al., 2012; Santos et al., 2011; Silva et al., 2013).

The gels obtained (supplemental Figure 1) 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 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 (Moraes et al., 2012; Neves et al., 2012; Santos et al., 2011; Silva et al., 2013).

2.6 Protein identification by mass spectrometry (LC- MS/ MS)

The protein *spots* were characterized by LC-MS/MS after being subjected to tryptic digestion and the elution of peptides according to the methodology described by Shevchenko et al. (2006) and Braga et al. (2015). The aliquots of the solutions containing the elute peptides were analyzed to obtain the mass spectra through the nanoAcquity UPLC system coupled to the Xevo G2 QTof mass spectrometer (Waters, Milford, MA, United States). The identification of proteins was performed by searching in database UniProt (www.uniprot.org) with the *Bos taurus* species. Proteins were considered depending on the respective theoretical and experimental isoelectric point and molecular mass, and for their scores (>60). After the identification of FASTA sequences the proteins, their sequences were analyzed by software OMICSBOX (BLAST2GO) (Conesa et al., 2005) and thus categorized by their molecular function, biological processes and cellular component with Gene Ontology (GO).

2.7 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*. Only proteins with significantly altered levels were excised for identification by MS. 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, test t) considering significance to determine the differentially expressed protein *spots* log2 FC values (t test, P value <0.05).

Following the average mode of background subtraction, individual spot intensity volume was normalized with total intensity volume (summation of the intensity volumes obtained from all *spots* in the same 2-DE gel). The normalized intensity volume values of individual protein *spots* were then used to determine differential protein expression among experimental groups.

2.8 Pathways enrichment analysis

The same KEGG-IDs were used to analyze metabolic pathways using the Kyoto Encyclopedia of Genes and Genomes function (KEGG pathways) and Reactome pathway enrichment analysis yielded similar results with on specific pathways affected, making it possible to map the expressions of proteins encoding enzymes found in the database.

3. Results

3.1 Image analysis and protein 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. 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

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

3.2 Proteins characterization by LC-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, 12 were classified as functional for the purpose of the study, which involve energy metabolism and inflammatory responses. Table 3 shows the differentially expressed protein profile in the cecum of Nellore beef cattle under different levels of starch and feed additives.

Table 0-3. Protein profile differentially expressed in Nellore cattle cecum fed with diets containing increasing starch levels (25, 35, and 45%) and additives (Monensin, Blend of essential oil + exogenous α-Amylase) using LC-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
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
ATP synthase subunit beta_ mitochondrial	P00829	533.0471	5.15/56283.53	5.49/47920
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 seven enzymes participating in the steps of the glycolysis and gluconeogenesis pathway was verified, such as: Triosephosphate isomerase (Step 1); Phosphoglycerate mutase (Step 2); Alpha-enolase (ENO1), Beta-enolase (ENO3) and Fructose-bisphosphate aldolase (ALDOB) (Step 4); Pyruvate Kinase (PKM) (Step 5) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). There was expression of three enzymes linked to pyruvate metabolism or 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 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 (UniProt, 2020).

3.3 Pathways enrichment and Reactome analysis

Using pathways enrichment and reactome analysis yielded similar results showed specific pathways affected. Differential expression found in all groups showed great observations. Metabolism of carbohydrates, pyruvate metabolism, Citric Acid (TCA) Cycle, respiratory electron transport, innate immune system and immune system were impacted in cecum tissues by different feeding strategies (Figure 1). Data statistics of Reactome Pathway Analysis have been provided in Supplemental Table 1.

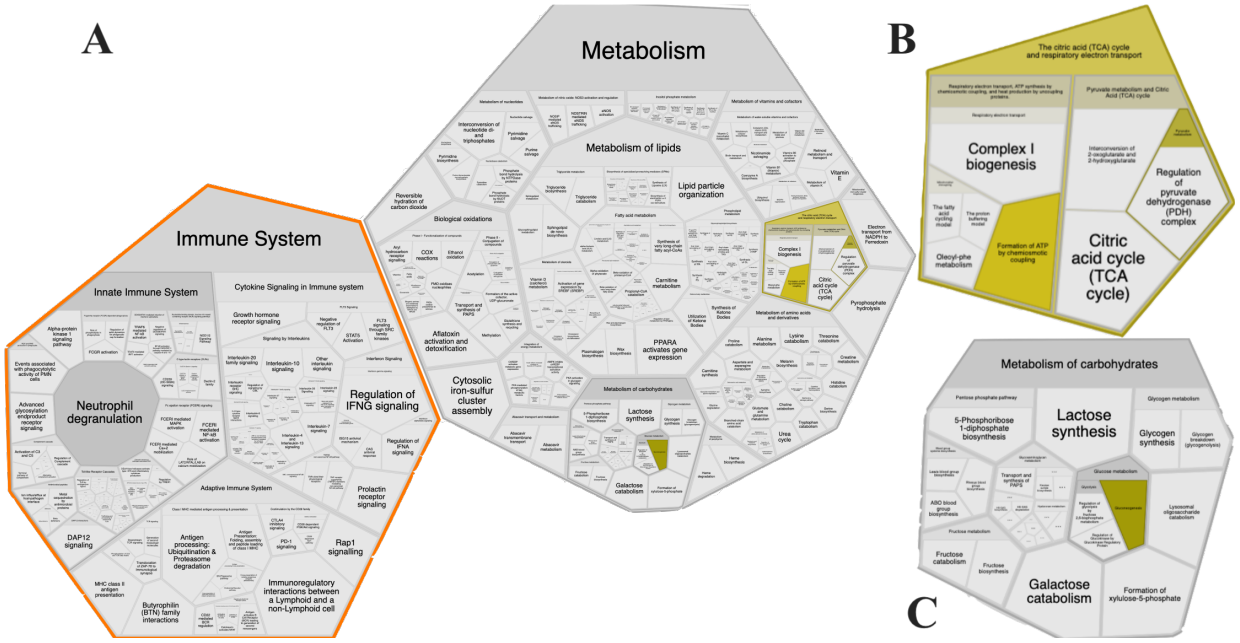

Figure 1. Affected pathways generated from KEGG ID input show that metabolism of carbohydrates, glycolysis, gluconeogenesis and immune system is impacted

Additionally, differential expression indicates similar encoding enzymes in glycolysis and gluconeogenesis pathways (Figure 2).

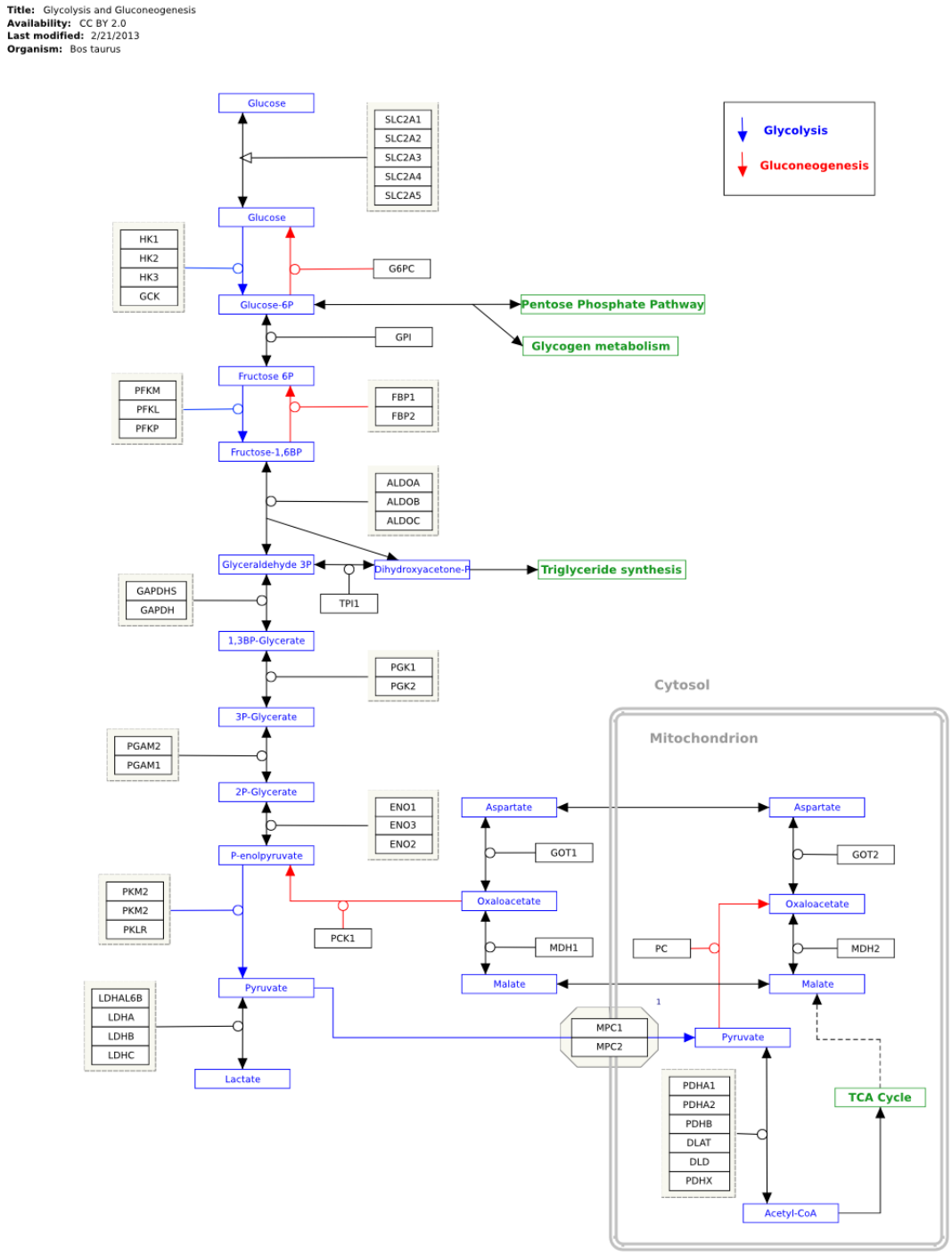

Figure 2. Expression protein profile encoding enzymes in glycolysis and gluconeogenesis pathway. KEGG key: EC 4.1.2.13: Fructose-bisphosphate aldolase (ALDOB); EC 5.3.1.1: Triosephosphate isomerase (TPI); EC 1.2.1.12: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH); EC 5.4.2.4: Phosphoglycerate mutase (PGAM); Alpha-enolase (ENO1); EC 4.2.1.11 Beta-enolase (ENO3); EC 2.7.1.40 Pyruvate Kinase (PKM); EC 1.1.1.27 L-lactate dehydrogenase (LDH)

4. Discussion

4.1. 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 (Figure 3), 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 play functions in energy metabolism.

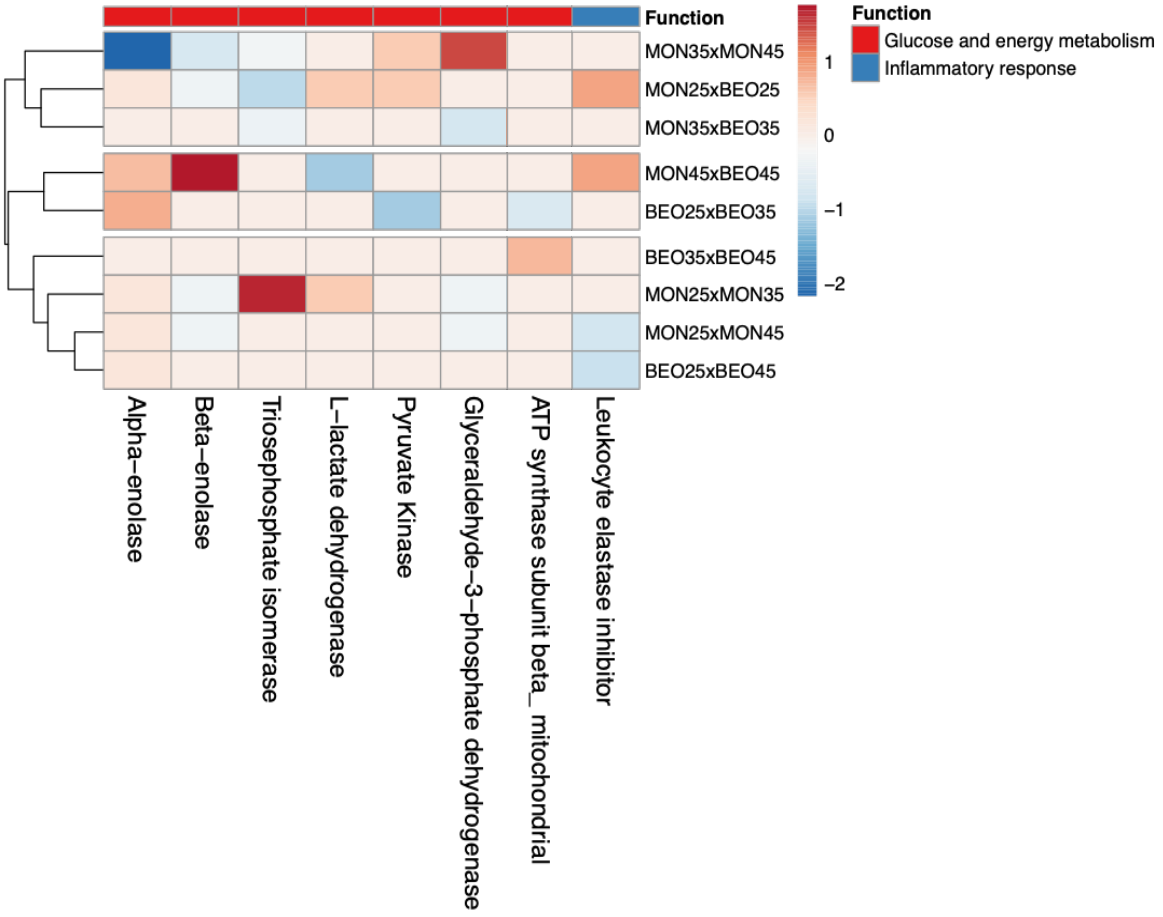

Figure 3. Heatmap of the differentially expressed proteins (ANOVA, P≤ 0.05) among the diets contending different starch levels and additives. Color-coded matrix showed the correlation coefficient of the *spots* expression values. Each row and column represent one group and protein, respective

Table 4. Expression values (test t, $P \leq 0.05$) in Nellore 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	35	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 L-lactate dehydrogenase A chain	∅/+	NS	NS	NS	NS	NS	NS	NS	NS
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
Glyceraldehyde-3-phosphate dehydrogenase	NS	-1,49	NS	∅/+	+/∅	∅/+	NS	NS	NS
ATP synthase subunit beta_ mitochondrial	NS	NS	NS	NS	NS	NS	∅/+	+/∅	NS
Inflammatory response									
Leukocyte elastase inhibitor	+/∅	NS	+/∅	NS	NS	-1,22	NS	NS	-1,29

The values are presented in the form log2FC (Fold Change) calculated in relation to the type of additives used, and subsequently the level of starch with the respective additives. NS: Not significant; +/∅: *spot* present in the first group in relation to the second; ∅/+: *spot* absent in the first group in relation to the second.

The animals fed with low starch in their diet (25%) associated with BEOα increased expression of the protein 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; all 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, thus, it may have a greater effect on post-rumen. In a similar study, Toseti et al. (2020) observed reduction fecal starch using the BEOα, suggesting a greater degradation of carbohydrates, because feed enzymes can remain active in the intestine and benefit the digestion of nutrients that escape rumen fermentation (Hristov et al., 1998). As demonstrated by Thomas et al. (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 ionophore 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. Higher concentrations of carbohydrates in the intestine together with the lesser effects of monensin may contribute to a greater accumulation of organic acids, in addition to that, the intestinal epithelium is much more vulnerable to the pH variation as compared to rumen (Sanz-Fernandez et al., 2020; Steele et al.,

2016), corroborating with this work that demonstrates the identification of proteins linked to immune responses. 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 increased epithelial injury (indicative damage)(Sanz-Fernandez et al., 2020; Tao et al., 2014), and upregulation of inflammatory, and subsequently to the lower expression of proteins associated with energy metabolism.

In view of the different feeding strategies, key enzymes were identified in the degradation of carbohydrates in the large intestine of cattle (Figure 2). 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 enzyme Glyceraldehyde-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 (UniProt, 2020). 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 (UniProt, 2020).

4.2. 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 (Choi et al., 2019) that consolidate the results of the above-mentioned authors, who reported less effect of ionophores in the hindgut. 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 diet (Chang et al., 2019; Liu et al., 2014). As well as, it was observed in this study, lower expression of proteins that participate in the energy metabolism when using higher levels of starch and thus can cause damage to the epithelium of the cecum.

5. 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 increased the expression of inflammatory responses and reduced the expression of proteins involved in energy metabolism probably caused by damage to the cecum epithelium.

Declaration of Competing Interest

Authors declare that have no conflict of interest.

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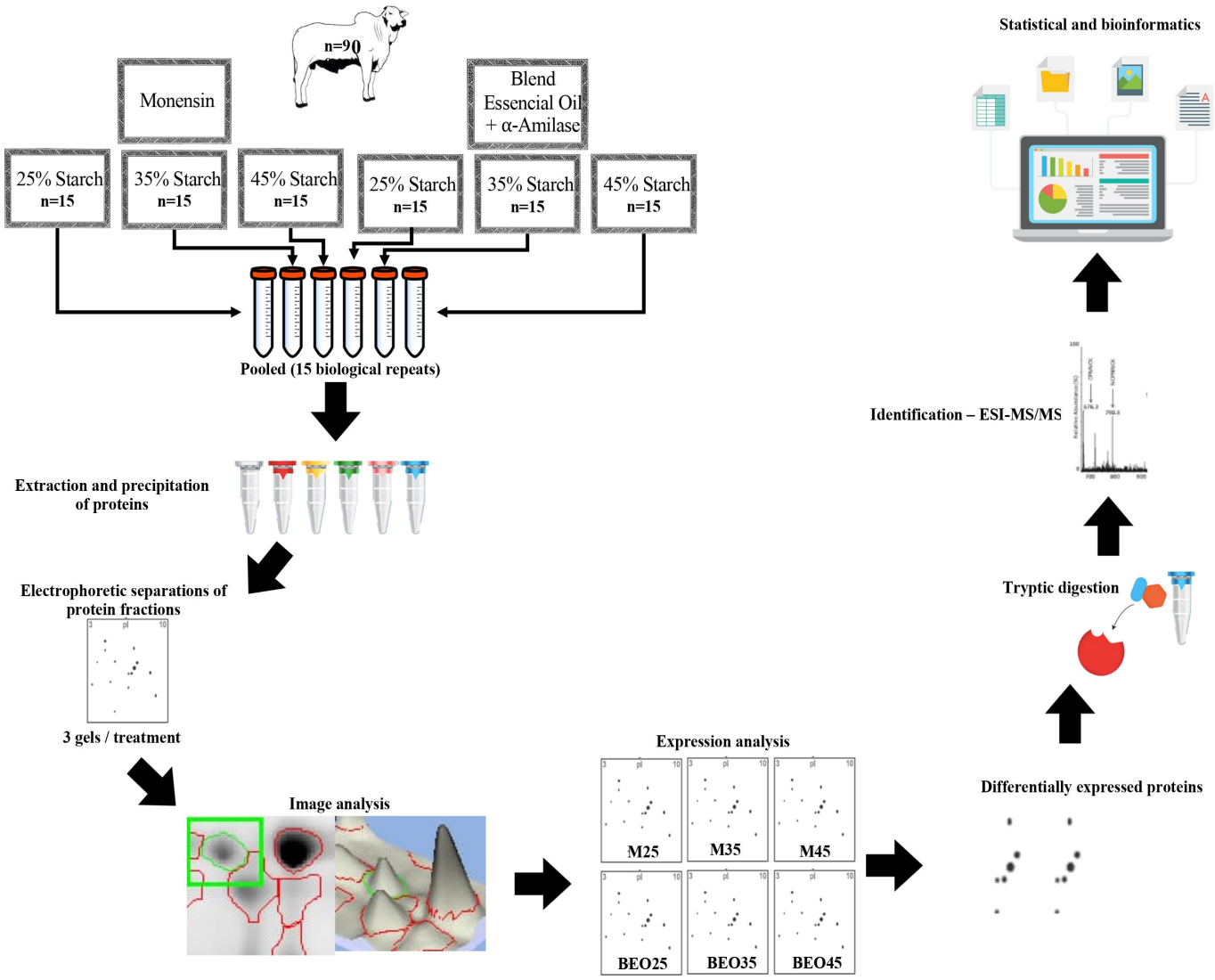
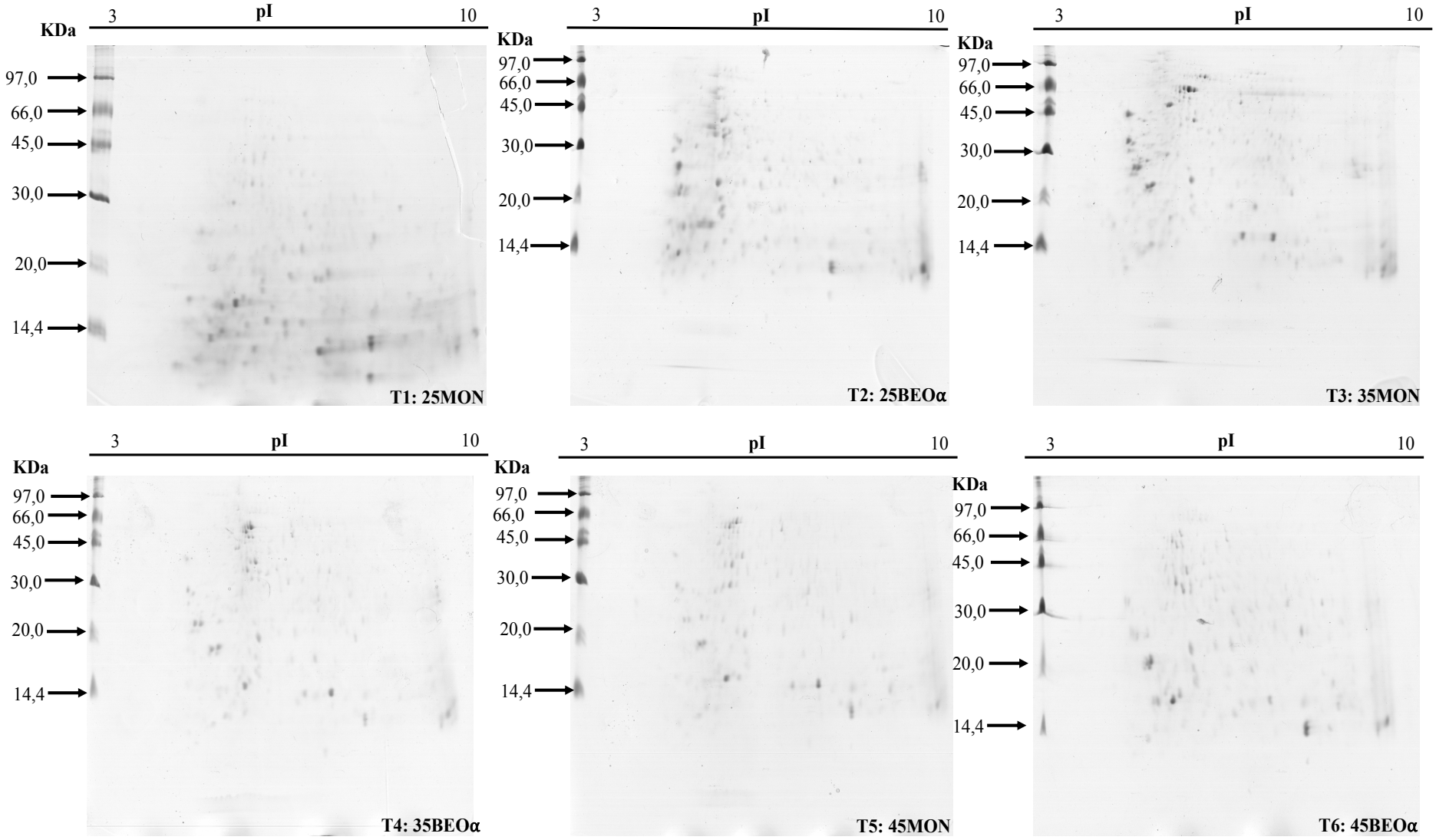
Supplemental Material

Supplemental Table 1. Values of Reactome Statistical analysis.

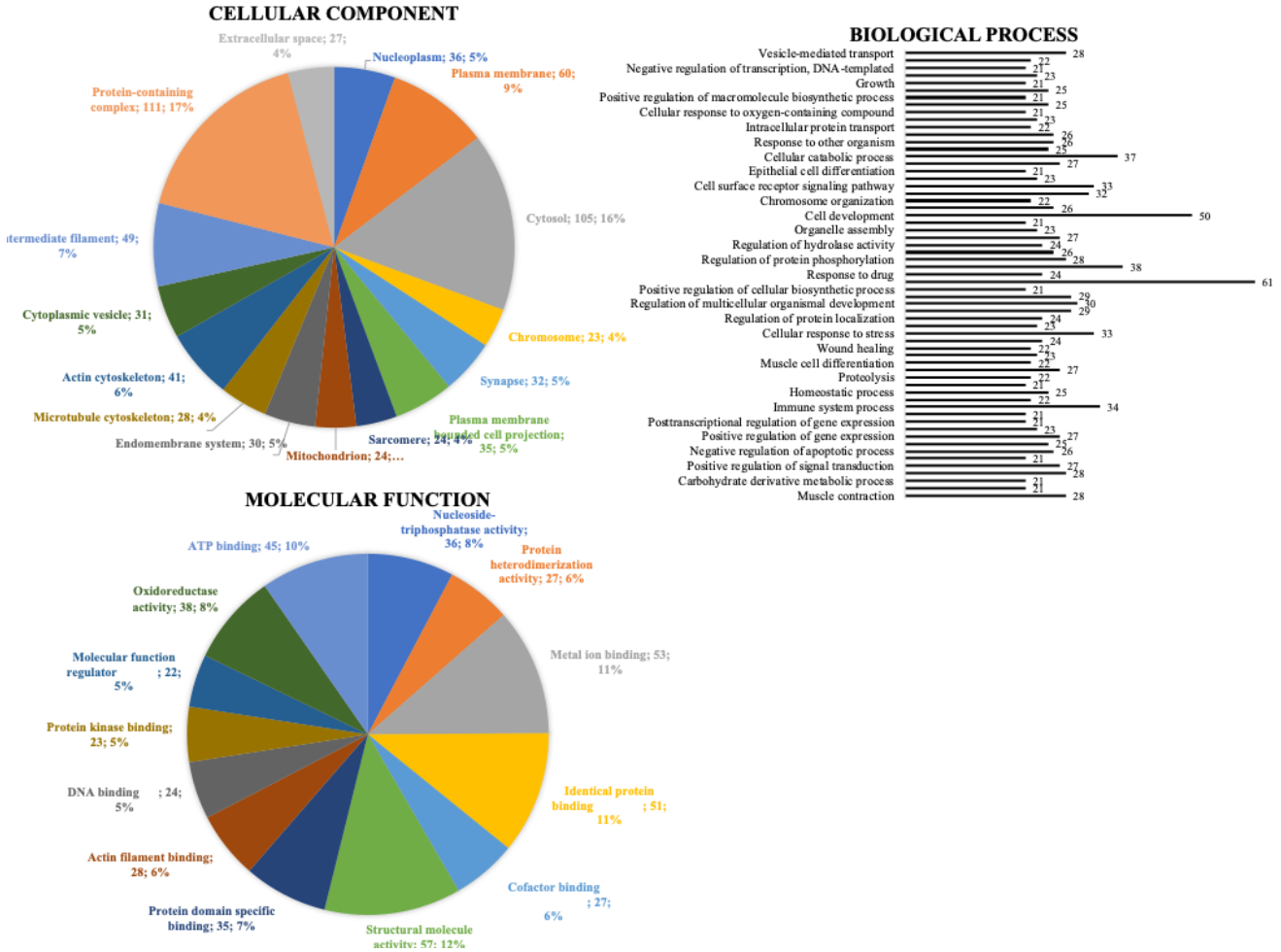
Pathway	**Raw P.value**	**FDR Adjusted P.Value**
Pyruvate Metabolism	3.2E-2	1.58E-1
Gluconeogenesis	3.51E-2	1.58E-1
Pyruvate Metabolism and Citric Acid (TCA) Cycle	5.62E-2	1.58E-1
Glycolysis	7.88E-2	1.58E-1
Glucose Metabolism	9.81E-2	1.7E-1
The Citric acid (TCA) cycle and respiratory electron transport	1.7E-1	1.7E-1
Metabolism Carbohydrate	2.73E-1	2.73E-1
Neutrophil degranulation	4.02E-1	4.02E-1
Metabolism	6.88E-1	6.88E-1
Innate Immune System	7.32E-1	7.32E-1
Immune System	9.27E-1	9.27E-1



Graphic Abstract 1



Supplemental Figure 1. Polyacrylamide gel electrophoresis images



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

CAPÍTULO 3

"Protein profiles identified by LC MS / MS demonstrate change in beta oxidation, ketogenesis and propionate metabolism in rumen epithelium with different additives"

Protein profiles identified by LC MS / MS demonstrate change in beta oxidation, ketogenesis and propionate metabolism in rumen epithelium with different additives

Abstract

Replacement of ionophore feed additives by essential oils demonstrate differences in energy metabolism. The mechanism of action of oils depends on the ruminal pH, therefore, feedlot diets with high starch inclusions tend to show greater effectiveness of these natural additives. The aim of this study is to map the proteome of the ruminal epithelium of feedlot Nellore cattle (n=60) with different additives (Monensin, Blend of essential oil + exogenous α-Amylase) and different levels of starch (25 and 45 %). Two-dimensional polyacrylamide gel electrophoresis was used to separate proteome of ruminal epithelium, then the differentially expressed protein *spots* were identified from the pI, molecular mass, volume and intensity of the *spots* and identified by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). Diets using an essential oil blend associated with exogenous amylase promoted greater expression of macromolecules from degradation of carbohydrates through the glycolytic and ketogenesis pathway. Fourteen proteins were identified in upregulation and the presence of proteins involved in glucose oxidation, and hydroxymethylglutaryl-CoA lyase that catalyzes part of metabolic intermediate metabolism, a key step in ketogenesis. Our results suggest that there was an increase in glycolysis from the oxidation of glyceraldehyde-3-phosphate, which participate in the first step of acetate and butyrate production and the oxidative decarboxylation of in the ruminal epithelium of Nellore feedlot. Monensin improve precursors of propionate, methylmalonyl-CoA mutase suggests greater synthesis of propionate via propionyl-CoA upon entry into citric acid cycle as succinyl-CoA, which can increase metabolizable energy and reduces feed intake.

Keywords: 2D-PAGE, essential oil, mass spectrometry, monensin, propionyl-CoA

1. Introduction

Plant secondary metabolites such as essential oils, tannins, saponins are of interest in ruminant nutrition as they have the potential to modify ruminal fermentation (NASCEM, 2016). In this way, the aim of the studies is to change the microbial population, reduce methane production and improve feed efficiency combined with the alternative use of antibiotic additives (Benchaar et al., 2008), recent studies with feed additives in livestock focus on replacing ionophores and growth promoters by alternative sources (Torres et al., 2021).

Plant extracts has effects antimicrobial potential and can be used in feedlot diet (Meschiatti et al., 2019; Toseti et al., 2020). The mechanism of action is due to its hydrophobic power, which interacts with bacterial cell membrane and increases permeability until membrane rupture and loss of cytoplasmatic constituents (Hassan et al., 2020; Torres et al., 2021) including electron transport, ion gradients, protein translocation, phosphorylation, and other enzyme-dependent reactions (Ultee et al., 1999; Dorman & Deans, 2000; Benchaar et. al., 2008). Replacement of monensin by essential oils shows some expected responses, greather dry matter intake (DMI), carcass dressing, total fatty acids attributed to DMI (Meschiatti et al., 2019; Torres et al., 2021).

In contrast, monensin demonstrates a greater 3% reduction in DMI (NASCEM, 2016), the committee also recommends that the use of monensin increases metabolizable energy by 2.3% due to the improvement in feed efficiency. Studies suggest that the increased synthesis of propionate with the use of monensin is responsible for improving feed efficiency (Ogunade et al., 2018). Due to hepatic beta oxidation, as ruminants are dependent on hepatic gluconeogenesis, and propionate stimulates greater synthesis of Acetyl CoA and generation of ATP, that induces hypophagia in ruminants (Allen, 2009).

Plant extracts effects on ruminal microbial fermentation may be pH-dependent and improve the ruminal fermentation profile in beef production systems when ruminal pH is low (Cardozo et al., 2005; Benchaar et al., 2008; Torres et al., 2021), therefore, feedlot diets high grain and low rumen pH can show differences in additives performance. The aim of this study is to characterize the differentially expressed proteins in the tissue of the rumen epithelium and to verify which alterations can be found in the metabolism due to the feed additive (Monensin, Blend of essential oil + exogenous α-Amylase) and different levels of starch (25 and 45 %).

2. Material and Methods

All procedures performed, animal care 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 São Paulo State University – UNESP, Botucatu – SP, under protocol n° 0107/2019.

2.1 Animals, facilities, treatments and collection

The experimental feedlot was conducted at Innovation and Applied Science Center of DSM Nutritional Products (I & AS Beef Center) (Rio Brilhante, Mato Grosso do Sul, Brazil). The animals passed for receiving program (weighing, deworming and vaccinating) and were submitted to a pre-adaptation period of 10 days in order to standardize their rumen population and adapt to the facilities and management. Nellore bulls (n = 60) (*Bos taurus indicus*), with an average body weight of ± 380 kg, from the grazing system were used. The diets were formulated using the LRNS system (Large Ruminant Nutrition System, (Fox et al., 2004)), level 2, meeting the nutritional requirements for daily weight gain between 1.5 and 1.7 kg.day.animal^{-1}. Animals were fed for 92 days and diets were offered *ad libitum* twice a day (8 a. m. and 3 p.m.). The experimental diets were composed of bagasse sugarcane in nature, ground corn, soybean hulls,

cottonseed, soybean, mineral-vitamin core, urea and additives. 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 (McIntosh et al., 2003), 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 and 560 mg kg^{-1} of dry matter, respectively. 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 (Table 1).

Table 1. Experimental diets containing increasing starch levels (25 and 45%) and additives (Monensin, Blend of essential oil + exogenous α-Amylase) in diets for Nellore cattle feedlot

Starch level (%)	Diets					
	Low			High		
	25			45		
Feedstuffs	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
Corn grain grind	300	330	360	300	470	640
Soybean meal	90	55	20	90	75	60
Whole cottonseed	60	80	100	60	80	100
Soybean hulls	150	235	320	150	75	0
Mineral and Vitamin supplement	50	50	50	50	50	50
	Nutrient Content (Dry matter, g kg^{-1})					
CP[3]	146	147	146	146	145	145
TDN[4]	660	680	690	660	720	770
DPI[5]	510	510	500	510	520	530
NDF[6]	437	424	412	437	316	252
peNFD[7]	360	300	250	360	280	220
Ca[8]	7.7	7.5	7.3	7.7	7.6	7.5
P[9]	3.1	2.8	2.5	3.1	3.6	3.7
Starch	209.5	230.8	**254.6**	209.5	372.8	**458.0**
NE Mcal/kg DM[10]	2.4	2.4	2.4	2.4	2.6	2.7

[1]Adap 1 = Adaptation 1, 0-7 days; [2]Adap 2 = Adaptation 2, 7 -14days, 14-92 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]Calcium (Ca); [9]Phosphor (P); [10]Net energy (NE).

The animals were transported to a commercial slaughterhouse where they were stunned by brain concussion using a captive dart gun, followed by bleeding hide removal and evisceration rumen 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. Samples were collected, so a pool was made from the homogenization of rumen tissue of the same treatment, wherein three animals per experimental unit (pen=5) were considered, i.e., 15 animals/group or 60 animals totals (15 animals x 4 groups).

2.2 Extraction, precipitation and quantification of proteins

To extract the protein fraction, the ruminal papillae were removed and 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 was collected and 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) following protocol described by Braga et al. (2015). Protein concentrations were determined by the Biuret method (Doumas et al., 1981) 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}.

2.3 Electrophoresis separation of proteins fraction

The 2D-PAGE separation following the protocols established by Braga et al., 2015. The proteins extracts were separated in first dimension using Ettan™ IPGphor™ 3 isoelectric

focusing system – IEF (GE Healthcare, Uppsala, Sweden). The electrical voltage used was established by the protocol described by Braga et al. (2015). After this steps, the strip was balanced in two stages of 15 minutes each, to keep the proteins in their reduced forms (Neves et al., 2012; Santos et al., 2011). Posteriorly, the second dimension of the electrophoretic process (SDS-PAGE) was performed in the molecular weight range of 14-97 kDa.

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 molecular mass standard (GE Healthcare, Uppsala, Sweden), with proteins of different molecular 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 run 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 fix solution and then the proteins were revealed using the colloidal Coomassie G-250 (USB, Cleveland, Ohio, United States) for 72 h and then removed by washing with ultrapure water (Moraes et al., 2012; Neves et al., 2012; Santos et al., 2011; Silva et al., 2013).

2.4 Image analysis

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 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

(Moraes et al., 2012; Neves et al., 2012; Santos et al., 2011; Silva et al., 2013). The polyacrylamide gels referring to the treatments used are described in Figure 1.

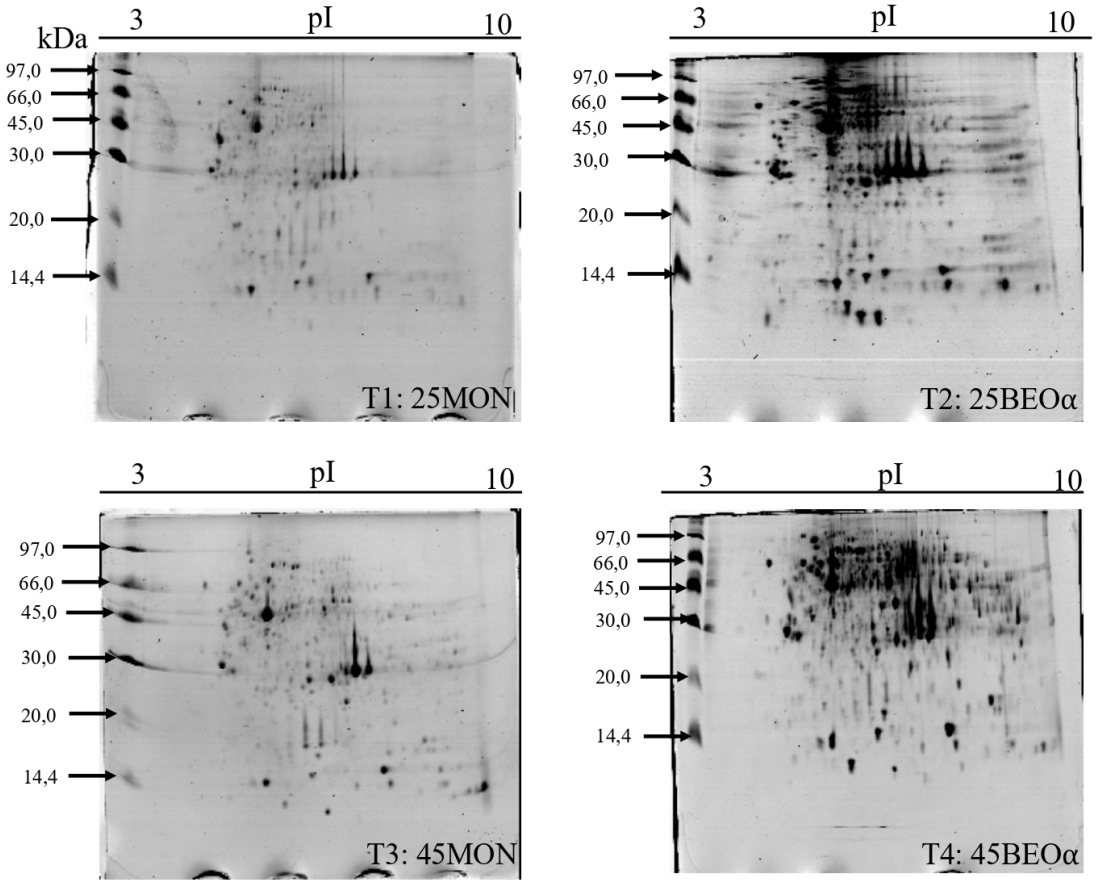


Figure 1. Polyacrylamide gel electrophoresis images of ruminal epithelium protein profile. T1: Treatment 1 = 25% starch + Monensin; T2: Treatment 2 = 25% starch + Blend Essential Oil and α-Amylase; T3: Treatment 3 = 45% starch + Monensin; T4: Treatment 4 = 45% starch + Blend Essential Oil and α-Amylase.

2.5 Protein identification by mass spectrometry (LC- MS/ MS)

The protein *spots* were characterized by LC-MS/MS after being subjected to tryptic digestion and the elution of peptides according to the methodology described by Shevchenko et al. (2006). The aliquots of the solutions containing the elute peptides were analyzed to obtain the mass spectra through the nanoAcquity UPLC system coupled to the Xevo G2 QTof mass

spectrometer (Waters, Milford, MA, United States). The identification of proteins was performed by searching in database UniProt (www.uniprot.org) with the *Bos taurus* species. Proteins were considered depending on the respective theoretical and experimental isoelectric point and molecular mass, and for their scores (>60). After the identification of FASTA sequences the proteins, their sequences were analyzed by software OMICSBOX (BLAST2GO) (Conesa et al., 2005) and thus categorized by their molecular function, biological processes and biochemical activities with Gene Ontology (GO).

2.6 Statistical analysis

The pen was considered the experimental unit, so a pool of samples was made from the homogenization of rumen tissue of the same treatment, wherein three animals per experimental unit (pen = 5) were considered, i.e., 15 animals/group or 60 animals totals (15 animals x 4 groups). The animals were randomly allocated to pens (7 animals/pen), with 12 m2 of area/animal and collective troughs (50 cm linear/animal). The factorial arrangement 2 x 2, and the fixed effects analyzed were: STARCH LEVEL (Low × High) and ADDITIVES (Monensin × Essential Oil Blend: CRINA® + Exogenous α-Amylase: Rumistar®); thus, the comparison between groups was by means of contrasts in order to verify differentially expressed protein *spots.* Only proteins with significantly altered levels were excised for identification by MS. 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, test t) considering significance to determine the differentially expressed protein *spots*.

Following the average mode of background subtraction, individual spot intensity volume was normalized with total intensity volume (summation of the intensity volumes

obtained from all *spots* in the same 2-DE gel). The normalized intensity volume values of individual protein *spots* were then used to determine differential protein expression among experimental groups.

2.7 String and network analysis

The String version 11.0 were performed to obtain protein-protein interaction from differentially abundant proteins using *Bos taurus*, making it possible to map the expressions of proteins encoding enzymes found in the database. It was created the interaction network from all proteins sequences identified. The GO terms were considered enriched at $P < 0.05$, corrected to the false discovery rate (FDR) (*P*-adjust <0.05).

3. Results

All proteins identified were showed in Supplemental Table 1. After identifying the profile of proteins characterized by mass spectrometry, the FASTA sequences (uniprot.org) were used to classify the proteins through Blast2GO at three different levels, cell component, molecular function and biological processes, described in Figure 2. 264 proteins were characterized by spectrometry mass. Thus, 32 proteins characterized in the proteome of the ruminal epithelium of Nellore cattle were considered. Using the FASTA sequence, the identified proteins were divided into their molecular function, cellular component and biological process (Figure 2)

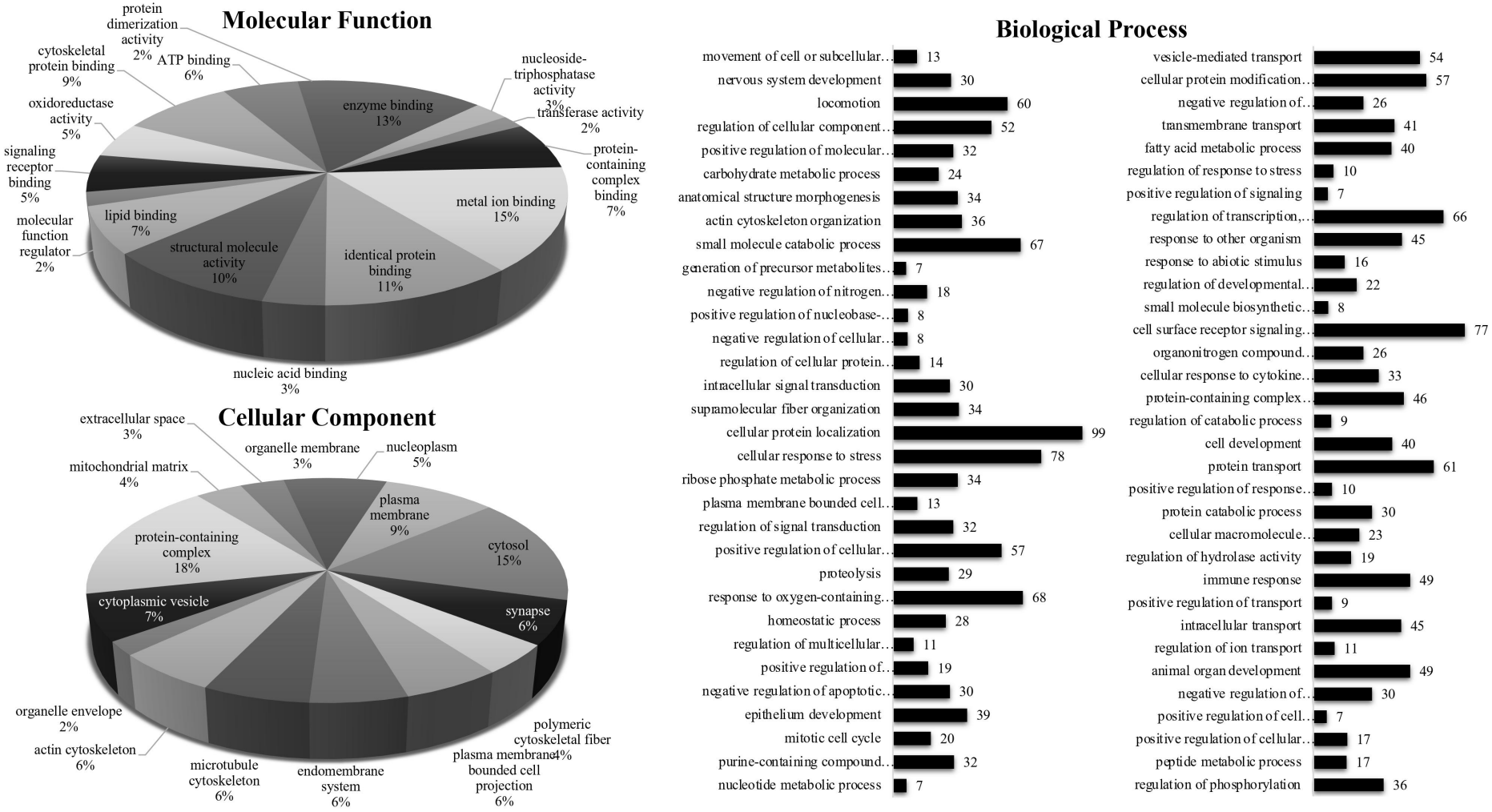


Figure 2. Proteins found in the ruminal epithelium were classified by molecular functions, cell component and biological process using Blast2GO.

The differentially expressed *spots* were standardized considering the highest Score Protein, pI and molecular mass (MM) closest to the theoretical and experimental results. Then, they were classified according to the purpose of the study. The proteins shown in Table 2 were clustering glucose and energy metabolism and fatty acid metabolic process.

Table 2. Proteins identified by LC/ MS-MS in Nellore Bulls rumen papillae fed with different starch level and additives.

Protein	Access	Score	pI/MM theoretical (Da)	pI/MM experimental (Da)
Glucose and Energy Metabolism				
Triosephosphate isomerase (TIM)	Q5E956	626.1260	6.45/26689.51	6.15/21015
Hydroxymethylglutaryl-CoA lyase	Q29448	510.5914	8.92/34167.90	7.69/28148
Malate dehydrogenase	Q32LG3	2714.0900	8.82/35668.50	9.31/36948
Glucose-6-phosphate isomerase (GPI)	Q3ZBD7	185.6869	7.33/62855.72	7.72/46722
L-lactate dehydrogenase B chain	Q5E9B1	962.9854	6.02/36723.24	8.78/28481
6-phosphogluconolactonase (6PGL)	Q2TBQ8	715.2087	5.57/27546.91	5.96/23491
UDP-glucose 6-dehydrogenase	P12378	207.9680	7.51/55136.32	7.52/51128
Eukaryotic translation initiation factor 6	Q9TU47	570.0528	4.61/26513.04	4.42/23206
D-3-phosphoglycerate dehydrogenase (3-PGDH)	Q5EAD2	331.7930	6.47/56451.50	6.05/49.759
Isocitrate dehydrogenase [NADP]	Q9XSG3	779.0844	6.13/46785.42	6.38/38800
Beta-enolase	Q3ZC09	795.1721	7.60/47096.01	6.94/49285
Citrate synthase, mitochondrial	Q29RK1	300.0121	8.16/51772.54	7.90/37470
L-lactate dehydrogenase A-like 6B	Q3T056	136.1171	8.91/41592.42	9.31/36948
L-lactate dehydrogenase A chain (LDH-A)	P19858	300.9528	8.12/36597.64	9.31/36948
Phosphoglycerate kinase 1	Q3T0P6	113.6219	8.48/44537.60	6.64/40022
L-xylulose reductase (XR)	Q1JP75	79.5362	7.74/25649.63	7.94/26480
Alpha-enolase	Q9XSJ4	1713.1130	6.37/47326.13	6.94/49285
Glyceraldehyde-3-phosphate dehydrogenase	Q2KJE5	1704.5970	8.32/43287.86	9.31/36948
Medium-chain specific acyl-CoA dehydrogenase	Q3SZB4	66.1058	8.31/46573.30	7.12/58276

Glyceraldehyde-3-phosphate dehydrogenase	P10096	929.1370	8.51/35868.09	9.31/36948
Fatty Acid Metabolic Process				
3-ketoacyl-CoA thiolase, hydrolase	Q3T0R7	660.7452	8.06/42131.21	8.28/40353
Fatty acid-binding protein	P10790	188.33	6.73/14778.91	5.32/13279
Acetyl-CoA acetyltransferase, mitochondrial	Q29RZ0	2625.8390	8.98/44889.16	9.05/46196
Fatty acid-binding protein 5	P55052	1225.4760	7.58/15074.33	7.96/14260
Methylmalonyl-CoA mutase	Q9GK13	120.6006	6.58/83234.71	6.72/68489
Short-chain specific acyl-CoA dehydrogenase	Q3ZBF6	2637.0740	8.82/44552.37	8.48/44131
Electron transfer flavoprotein subunit alpha, mitochondrial (Alpha-ETF)	Q2KJE4	811.8699	8.77/34961.43	7.69/28148
Enoyl-CoA hydratase, mitochondrial	Q58DM8	577.2590	8.82/31243.31	7.69/28148
Isovaleryl-CoA dehydrogenase, mitochondrial (IVD)	Q3SZI8	520.0278	7.10/46497.56	6.22/45000
V-type proton ATPase subunit B	P31407	242.2612	5.33/56746.76	5.93/57994
Alcohol dehydrogenase class-3	Q3ZC42	106.5743	7.46/39677.13	7.99/34794
Fatty acid-binding protein	P48035	5497.8350	5.52/14677.89	5.32/13279

The expression values of differentially protein were showed in Table 3, and protein ID were performed with String, version 11.0 (string-db.org) using the available *Bos taurus* genome. To obtain a more robust interaction network, protein of all treatments were combined (Table 4).

Table 3. Expression values (test t, P≤ 0.05) in protein profile of Nellore Bulls epithelium rumen fed starch levels (Low=25 % and High= 45%) and additives (Monensin and Blend Essential Oil + α-Amylase).

Protein	Starch Level				Additives			
	Low		High		MON		BEO	
	MON	BEO	MON	BEO	Low	High	Low	High
Glucose and Energy Metabolism								
Triosephosphate isomerase (TIM)	∅	+	ns		ns		ns	
Hydroxymethylglutaryl-CoA lyase	-1,14	1,14	ns		ns		ns	
Malate dehydrogenase	ns		ns		∅	+	ns	
Glucose-6-phosphate isomerase (GPI)	∅	+	∅	+	ns		ns	
L-lactate dehydrogenase B chain	∅	+	∅	+	ns		ns	
6-phosphogluconolactonase (6PGL)	∅	+	ns		ns		ns	
UDP-glucose 6-dehydrogenase	∅	+	∅	+	ns		ns	
Eukaryotic translation initiation factor 6	∅	+	ns		ns		∅	+
D-3-phosphoglycerate dehydrogenase (3-PGDH)	ns		2,21	-2,21	-1,79	1,79	ns	
Isocitrate dehydrogenase [NADP]	3,35	-3,35	1,15	-1,15	3,83	-3,83	+	∅
Beta-enolase	ns		ns		-1,48	1,48	-1,35	1,35
Citrate synthase, mitochondrial	∅	+	∅	+	ns		ns	
L-lactate dehydrogenase A-like 6B	∅	+	∅	+	ns		∅	+
L-lactate dehydrogenase A chain (LDH-A)	∅	+	∅	+	ns		∅	+
Phosphoglycerate kinase 1	ns		ns		+	∅	ns	
L-xylulose reductase (XR)	ns		ns		+	∅	2,42	-2,42
Alpha-enolase	ns		ns		-1,48	1,48	-1,27	1,27
Glyceraldehyde-3-phosphate dehydrogenase	ns		ns		∅	+	ns	
Medium-chain specific acyl-CoA dehydrogenase	ns		ns		ns		15,09	-15,09
Glyceraldehyde-3-phosphate dehydrogenase	ns		∅	+	∅	+	ns	
Fatty Acid Metabolic Process								
3-ketoacyl-CoA thiolase, hydrolase	ns		1,26	-1,26	-1,26	1,26	-1,47	1,47
Fatty acid-binding protein	ns		1,52	-1,52	ns		ns	
Acetyl-CoA acetyltransferase, mitochondrial	ns		2,21	-2,21	-1,79	1,79	ns	

Fatty acid-binding protein 5	∅	+	ns		∅	+	+	∅		
Methylmalonyl-CoA mutase	3,60	-3,60	ns		ns		ns			
Short-chain specific acyl-CoA dehydrogenase	ns		1,61	-1,61	ns		ns			
Electron transfer flavoprotein subunit alpha	ns		ns		ns		+	∅		
Enoyl-CoA hydratase, mitochondrial	ns		1,91	-1,91	ns		ns			
Isovaleryl-CoA dehydrogenase, mitochondrial (IVD)	ns		+	∅	ns		ns			
V-type proton ATPase subunit B	ns		ns		ns		-1.96	1,96		
Alcohol dehydrogenase class-3	∅	+	ns		ns		ns			
Fatty acid-binding protein	2,07	-2,07	1,28	-1,28	1,65	-1,65	ns			

The values are presented in the form log2FC (Fold Change) calculated in relation to the level of starch and type of additives used. ns: Not significant; +: *spot* present in the group. ∅: *spot* absent in the first group

Using proteins sequence were found macromolecules linked to carbohydrate metabolism, responsible for energy biosynthesis through glycolysis (GO: 0006096), tricarboxylic acid cycle (GO: 00066099), gluconeogenesis (GO: 0006094), carbohydrate metabolic process (GO: 0005975) and the biosynthetic process of carbohydrate derivatives (GO: 1901137) begins with the metabolism of a carbohydrate to generate pyruvate, which can be converted to acetyl-coenzyme A, combining with oxaloacetate to form citrate, which undergoes successive transformations into isocitrate, 2-oxoglutarate, succinyl-CoA, succinate, fumarate, malate or intermediate groups of the glycolytic pathway, as well as ethanol and lactate (Supplemental Figure 1) (string-db.org).

Table 4. Biological Process related to differentially expressed protein in beef cattle rumen epithelium

GO-Term	Description	*P*-adjust (FDR)[1]
	Carbohydrate Metabolic Process	
GO:0006096	Glycolytic Process	2.43e-15
GO:0006099	Tricarboxilic Acid Cycle	6.73e-06
GO:0006094	Gluconeogenesis	0.00045
GO:0005975	Carbohydrate Metabolic Process	4.69e-27
GO:1901137	Carbohydrate derivate biosynthetic Process	1.61e-11
	Fatty Acid Metabolic Process	
GO:0009083	Branched-chain Amino Acid Catabolic Process	0.00015
GO:0006635	Fatty Acid Beta Oxidation	1.49e-08
GO:0046395	Fatty Acid Metabolic Process	8.57e-10
GO:0005504	Fatty Acid Binding	2.08e-06

[1] P-Adjust: *P*-Value corrected to multiple tests by false discovery rate (FDR).

Proteins linked to the metabolic process of fatty acids were identified (Supplemental Figure 2), responsible by branched-chain amino acid catabolic process (GO:0009083), fatty acid beta oxidation (GO:0006635), fatty acid metabolic process (GO:0046395), fatty acid binding (GO:0005504) which chemical reactions and pathways resulting in the breakdown of amino acids containing a branched carbon skeleton, comprising isoleucine, leucine, valine, and fatty acid oxidation process that results in the complete oxidation of a long-chain fatty acid.

4. Discussion

About 70% of volatile fatty acids produced in the rumen are absorbed via the epithelium, it is responsible for physiologically important functions, such as absorption, transport, volatile fatty acids (VFA) metabolism and protection (Baldwin, 1998). The metabolism of VFA occurs in the intraepithelial, the addition of ester Acyl-SCoA synthetase present in the mitochondrial matrix of the epithelial cells so that they are oxidized in the Krebs Cycle (Penner et al., 2011).

4.1 Upregulation and proteins expressed in the cattle epithelium fed essential oil

Diets using an essential oil blend associated with exogenous amylase promoted greater expression of macromolecules from glycolysis pathway. 14 proteins were identified in upregulation and the presence of proteins involved in glucose oxidation ($P<0.05$). Among these proteins that participate in the degradation of carbohydrates, in step 1 of the glycolysis pathway were identified Triosephosphate isomerase (TIM) is an extremely efficient metabolic enzyme that catalyzes the interconversion between dihydroxyacetone phosphate (DHAP), D-glyceraldehyde-3-phosphate (G3P) is a key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate, UDP-glucose 6-dehydrogenase synthesizes UDP-alpha-D-glucuronate from UDP-alpha-D-glucose and Citrate synthase synthesizes isocitrate from oxaloacetate, it is part of the pathway tricarboxylic acid cycle. Glucose-6-phosphate isomerase (GPI) catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate, the second step in glycolysis and 6-phosphogluconolactonase (6PGL) the subpathway that synthesizes D-ribulose 5-phosphate from D-glucose 6-phosphate (oxidative stage) (Uniprot, 2021). Thus, our results suggest that there was an increase in glycolysis from the oxidation of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate coupled with the reduction of NAD + to NADH, which participate in the

first step of acetate and butyrate production and the oxidative decarboxylation of pyruvate (Ungerfeld, 2020).

Mitochondrial 3-hydroxymethyl-3-methylglutaryl-CoA lyase that catalyzes a cation-dependent cleavage of (S)-3-hydroxy-3-methylglutaryl-CoA into acetyl-CoA and acetoacetate itself part of metabolic intermediate metabolism, a key step in ketogenesis. Ketone bodies (beta-hydroxybutyrate, acetoacetate and acetone) are essential as an alternative source of energy to glucose (Uniprot, 2021). When evaluating the effects of the plant extract and the mixture of active compounds of essential oil (BEO) on the parameters of in vitro ruminal fermentation, it was found that there was greater fermentation of butyrate when compared to monensin or increasing doses of BEO (Mirzaei-Alamouuti et al., 2016; Joch et al., 2019), as opposed to acetate and propionate, butyrate after activated in butyryl-SCoA is converted into ketone bodies mainly into 3-methylglutaryl CoA synthase. Drong et al (2016) corroborate that ketogenesis can happen due to lower glucose production in the liver via propionate and increased butyrate.

L-Lactate dehydrogenase A chain, L-lactate dehydrogenase A-like 6B and L-lactate dehydrogenase B chain are protein is involved in step 1 of the subpathway that synthesizes (S)-lactate from pyruvate, it is part of the pathway pyruvate fermentation to lactate (Uniprot, 2021). BEO decrease methane fermentation (Garcia et al., 2020), and although there is a higher DMI, the use of BEO does not present a greater production of methane when replacing monensin. The greater expression of proteins that participate in lactate metabolism can be attributed to part of this energy can be released for lactate that would be used in the production of methane gas, reduced compounds intermediate of fermentation pathways that incorporate [H] in their formation, and are released by some microbial cells and taken up by others e.g., formate, ethanol, lactate and succinate (Ungerfeld, 2020).

4.2 Upregulation and proteins expressed in the cattle epithelium fed monensin

It was found lower expression of proteins to glycolytic metabolic ($P<0.05$) in the ruminal epithelium of cattle fed monensin as a feed additive. D-3-phosphoglycerate dehydrogenase (3-PGDH) and Isocitrate dehydrogenase [NADP] were upregulation expression. Proteins that participate in the metabolic process of fatty acids had greater expression ($P<0.05$) in the epithelium of animals fed with monensin. 3-ketoacyl-CoA thiolase hydrolase is one of the enzymes that catalyzes the last step of the mitochondrial beta-oxidation pathway, an aerobic process breaking down fatty acids into acetyl-CoA. Also catalyzes the condensation of two acetyl-CoA molecules into acetoacetyl-CoA and could be involved in the production of ketone bodies. Fatty acid-binding protein are thought to play a role in the intracellular transport of long-chain fatty acids and their acyl-CoA esters, Acetyl-CoA acetyltransferase is one of the enzymes that catalyzes the last step of the mitochondrial beta-oxidation pathway, an aerobic process breaking down fatty acids into acetyl-CoA. Methylmalonyl-CoA mutase catalyzes the reversible isomerization of methylmalonyl-CoA to succinyl-CoA (3-carboxy propionyl-CoA), a key intermediate of the tricarboxylic acid cycle, Short-chain specific acyl-CoA dehydrogenase is one of the acyl-CoA dehydrogenases that catalyze the first step of mitochondrial fatty acid beta-oxidation, an aerobic process breaking down fatty acids into acetyl-CoA and allowing the production of energy, Enoyl-CoA hydratase is involved in the pathway fatty acid beta-oxidation,and Isovaleryl-CoA dehydrogenase catalyzes the conversion of isovaleryl-CoA/3-methylbutanoyl-CoA to 3-methylbut-2-enoyl-CoA as an intermediate step in the leucine (Leu) catabolic pathway. Also, able to catalyze the oxidation of another saturated short-chain acyl-CoA (Uniprot, 2021).

Higher expression of Methylmalonyl-CoA mutase suggests greater synthesis of propionate via propionyl-CoA upon entry into citric acid cycle (CAC) as succinyl-CoA. The existence of significant propionyl-CoA synthetase activity in rumen epithelium indicates that this tissue is potentially able of metabolizing propionate (Ash and Baird, 1973). The increase

in ruminal propionate when using monensin can be explained by the reduction in microbial diversity in the rumen when compared to the use of BEO (Weimer et al., 2008; Schären et al., 2017; Torres et al., 2021). The smaller diversity of microorganisms allows for greater efficiency of the ruminal microbiota related to a lower number of metabolic pathways and competition for substrate (Li et al., 2019). Therefore, lower dry matter intake of monensin-fed animals due to increased portal propionate concentration that stimulates insulin release by pancreatic cells (Manns et al., 1967).

5. Conclusion

The use of blend essential oil + α-Amylase as an additive in diets promotes greater degradation of carbohydrates through the glycolytic and ketogenesis pathway, our results suggest that there was an increase in glycolysis from the oxidation of glyceraldehyde-3-phosphate, which participate in the first step of acetate and butyrate production and the oxidative decarboxylation of in the ruminal epithelium of Nellore feedlot. In contrast, monensin provides greater synthesis of propionate precursors and beta oxidation, which can increase metabolizable energy and reduces feed intake.

Declaration of Competing Interest

Authors declare that have no conflict of interest.

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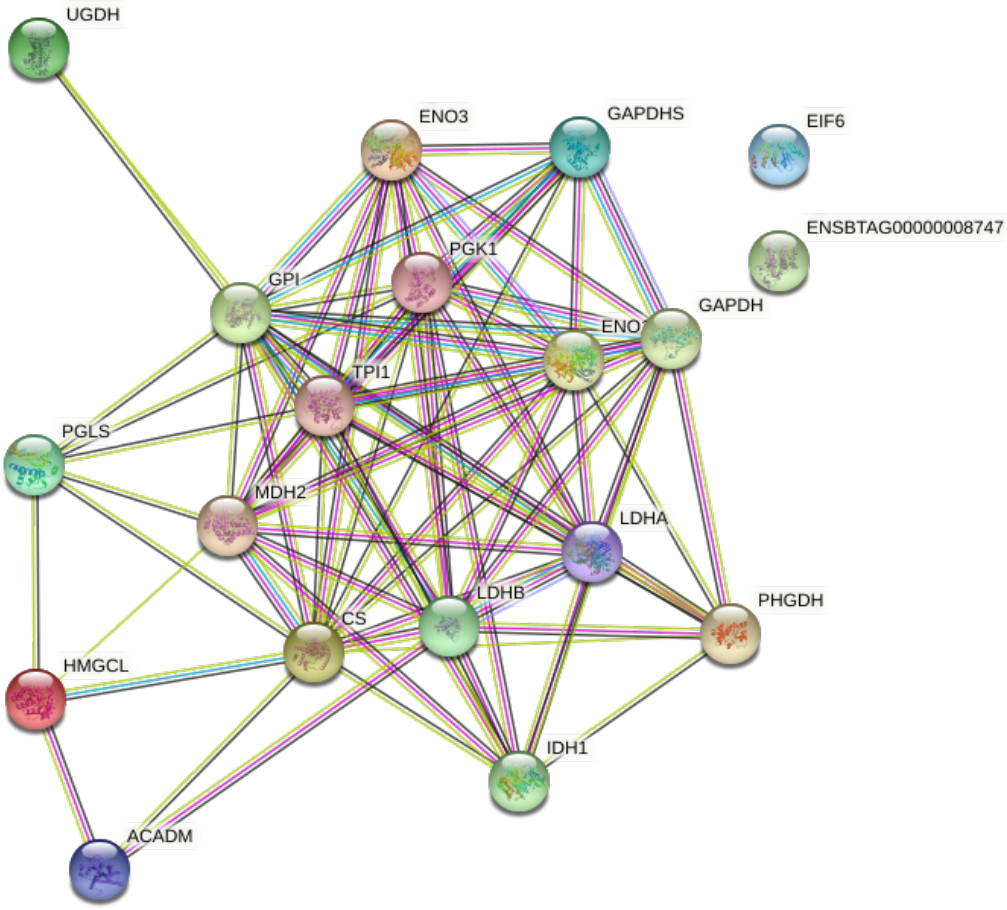
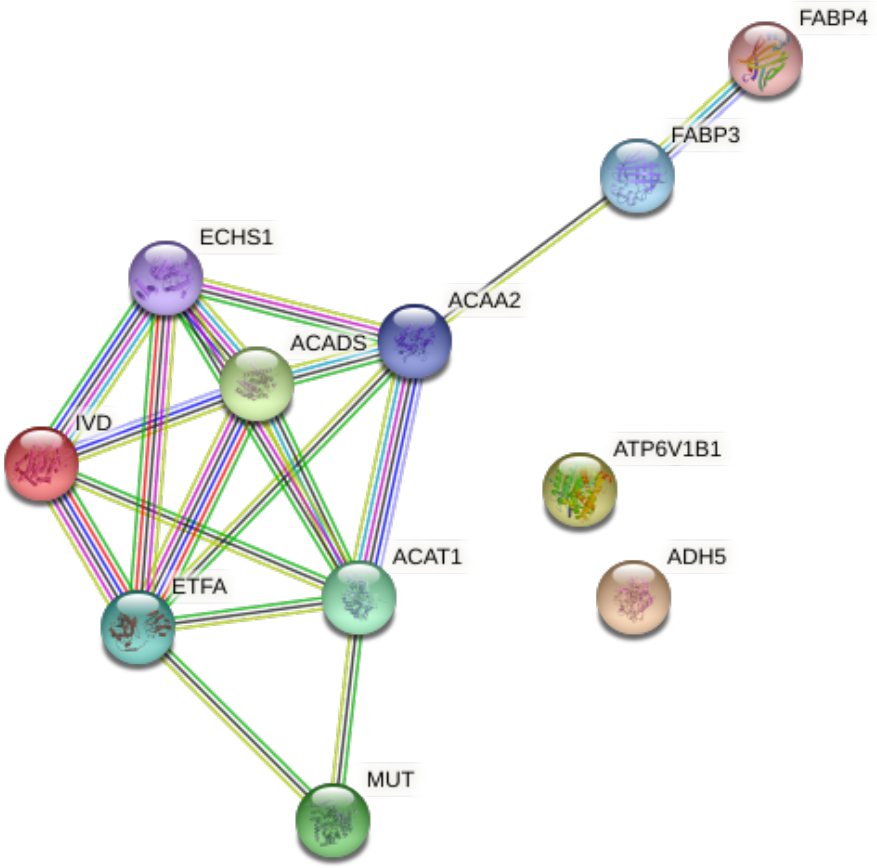
Supplementary Material



Supplemental Figure 1. Protein-protein interaction of differentially proteins expressed involved glucose and energy metabolism of rumen protein profile. Medium-chain specific acyl-CoA dehydrogenase (ACADM), Citrate synthase (CS), Eukaryotic translation initiation fator (EIF6), Alpha-enolase (ENO1), Beta-enolase (ENO3), Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Glyceraldehyde-3-phosphate dehydrogenase (GAPDHS), Glucose-6-phosphate isomerase (GPI), Hydroxymethylglutaryl-CoA lyase, mitochondrial (HMGOL), Isocitrate dehydrogenase (IDH1), L actate dehydrogenase A (LDHA), lactate dehydrogenase B (LDHB), Malate dehydrogenase (MDH2), Phosphoglycerate kinase 1 (PGK1), 6-phosphogluconolactonase; (PGLS), D-3-phosphoglycerate dehydrogenase (PHGDH), triosephosphate isomerase (TPI1), UDP-glucose 6-dehydrogenase (UGDH).



Supplemental Figure 2. Protein-protein interaction of differentially proteins expressed involved fatty acids metabolism of rumen protein profile. Methylmalonyl-CoA mutase (MUT), Electron transfer flavoprotein subunit alpha (ETFA), Acetyl-CoA acetyltransferase (ACAT1), Alcohol dehydrogenase (ADH5), Isovaleryl-CoA dehydrogenase (IVD), V-type proton ATPase subunit B, (ATP6VB1), Short-chain specific acyl-CoA dehydrogenase (ACADS), 3-ketoacyl-CoA thiolase (ACAA2), Enoyl-CoA hydratase (ECHS1), Fatty acid-binding protein, (FABP3), Fatty acid-binding protein (FABP4).

Supplemental Material

Supplemental Table 1. Proteins sequences identified by LC-MS/MS in rumen epithelium

Number	Entry	Entry name	Protein names	Mass	Gene names
1	P11116	LEG1_BOVIN	Galectin-1 (Gal-1) (	14,744	LGALS1
2	O97680	THIO_BOVIN	Thioredoxin (Trx)	11,813	TXN
3	P48035	FABP4_BOVIN	Fatty acid-binding protein	14,678	FABP4
4	P02690	MYP2_BOVIN	Myelin P2 protein	14,950	PMP2
5	P10790	FABPH_BOVIN	Fatty acid-binding protein, heart (	14,779	FABP3
6	Q1LZA1	CAH1_BOVIN	Carbonic anhydrase 1	28,822	CA1
7	P06394	K1C10_BOVIN	Keratin, type I cytoskeletal 10	54,848	KRT10
8	Q32KN8	TBA3_BOVIN	Tubulin alpha-3 chain	49,926	TUBA3
9	P81947	TBA1B_BOVIN	Tubulin alpha-1B chain	50,152	
10	Q3ZCJ7	TBA1C_BOVIN	Tubulin alpha-1C chain	49,857	TUBA1C
11	Q2HJ86	TBA1D_BOVIN	Tubulin alpha-1D chain [	50,283	TUBA1D
12	Q27975	HS71A_BOVIN	Heat shock 70 kDa protein 1A (Heat shock 70 kDa protein 1) (HSP70.1)	70,259	HSPA1A HSP70-1
13	Q27965	HS71B_BOVIN	Heat shock 70 kDa protein 1B (Heat shock 70 kDa protein 2) (HSP70.2)	70,228	HSPA1B HSP70-2
14	P00921	CAH2_BOVIN	Carbonic anhydrase 2	29,114	CA2
15	Q3T165	PHB_BOVIN	Prohibitin	29,804	PHB
16	Q2KJ32	SBP1_BOVIN	Methanethiol oxidase (MTO)	52,555	SELENBP1 SBP
17	P0CB32	HS71L_BOVIN	Heat shock 70 kDa protein 1-like (Heat shock 70 kDa protein 1L)	70,389	HSPA1L
18	Q5E987	PSA5_BOVIN	Proteasome subunit alpha type-5	26,411	PSMA5
19	Q2KIE6	HMCS2_BOVIN	Hydroxymethylglutaryl-CoA synthase	56,895	HMGCS2
20	P00586	THTR_BOVIN	Thiosulfate sulfurtransferase	33,296	TST
21	Q9TS87	TAGL_BOVIN	Transgelin (25 kDa F-actin-binding protein)	22,599	TAGLN SM22
22	P04272	ANXA2_BOVIN	Annexin A2	38,612	ANXA2 ANX2
23	P63258	ACTG_BOVIN	Actin, cytoplasmic 2 (	41,793	ACTG1 ACTG
24	P60712	ACTB_BOVIN	Actin, cytoplasmic 1 (	41,737	ACTB
25	Q3ZC07	ACTC_BOVIN	Actin, alpha cardiac muscle 1	42,019	ACTC1 ACTC

26	Q5E9B5	ACTH_BOVIN	Actin, gamma-enteric smooth muscle	41,877	ACTG2
27	P62739	ACTA_BOVIN	Actin, aortic smooth muscle (Alpha-actin-2)	42,009	ACTA2
28	P68138	ACTS_BOVIN	Actin, alpha skeletal muscle (Alpha-actin-1)	42,051	ACTA1 ACTA
29	Q08D91	K2C75_BOVIN	Keratin, type II cytoskeletal 75 (Cytokeratin-75)	59,036	KRT75
30	Q5XQN5	K2C5_BOVIN	Keratin, type II cytoskeletal 5 (Cytokeratin-5)	62,937	KRT5
31	Q29S21	K2C7_BOVIN	Keratin, type II cytoskeletal 7 (Cytokeratin-7)	51,578	KRT7
32	O77834	PRDX6_BOVIN	Peroxiredoxin-6	25,067	PRDX6 AOP2 GPX PHGPX
33	P19120	HSP7C_BOVIN	Heat shock cognate 71 kDa protein	71,241	HSPA8 HSC70
34	Q17QQ2	TPMT_BOVIN	Thiopurine S-methyltransferase	28,335	TPMT
35	Q0VCN1	NMRL1_BOVIN	NmrA-like family domain-containing protein 1	33,154	NMRAL1
36	P37980	IPYR_BOVIN	Inorganic pyrophosphatase	32,814	PPA1 PP
37	P31800	QCR1_BOVIN	Cytochrome b-c1 complex subunit 1	52,736	UQCRC1
38	Q3MHX5	SUCB2_BOVIN	Succinate--CoA ligase	46,691	SUCLG2
39	P02769	ALBU_BOVIN	Albumin (BSA)	69,293	ALB
40	P31081	CH60_BOVIN	60 kDa heat shock protein	61,108	HSPD1 HSP60
41	Q5E9E2	MYL9_BOVIN	Myosin regulatory light polypeptide 9	19,865	MYL9 MYRL2
42	A4IF97	ML12B_BOVIN	Myosin regulatory light chain 12B	19,692	MYL12B MRLC2 MYLC2B
43	A5D7D1	ACTN4_BOVIN	Alpha-actinin-4 (Non-muscle alpha-actinin 4)	104,928	ACTN4
44	Q3B7N2	ACTN1_BOVIN	Alpha-actinin-1	102,980	ACTN1
45	Q0III9	ACTN3_BOVIN	Alpha-actinin-	103,151	ACTN3
46	Q3ZC55	ACTN2_BOVIN	Alpha-actinin-2	103,779	ACTN2
47	Q3ZBT1	TERA_BOVIN	Transitional endoplasmic reticulum ATPase (TER ATPase)	89,330	VCP
48	P48616	VIME_BOVIN	Vimentin	53,728	VIM
49	O62654	DESM_BOVIN	Desmin	53,532	DES
50	P02548	NFL_BOVIN	Neurofilament light polypeptide (NF-L)	62,646	NEFL
51	Q08DH7	AINX_BOVIN	Alpha-internexin (Alpha-Inx)	55,396	INA

52	O77788	NFM_BOVIN	Neurofilament medium polypeptide (NF-M)	103,210	NEFM NEF3 NFM
53	A6QQJ3	PERI_BOVIN	Peripherin	53,631	PRPH
54	P05786	K2C8_BOVIN	Keratin, type II cytoskeletal 8	53,627	KRT8
55	Q9TU47	IF6_BOVIN	Eukaryotic translation initiation factor 6	26,513	EIF6 ITGB4BP
56	Q2TBG8	UCHL3_BOVIN	Ubiquitin carboxyl-terminal hydrolase isozyme L3	26,182	UCHL3
57	Q9BGI1	PRDX5_BOVIN	Peroxiredoxin-5, mitochondrial	23,253	PRDX5
58	Q9XSG3	IDHC_BOVIN	Isocitrate dehydrogenase [NADP]	46,785	IDH1 ICDH
59	Q5KR47	TPM3_BOVIN	Tropomyosin alpha-3 chain (Gamma-tropomyosin)	32,819	TPM3
60	Q5KR49	TPM1_BOVIN	Tropomyosin alpha-1 chain (Alpha-tropomyosin) (Tropomyosin-1)	32,695	TPM1
61	Q5KR48	TPM2_BOVIN	Tropomyosin beta chain (Beta-tropomyosin)	32,837	TPM2
62	P02070	HBB_BOVIN	Hemoglobin subunit beta (Beta-globin)	15,954	HBB
63	P02081	HBBF_BOVIN	Hemoglobin fetal subunit beta (Beta-globin, fetal)	15,859	
64	P79105	S10AC_BOVIN	Protein S100-A12 (Calcium-binding protein in amniotic fluid 1) (CAAF1)	10,685	S100A12 CAAF1
65	P10096	G3P_BOVIN	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	35,868	GAPDH GAPD
66	Q5E9F7	COF1_BOVIN	Cofilin-1 (Cofilin, non-muscle isoform)	18,519	CFL1
67	Q148F1	COF2_BOVIN	Cofilin-2 (Cofilin, muscle isoform)	18,737	CFL2
68	P62935	PPIA_BOVIN	Peptidyl-prolyl cis-trans isomerase A (PPIase A)	17,869	PPIA
69	A4FV72	PPIE_BOVIN	Peptidyl-prolyl cis-trans isomerase E (PPIase E) (	33,461	PPIE
70	A7YY28	ABHEB_BOVIN	Protein ABHD14B	22,455	ABHD14B
71	P35705	PRDX3_BOVIN	Thioredoxin-dependent peroxide reductase, mitochondrial	28,195	PRDX3 AOP1
72	Q3T149	HSPB1_BOVIN	Heat shock protein beta-1 (HspB1)	22,393	HSPB1 HSP27
73	A5D989	EF1D_BOVIN	Elongation factor 1-delta (EF-1-delta)	31,142	EEF1D
74	Q3MHM5	TBB4B_BOVIN	Tubulin beta-4B chain (Tubulin beta-2C chain)	49,831	TUBB4B TUBB2C
75	Q2KJD0	TBB5_BOVIN	Tubulin beta-5 chain	49,671	TUBB5
76	Q3ZBU7	TBB4A_BOVIN	Tubulin beta-4A chain (Tubulin beta-4 chain)	49,586	TUBB4A TUBB4
77	Q6B856	TBB2B_BOVIN	Tubulin beta-2B chain	49,953	TUBB2B
78	Q2T9S0	TBB3_BOVIN	Tubulin beta-3 chain	50,433	TUBB3
79	Q2HJ81	TBB6_BOVIN	Tubulin beta-6 chain	49,900	TUBB6

80	Q76LV1	HS90B_BOVIN	Heat shock protein HSP 90-beta	83,253	HSP90AB1 HSPCB
81	Q76LV2	HS90A_BOVIN	Heat shock protein HSP 90-alpha	84,731	HSP90AA1 HSP90A HSPCA
82	Q2TBI4	TRAP1_BOVIN	Heat shock protein 75 kDa	79,381	TRAP1 HSP75
83	Q95M18	ENPL_BOVIN	Endoplasmin (94 kDa glucose-regulated protein) (GRP-94)	92,427	HSP90B1 GRP94 TRA1
84	A5PJK0	SPB10_BOVIN	Serpin B10	45,196	SERPINB10
85	Q17QG2	NUDC_BOVIN	Nuclear migration protein nudC (Nuclear distribution protein C homolog)	38,243	NUDC
86	P80209	CATD_BOVIN	Cathepsin D	42,491	CTSD
87	Q58DA7	GLRX3_BOVIN	Glutaredoxin-3 (PKC-interacting cousin of thioredoxin) (PICOT)	37,298	GLRX3 TXNL2
88	Q95140	RLA0_BOVIN	60S acidic ribosomal protein P0 (60S ribosomal protein L10E)	34,371	RPLP0
89	P13214	ANXA4_BOVIN	Annexin A4 (35-beta calcimedin)	35,889	ANXA4 ANX4
90	Q95L54	ANXA8_BOVIN	Annexin A8 (Annexin VIII)	36,787	ANXA8
91	Q2HJD7	3HIDH_BOVIN	3-hydroxyisobutyrate dehydrogenase, mitochondrial (HIBADH) (	35,410	HIBADH
92	P55859	PNPH_BOVIN	Purine nucleoside phosphorylase (PNP)	32,037	PNP NP
93	Q2TBQ8	6PGL_BOVIN	6-phosphogluconolactonase (6PGL)	27,547	PGLS
94	Q58DM8	ECHM_BOVIN	Enoyl-CoA hydratase, mitochondrial	31,243	ECHS1
95	Q9BGI2	PRDX4_BOVIN	Peroxiredoxin-4	30,741	PRDX4
96	Q5E947	PRDX1_BOVIN	Peroxiredoxin-1)	22,210	PRDX1
97	A1L595	K1C17_BOVIN	Keratin, type I cytoskeletal 17	48,712	KRT17
98	P08728	K1C19_BOVIN	Keratin, type I cytoskeletal 19	43,885	KRT19
99	Q9N0V4	GSTM1_BOVIN	Glutathione S-transferase Mu 1	25,635	GSTM1 GSTM
100	Q3T054	RAN_BOVIN	GTP-binding nuclear protein Ran (GTPase Ran) (	24,423	RAN
101	Q9TTK8	KCRU_BOVIN	Creatine kinase U-type, mitochondrial	46,897	CKMT1
102	Q3ZBP1	KCRS_BOVIN	Creatine kinase S-type, mitochondrial	47,231	CKMT2
103	Q29RK1	CISY_BOVIN	Citrate synthase, mitochondrial	51,773	CS
104	P14568	ASSY_BOVIN	Argininosuccinate synthase	46,417	ASS1
105	Q2KIW6	PRS10_BOVIN	26S proteasome regulatory subunit 10B	44,074	PSMC6
106	Q3ZBF6	ACADS_BOVIN	Short-chain specific acyl-CoA dehydrogenase	44,552	ACADS
107	Q3ZC42	ADHX_BOVIN	Alcohol dehydrogenase class-3	39,677	ADH5
108	Q3T0R7	THIM_BOVIN	3-ketoacyl-CoA thiolase, hydrolase	42,131	ACAA2

109	Q0P5J4	K1C25_BOVIN	Keratin, type I cytoskeletal 25	49,313	KRT25
110	Q148H6	K1C28_BOVIN	Keratin, type I cytoskeletal 28	50,775	KRT28
111	Q0P5J6	K1C27_BOVIN	Keratin, type I cytoskeletal 27	49,907	KRT27
112	P38657	PDIA3_BOVIN	Protein disulfide-isomerase A3	56,930	PDIA3 GRP58
113	Q2TBR0	PCCB_BOVIN	Propionyl-CoA carboxylase beta chain	58,311	PCCB
114	A6QLP2	SAHH3_BOVIN	Adenosylhomocysteinase 3 (AdoHcyase 3)	66,774	AHCYL2
115	O62768	TRXR1_BOVIN	Thioredoxin reductase 1, cytoplasmic (TR)	54,770	TXNRD1
116	Q6B855	TKT_BOVIN	Transketolase (TK)	67,906	TKT TKT1
117	Q08E20	ESTD_BOVIN	S-formylglutathione hydrolase (FGH)	31,548	ESD
118	P46193	ANXA1_BOVIN	Annexin A1	38,952	ANXA1 ANX1
119	Q5E9E1	PDLI1_BOVIN	PDZ and LIM domain protein 1	35,881	PDLIM1
120	Q3T145	MDHC_BOVIN	Malate dehydrogenase	36,438	MDH1
121	P52897	PGFS2_BOVIN	Prostaglandin F synthase 2 (PGF 2)	36,742	
122	P05980	PGFS1_BOVIN	Prostaglandin F synthase 1 (PGF 1) (	36,720	
123	Q2HJ58	PRPS1_BOVIN	Ribose-phosphate pyrophosphokinase 1	34,834	PRPS1
124	P52898	DDBX_BOVIN	Dihydrodiol dehydrogenase 3	36,784	
125	Q5E9B1	LDHB_BOVIN	L-lactate dehydrogenase B chain	36,724	LDHB
126	P19858	LDHA_BOVIN	L-lactate dehydrogenase A chain (LDH-A)	36,598	LDHA
127	Q3SYV4	CAP1_BOVIN	Adenylyl cyclase-associated protein 1 (CAP 1)	51,273	CAP1
128	Q07536	MMSA_BOVIN	Methylmalonate-semialdehyde	58,063	ALDH6A1 MMSDH
129	P19483	ATPA_BOVIN	ATP synthase subunit alpha	59,720	ATP5F1A ATP5A1 ATP5A2
130	P12378	UGDH_BOVIN	UDP-glucose 6-dehydrogenase (UDP-Glc dehydrogenase)	55,136	UGDH
131	Q3ZBD7	G6PI_BOVIN	Glucose-6-phosphate isomerase (GPI)	62,855	GPI
132	Q2NKZ1	TCPH_BOVIN	T-complex protein 1 subunit eta (TCP-1-eta)	59,443	CCT7
133	Q29465	SYYC_BOVIN	Tyrosine--tRNA ligase, cytoplasmic (	59,149	YARS1 TYRS YARS
134	Q29443	TRFE_BOVIN	Serotransferrin (Transferrin)	77,753	TF
135	P31039	SDHA_BOVIN	Succinate dehydrogenase	72,944	SDHA SDH2 SDHFP1
136	A6QP57	TGM3_BOVIN	Protein-glutamine gamma-glutamyltransferase E	76,791	TGM3
137	P82292	Z13_BOVIN	Spermadhesin Z13	13,383	

138	P10895	PLCD1_BOVIN	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase delta-1	86,073	PLCD1
139	Q9GK13	MUTA_BOVIN	Methylmalonyl-CoA mutase, mitochondrial (MCM)	83,235	MMUT MCM MUT
140	Q2KJH9	AL9A1_BOVIN	4-trimethylaminobutyraldehyde dehydrogenase (TMABA-DH)	53,977	ALDH9A1
141	Q2KJC9	AL7A1_BOVIN	Alpha-aminoadipic semialdehyde dehydrogenase (Alpha-AASA)	58,582	ALDH7A1
142	P11179	ODO2_BOVIN	Dihydrolipoyllysine-residue succinyltransferase	48,973	DLST
143	P61157	ARP3_BOVIN	Actin-related protein 3 (Actin-2)	47,371	ACTR3 ARP3
144	P34933	HSP72_BOVIN	Heat shock-related 70 kDa protein 2	69,740	HSPA2 HSP70-3 HSPA3
145	Q0VCX2	BIP_BOVIN	Endoplasmic reticulum chaperone BiP	72,400	HSPA5 GRP78
146	P52556	BLVRB_BOVIN	Flavin reductase (NADPH)	22,132	BLVRB
147	Q3ZCH0	GRP75_BOVIN	Stress-70 protein	73,742	HSPA9
148	Q9TRY0	FKBP4_BOVIN	Peptidyl-prolyl cis-trans isomerase FKBP4 (PPIase FKBP4)	51,529	FKBP4
149	P61603	CH10_BOVIN	10 kDa heat shock protein	10,932	HSPE1
150	P01966	HBA_BOVIN	Hemoglobin subunit alpha (Alpha-globin)	15,184	HBA
151	P81948	TBA4A_BOVIN	Tubulin alpha-4A chain (Alpha-tubulin 1)	49,924	TUBA4A TUBA1
152	Q2YDE4	PSA6_BOVIN	Proteasome subunit alpha type-6	27,399	PSMA6
153	Q3ZBV9	DHR11_BOVIN	Dehydrogenase/reductase SDR	27,811	DHRS11 SDR24C1
154	Q3T094	ETHE1_BOVIN	Persulfide dioxygenase ETHE1	27,900	ETHE1
155	P50397	GDIB_BOVIN	Rab GDP dissociation inhibitor beta (Rab GDI beta) (	50,488	GDI2 RABGDIB
156	P21856	GDIA_BOVIN	Rab GDP dissociation inhibitor alpha (Rab GDI alpha)	50,566	GDI1 RABGDIA
157	Q148J6	ARPC4_BOVIN	Actin-related protein 2/3 complex subunit 4	19,667	ARPC4
158	P48644	AL1A1_BOVIN	Retinal dehydrogenase 1 (RALDH 1)	54,806	ALDH1A1
159	P35466	S10A4_BOVIN	Protein S100-A4 (Metastasin)	11,807	S100A4 CAPL
160	Q3T114	RIDA_BOVIN	2-iminobutanoate/2-iminopropanoate deaminase	14,272	RIDA
161	P55052	FABP5_BOVIN	Fatty acid-binding protein 5	15,074	FABP5
162	P02584	PROF1_BOVIN	Profilin-1 (Profilin I)	15,057	PFN1
163	Q3ZCK9	PSA4_BOVIN	Proteasome subunit alpha type-4	29,484	PSMA4
164	P08166	KAD2_BOVIN	Adenylate kinase 2, mitochondrial (AK 2)	26,497	AK2
165	Q1JP75	DCXR_BOVIN	L-xylulose reductase (XR)	25,650	DCXR
166	Q2HJB8	TBA8_BOVIN	Tubulin alpha-8 chain (Alpha-tubulin 8)	50,054	TUBA8

167	P79136	CAPZB_BOVIN	F-actin-capping protein subunit beta (CapZ beta)	33,741	CAPZB
168	P15497	APOA1_BOVIN	Apolipoprotein A-I (Apo-AI)	30,276	APOA1
169	Q3SZ57	FETA_BOVIN	Alpha-fetoprotein (Alpha-1-fetoprotein)	68,588	AFP
170	Q3SZI8	IVD_BOVIN	Isovaleryl-CoA dehydrogenase, mitochondrial (IVD) (	46,498	IVD
171	P56966	GGPPS_BOVIN	Geranylgeranyl pyrophosphate synthase (GGPP synthase)	34,900	GGPS1
172	P49410	EFTU_BOVIN	Elongation factor Tu, mitochondrial (EF-Tu)	49,398	TUFM
173	Q2HJ74	GATM_BOVIN	Glycine amidinotransferase, mitochondrial	48,357	GATM
174	Q5EAC2	GSHB_BOVIN	Glutathione synthetase (GSH synthetase) (GSH-S)	52,066	GSS
175	Q5E946	PARK7_BOVIN	Parkinson disease protein 7 homolog (Maillard deglycase)	20,035	PARK7
176	P00435	GPX1_BOVIN	Glutathione peroxidase 1 (GPx-1)	22,659	GPX1
177	Q5E956	TPIS_BOVIN	Triosephosphate isomerase (TIM)	26,690	TPI1
178	Q3ZBF7	TEBP_BOVIN	Prostaglandin E synthase 3	18,697	PTGES3
179	Q28035	GSTA1_BOVIN	Glutathione S-transferase A1	25,452	GSTA1
180	O18879	GSTA2_BOVIN	Glutathione S-transferase A2	25,717	GSTA2
181	Q29RV1	PDIA4_BOVIN	Protein disulfide-isomerase A4	72,526	PDIA4
182	P13620	ATP5H_BOVIN	ATP synthase subunit d	18,692	ATP5PD ATP5H
183	Q5E9F9	PRS7_BOVIN	26S proteasome regulatory subunit 7	48,634	PSMC2
184	P11178	ODBA_BOVIN	2-oxoisovalerate dehydrogenase subunit alpha	51,678	BCKDHA
185	Q9XSJ4	ENOA_BOVIN	Alpha-enolase	47,326	ENO1
186	Q3ZC09	ENOB_BOVIN	Beta-enolase	47,096	ENO3
187	Q3ZCI9	TCPQ_BOVIN	T-complex protein 1 subunit theta (TCP-1-theta)	59,609	CCT8
188	Q3SX14	GELS_BOVIN	Gelsolin (Actin-depolymerizing factor) (ADF)	80,731	GSN
189	Q3SZU4	CDO1_BOVIN	Cysteine dioxygenase type 1	23,013	CDO1
190	Q3T0X9	DPOD4_BOVIN	DNA polymerase delta subunit 4	12,342	POLD4
191	A5PJZ2	PPM1L_BOVIN	Protein phosphatase 1L	41,042	PPM1L PP2CE
192	A4FUA8	CAZA1_BOVIN	F-actin-capping protein subunit alpha-1 (CapZ alpha-1)	32,932	CAPZA1
193	Q5E997	CAZA2_BOVIN	F-actin-capping protein subunit alpha-2 (CapZ alpha-2)	32,979	CAPZA2
194	Q3T0P6	PGK1_BOVIN	Phosphoglycerate kinase 1	44,538	PGK1
195	P00829	ATPB_BOVIN	ATP synthase subunit beta	56,284	ATP5F1B ATP5B
196	P28801	GSTP1_BOVIN	Glutathione S-transferase P	23,613	GSTP1

197	Q3T0Q4	NDKB_BOVIN	Nucleoside diphosphate kinase B (NDK B)	17,316	NME2
198	P52174	NDKA1_BOVIN	Nucleoside diphosphate kinase A 1 (NDK A 1)	17,261	NME1-1
199	P52175	NDKA2_BOVIN	Nucleoside diphosphate kinase A 2	17,298	NME1-2
200	P13272	UCRI_BOVIN	Cytochrome b-c1 complex subunit Rieske, mitochondrial	29,547	UQCRFS1
201	Q5E958	RS8_BOVIN	40S ribosomal protein S8	24,205	RPS8
202	Q32LG3	MDHM_BOVIN	Malate dehydrogenase, mitochondrial	35,668	MDH2
203	Q2KJE5	G3PT_BOVIN	Glyceraldehyde-3-phosphate dehydrogenase	43,288	GAPDHS
204	O97764	QOR_BOVIN	Zeta-crystallin	35,383	CRYZ
205	Q5E9B7	CLIC1_BOVIN	Chloride intracellular channel protein 1	26,992	CLIC1
206	Q29RZ0	THIL_BOVIN	Acetyl-CoA acetyltransferase, mitochondrial	44,889	ACAT1
207	Q3T108	PSB4_BOVIN	Proteasome subunit beta type-4	29,031	PSMB4
208	Q645M6	FADD_BOVIN	FAS-associated death domain protein	23,002	FADD
209	Q2KJE4	ETFA_BOVIN	Electron transfer flavoprotein subunit alpha, mitochondrial (Alpha-ETF)	34,961	ETFA
210	Q02337	BDH_BOVIN	D-beta-hydroxybutyrate dehydrogenase	38,391	BDH1 BDH
211	Q29448	HMGCL_BOVIN	Hydroxymethylglutaryl-CoA lyase	34,168	HMGCL
212	A6QLY4	ISOC1_BOVIN	Isochorismatase domain-containing protein 1	32,116	ISOC1
213	Q3MHF7	MTAP_BOVIN	S-methyl-5'-thioadenosine phosphorylase ((MTAPase)	31,256	MTAP
214	Q32KP9	NTF2_BOVIN	Nuclear transport factor 2 (NTF-2)	14,478	NUTF2
215	Q76I81	RS12_BOVIN	40S ribosomal protein S12	14,515	RPS12
216	P62992	RS27A_BOVIN	Ubiquitin-40S ribosomal protein S27a	17,965	RPS27A UBA80 UBCEP1
217	P63048	RL40_BOVIN	Ubiquitin-60S ribosomal protein L40 (	14,728	UBA52 UBCEP2
218	P0CH28	UBC_BOVIN	Polyubiquitin-C [Cleaved into: Ubiquitin-related; Ubiquitin]	77,570	UBC
219	P0CG53	UBB_BOVIN	Polyubiquitin-B [Cleaved into: Ubiquitin]	34,308	UBB
220	P02510	CRYAB_BOVIN	Alpha-crystallin B chain (Alpha(B)-crystallin)	20,037	CRYAB CRYA2
221	A4FUZ0	KRT83_BOVIN	Keratin, type II cuticular Hb3	53,989	KRT83
222	Q148H4	KRT81_BOVIN	Keratin, type II cuticular Hb1 (Keratin-81)	54,613	KRT81
223	Q3ZCJ2	AK1A1_BOVIN	Aldo-keto reductase family 1 member A1	36,617	AKR1A1
224	A5PKH3	FAAA_BOVIN	Fumarylacetoacetase (FAA) (EC 3.7.1.2) (Beta-diketonase) (Fumarylacetoacetate hydrolase)	46,156	FAH

225	Q17QJ1	ACSF2_BOVIN	Medium-chain acyl-CoA ligase ACSF2, mitochondrial (EC 6.2.1.2)	68,201	ACSF2
226	Q2KIM0	FUCO_BOVIN	Tissue alpha-L-fucosidase	54,089	FUCA1
227	Q29RH3	NUD12_BOVIN	NAD-capped RNA hydrolase NUDT12 (DeNADding enzyme NUDT12)	50,119	NUDT12
228	P20000	ALDH2_BOVIN	Aldehyde dehydrogenase, mitochondrial	56,653	ALDH2
229	Q3ZBM5	SNX5_BOVIN	Sorting nexin-5	46,819	SNX5
230	A5PJI7	EI2BG_BOVIN	Translation initiation factor eIF-2B subunit gamma	50,218	EIF2B3
231	Q05443	LUM_BOVIN	Lumican (Corneal keratan sulfate proteoglycan 37B core protein)	38,756	LUM LDC
232	Q5EAD2	SERA_BOVIN	D-3-phosphoglycerate dehydrogenase (3-PGDH)	56,452	PHGDH
233	Q3SZ20	GLYM_BOVIN	Serine hydroxymethyltransferase	55,605	SHMT2
234	A2VE99	SEP11_BOVIN	Septin-11	48,992	SEPTIN11 SEPT11
235	Q3SZB4	ACADM_BOVIN	Medium-chain specific acyl-CoA dehydrogenase	46,573	ACADM
236	Q3ZBZ8	STIP1_BOVIN	Stress-induced-phosphoprotein 1 (STI1)	62,482	STIP1
237	P53619	COPD_BOVIN	Coatomer subunit delta (Archain)	57,274	ARCN1 COPD
238	O02675	DPYL2_BOVIN	Dihydropyrimidinase-related protein 2 (DRP-2)	62,278	DPYSL2
239	Q3SZV7	HEMO_BOVIN	Hemopexin	52,209	HPX
240	P45879	VDAC1_BOVIN	Voltage-dependent anion-selective channel protein 1 (VDAC-1)	30,741	VDAC1
241	Q9MZ13	VDAC3_BOVIN	Voltage-dependent anion-selective channel protein 3 (VDAC-3)	30,739	VDAC3
242	Q2TBV3	ETFB_BOVIN	Electron transfer flavoprotein subunit beta (Beta-ETF)	27,699	ETFB
243	Q3T056	LDH6B_BOVIN	L-lactate dehydrogenase A-like 6B	41,592	LDHAL6B
244	P23004	QCR2_BOVIN	Cytochrome b-c1 complex subunit 2	48,149	UQCRC2
245	A6QLQ8	ENDOU_BOVIN	Poly(U)-specific endoribonuclease	47,188	ENDOU
246	P31407	VATB1_BOVIN	V-type proton ATPase subunit B	56,747	ATP6V1B1 ATP6B1
247	P17248	SYWC_BOVIN	Tryptophan--tRNA ligase	53,812	WARS1 WARS
248	P12799	FIBG_BOVIN	Fibrinogen gamma-B chain (Gamma')	50,244	FGG
249	Q0P5A1	DCTN3_BOVIN	Dynactin subunit 3	21,192	DCTN3
250	P41976	SODM_BOVIN	Superoxide dismutase [Mn], mitochondrial	24,638	SOD2
251	P62261	1433E_BOVIN	14-3-3 protein epsilon (14-3-3E)	29,174	YWHAE
252	Q0VC36	1433S_BOVIN	14-3-3 protein sigma (Stratifin)	27,849	SFN
253	P68252	1433G_BOVIN	14-3-3 protein gamma (Protein kinase C inhibitor protein 1) (	28,253	YWHAG
254	P68509	1433F_BOVIN	14-3-3 protein eta (Protein kinase C inhibitor protein 1)	28,212	YWHAH

255	P68250	1433B_BOVIN	14-3-3 protein beta/alpha	28,081	YWHAB
256	Q3SZI4	1433T_BOVIN	14-3-3 protein theta	27,764	YWHAQ
257	P63103	1433Z_BOVIN	14-3-3 protein zeta/delta	27,745	YWHAZ
258	P11966	ODPB_BOVIN	Pyruvate dehydrogenase E1 component subunit beta	39,126	PDHB
259	P81287	ANXA5_BOVIN	Annexin A5 (Anchorin CII)	36,089	ANXA5 ANX5
260	Q28115	GFAP_BOVIN	Glial fibrillary acidic protein (GFAP)	49,512	GFAP
261	Q148H7	K2C79_BOVIN	Keratin, type II cytoskeletal 79 (	57,721	KRT79
262	A6QNX5	K2C78_BOVIN	Keratin, type II cytoskeletal 78	57,464	KRT78
263	P19803	GDIR1_BOVIN	Rho GDP-dissociation inhibitor 1 (Rho GDI 1)	23,421	ARHGDIA
264	P26452	RSSA_BOVIN	40S ribosomal protein SA	32,884	RPSA LAMR1

CAPÍTULO 4

IMPLICAÇÕES

Com a necessidade de dietas com alta densidade energética, progressivamente pesquisas destinadas aos efeitos do uso de carboidratos fermentescíveis e aditivos alimentares serão necessárias. O presente estudo demonstra grande contribuição para elucidação do metabolismo de carboidrato e uso de aditivos no aproveitamento ruminal e cecal.

Foi comprovada a hipótese apresentada, encontrou-se macromoléculas envolvidas na degradação de carboidratos em tecidos do ceco bovino, dietas com maiores teores de amido demonstrando que o aproveitamento de energia a nível de ceco é afetado tanto para o nível de amido e/ou tipo de aditivo incorporado. Para o estudo do rúmen, os dados utilizando a proteômica com técnica 2D-PAGE demostram que a utilização de diferentes aditivos nas dietas de bovinos confinados proporciona distintas rotas e síntese energética. Destaca-se que talvez a utilização de fitas com pH 4-7 poderia favorecer melhor separação dos *spots* proteicos no tecido ruminal.

O trabalho fortalece a necessidade de estudos posteriores para melhor entendimento da digestão e absorção de amido e efeitos de aditivos pós rúmen e comprova elucida como a síntese pode ocorrer no rúmen. Para novos estudos é necessário que haja redução de grupos ou tratamentos, pois, dificulta a análise e conclusão na expressão de proteínas em estudos utilizando eletroforese bidimensional, em contrapartida, essa técnica aliada a Shotgun (Gel Free) pode dar robustez a estudos futuros.