MARIANA MESCOUTO LOPES

EFFECTS OF B VITAMINS AND HYDROXY TRACE MINERALS SUPPLEMENTATION ON HEPATIC METABOLISM OF BEEF CATLE AT FINISHING PHASE

Dissertation submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Magister Scientiae*.

Adviser: Márcio de Souza Duarte

Co-adviser: Tiago Antônio de Oliveira Mendes

VIÇOSA - MINAS GERAIS
2021

Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T
L864e
2021

Lopes, Mariana Mescouto, 1996-
Effects of B vitamins and hydroxy trace minerals supplementation on hepatic metabolism of beef cattle at finishing phase / Mariana Mescouto Lopes. – Viçosa, MG, 2021.
26 f. : il. ; 29 cm.

Orientador: Márcio de Souza Duarte.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Bovinos de corte. 2. Complexo de vitamina B. 3. Minerais na nutrição animal. 4. Metabolismo energético. 5. Proteômica. I. Universidade Federal de Viçosa. Departamento de Zootecnia. Programa de Pós-Graduação em Zootecnia. II. Título.

CDD 22. ed. 636.20852

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APPROVED: February 18, 2021.

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ACKNOWLEDGMENTS

I would like to thank the Universidade Federal de Viçosa, particularly the Animal Science Department, to contributed to my academic and professional growth,

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

To Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), INCT Ciência Animal and Trouw Nutrition, Campinas, SP, Brazil, for the financial support that allowed me to conclued this reasearch,

To my family: Simone, Carlos, Camila, Renata, Enzo e Raul, for all support,

To my friends from BH and Viçosa who believed in me

To my adviser, Professor Márcio de Souza Duarte, for patience, orientation, and friendship through all these years!

To my co-adviser, Tiago Antônio de Oliveira Mendes, for all help and guidance,

To Professor Nick Serão for all shared knowledge and contribution in this work,

To Professor Mateus Pies Gionbelli, and Tiago for participating in my dissertation defense,

To Josiane Lage for trusting me with this work, all support, and guidelines,

To all other professors, employees and students from the Animal Science Department,

To my friends from LABTEC and group of Muscle Biology and Nutrigenomics for all companionship, patience, and help,

To all who participated and contributed to the development of this master's project.

Thank you so much!

ABSTRACT

LOPES, Mariana Mescouto, M.Sc., Universidade Federal de Viçosa, February, 2021. **Effects of B vitamins and hydroxy trace minerals supplementation on hepatic metabolism of beef cattle at finishing phase.** Adviser: Márcio de Souza Duarte. Co-adviser: Tiago Antônio de Oliveira Mendes.

Vitamin B and trace minerals are crucial molecular signals involved in many biological pathways of energy metabolism, immune response, and others, being their bioavailability compromised in high-producing ruminant animals. Despite the current knowledge of the effects of vitamin B complex and trace minerals on animal performance, their use as a rumen-protected form and its impact on liver metabolism in finishing beef cattle is poorly known. The present study aimed to assess the effects of rumen-protected B-vitamin blend and hydroxy trace minerals on the hepatic proteome. A total of 20 non-castrated Nellore males with 353 ± 43 kg of initial body weight were randomly assigned to one of the following treatments: CTRL – inorganic trace minerals without supplementation of protected vitamin B blend; SUP – supplementation of hydroxy trace minerals (Cu and Zn) and protected vitamin B blend (B5, B6, B7, B9, and B12). All animals were fed whit the same level of the experimental diet for 106 days and liver biopsy was performed at the end of the experimental period. We use shotgun proteomics combined with biological and network analyses of the protein differentially abundant between treatments, showing 37 proteins differentially abundant ($P < 0.10$) between treatment groups, where all proteins were up-regulated in the SUP treatment. These proteins were related to protein folding ($P = 0.04$), mitochondrial respiratory chain complex I ($P = 0.01$), and IV ($P = 0.01$), chaperonin-containing T-complex 2 ($P = 0.01$), glutathione metabolism ($P < 0.01$) and others linked to oxidative stress response. These results indicate that rumen-protect vitamin B and hydroxyl trace minerals supplementation during the finishing phase alters the abundance of proteins associated with the electron transport chain and other oxidation-reduction pathways, boosting the production of reactive oxygen species. Such alteration appears to modulate proteins linked to oxidative damage response to maintain cellular homeostasis.

Keywords: Beef cattle. Proteomics. Vitamin B. Hydroxy trace minerals. Liver metabolism

RESUMO

LOPES, Mariana Mescouto, M.Sc., Universidade Federal de Viçosa, fevereiro de 2021. **Efeitos dos fornecimento de hidroximinerais e vitaminas do complexo B sobre o metabolismo hepático em bovinos de corte durante a fase de terminação.** Orientador: Márcio de Souza Duarte. Coorientador: Tiago Antônio de Oliveira Mendes.

Vitaminas do complexo B e micro minerais são descritos como fatores cruciais envolvidos em diversas vias biológicas do metabolismo energético, resposta imune e outras, apresentando sua biodisponibilidade comprometida em animais de alta produção. Apesar do conhecimento atual dos efeitos das vitaminas do complexo B e micro minerais no desempenho animal, seu uso como forma protegida da degradação ruminal e impacto no metabolismo do fígado em bovinos de corte na fase de terminação é pouco conhecido. O objetivo do presente estudo, então, foi compreender os efeitos da suplementação de vitaminas do complexo B protegidas e hidroxi micro minerais durante a fase de terminação no proteoma hepático. Para isto, foi utilizado 20 machos Nelore não-castrados com peso inicial de 353 ± 43 kg distribuidos aleatoriamente em um dos seguintes tratamentos: CTRL – fornecimento de micro minerais orgânicos sem a suplementação de vitaminas do complexo B protegidas; SUP – suplementação de hidroxi micro minerais (Cu e Zn) e de vitaminas do complexo B protegidas (B5, B6, B7, B9, e B12). Todos animais foram submetidos à mesma quantidade das dietas expermentais por um período de 106 dias e, ao final do período experimental, foi realizada biópsia do fígado. Utilizamos a técnica de proteômica *shotgun* combinada com análises de interação de proteínas e de vias biológicas das proteínas diferencialmente abundantes, identificando 37 proteínas mais abundantes no tratamento SUP ($P < 0,10$). Estas foram relacionadas ao dobramento de proteínas ($P = 0,04$), complexo I ($P = 0,01$) e IV ($P = 0,01$) da cadeia transportadora de elétrons, chaperonas moleculares ($P = 0,01$), metabolismo de glutationa ($P < 0,01$) e outras envolvidas na resposta ao estresse oxidativo. Estes resultados indicam que a suplementação de vitamina B e hidroxi minerais protegidos da degradação ruiminal durante a fase de terminação altera a abundância de proteinas associadas a cadeia transportadora de elétrons e outras vias de oxidação-redução, aumentando a produção de espécies reativas de oxigênio. Tais alterações parecem modular a abundância de proteínas ligadas a resposta à danos oxidativos, mantendo, assim, a homeostase celular.

Palavras-chave: Bovinos de corte. Proteômica. Vitaminas B. Hidroximinerais. Metabolismo hepático

SUMMARY

1. CHAPTER I

1.1.General introduction

In the ruminant industry, the substitution of forage as a basal nutritional resource with an elevated level of concentrate can be economically valuable in certain situations, however, it might occur the intensification of subclinical diseases [1]. Moreover, high-producing animals present increased nutrient requirements, due to higher metabolic demand and alterations in the ruminal environment [2]. Therefore, to optimize animal growth and health, is indispensable the knowledge of biological mechanisms underlying nutrient utilization and metabolism for the development of precise feeding strategies [3].

Greater performing and productive cattle lead to greater nutritional demands for energy, macro and micronutrients. For any animal to maintain long-term production, mineral supplementation is necessary [4]. Trace minerals are the smallest components of the diet, but vital for animal health and development due to their involvement in immunity, fertility, metabolism, and production [5]. For example, zinc is a component of the superoxide dismutase enzyme (SOD), eliminating reactive species of oxygen [6]; it participates in protein domains (zinc fingers), holding the structure of transcription factors, which promote the recognition of specific DNA regions to initiate or inhibit transcription [7]; it is linked to the activity of hormones related to appetite control, such as cholecystokinin [8] and leptin [9]. Copper also plays a role in animal growth and health, once is component of several enzymes, such as cytochrome oxidase metalloenzyme (CCO), which is essential for cellular respiration [10], and lysyl oxidase, forming the cross-links of collagen and elastin [11]. Furthermore, low copper levels diets decrease reproductive rates [12], impair the functionality of neutrophils and monocytes [13], and antibody production [14].

There are different sources of trace minerals differing due to the type of chemical bond formed with the ion [5]. Inorganic minerals have ionic bond to an inorganic salt; organic minerals are covalently bonded to any component that has carbon in its composition; and, finally, hydroxyl minerals form covalent bond between hydroxyl groups [15]. Since ionic bonds are "weaker" than covalent bonds, the reactivity of ions from inorganic sources is higher, which leads to their dissociation in the rumen and diminishes the stability of some nutrients, such as vitamin E [16].

The B vitamins are organic compounds required in small amounts, but essential for life as they act as enzymatic cofactors, stimulating the metabolism of carbohydrates, lipids, and amino acids [17]. For example, biotin (B7) is a cofactor for five cellular carboxylases, which

plays a role mostly in lipid metabolism and gluconeogenesis, and it is essential for control genome expression through biotinylation [18]. Pantothenic acid is the precursor of coenzyme A (CoA), an essential enzyme for the tricarboxylic acid cycle (TCA), fatty acid oxidation, leucine metabolism, and for the first step of cholesterol and fatty acid biosynthesis [19]. Pyridoxine (B6), folate (B9), and cobalamin (B12) are responsible for maintaining the one-carbon transfer cycles, donating methyl groups required for mitochondrial protein and nucleic acid synthesis, and play a key role in glutathione biosynthesis [20].

Several studies in the past decade led to the general concept that the concentrations found in basal nutritional resources and the ability of ruminal bacteria to synthesize these vitamins are enough to meet ruminant requirements [21,22]. However, 95% of the B vitamins offered in the diet are degraded by rumen bacteria, for that reason intensive production systems highlight the importance of vitamin supplementation to fulfill the animal's requirements [23]. So, the use of protected B vitamins from rumen degradation can be interesting to meet the metabolism demand of high production animals.

While hydroxy TM and B vitamins have been shown to have impacts on health and development in beef cattle and other species, little research has been done to evaluate the effects of these micronutrients supplementation in calf hepatic metabolism. Therefore, the objective of this study was to compare the effects of hydroxy trace minerals and rumen-protected B vitamins in the liver proteome of finishing beef cattle.

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1. CHAPTER II

Proteomic analysis of liver from finishing beef cattle supplemented with rumen-protected B vitamin blend and hydroxy trace minerals

2.1. Introduction

Micronutrients, like vitamins, carotenoids, and minerals, acts as signals that regulate gene expression and, subsequently, the mRNA, proteins, and metabolites levels [1] and lack of these bioactive compounds may lead to alteration of DNA methylation patterns, impacting gene expression and protein abundance [2]. In this sense, vitamin B complex molecules play a vital role in the activation of several enzymes that constitutes biological pathways of carbohydrate, lipid, protein, and one-carbon metabolism, besides contributing to antioxidant defense [3]. B vitamins are water-soluble vitamins, hence are not stored, and are synthesized by ruminal bacteria, leading to a belief that it's unnecessary to supplement [4]. However, as a result caused by the changes in the ruminal parameters due to greater concentrate intake, several studies have been proved to be beneficial B vitamin supplementation to attend to the requirements of high-producing animals, especially when provided in a rumen-protected form [5-8]. As such, to increase the intake of these vitamins in the intestine, encapsulation technology in a lipid matrix might be employed.

Besides vitamins, trace minerals (TM) are fundamentals nutritional compounds that participate in most biochemical reactions in the body, acting as a significant part of the development and health of domestic animals [9]. TM have multiple roles as a component of metalloenzymatic complexes, controlling gene expression, appetite, fat metabolism, and immune response (zinc); and contributes to hemoglobin formation, growth, and antioxidant defense (cooper) [10,11].

The establishment of the mineral requirement in the livestock is a challenging procedure, once it must examine the type, quality, and processing of the dietetical ingredients due to the interaction of TM with other feedstuff, impacting nutrient balance [12]. The higher reactivity of ions from inorganic sources leads to their dissociation in the rumen, consequently, reducing their bioavailability [13]. Therefore, less reactive ion sources, like hydroxy minerals, may increase the digestibility of vitamins, lipids, and enzymes, reducing ruminal TM dissociation and improves mineral intake due it greater palatability [14-16]. However, the knowledge about the effects of supplementing calves at high growth rates with hydroxy sourced TM is limited.

Despite the current knowledge of the effects of vitamin B complex and trace minerals on animal performance, their impact on liver metabolism in finishing beef cattle is poorly known. The liver is a complex organ fundamental for all metabolic processes, which is responsible for the distribution of energy and integrates signals that respond to hunger or satiety,

processing, and directing nutrients to the body [17,18], and ultimately affects animal performance. Thus, in the current study, we investigated the effects of hydroxy trace minerals and rumen-protected B-vitamin blend in the liver proteome of beef cattle at the finishing phase in pasture with high concentrate.

2.2. Material and Methods

All the protocols related to animal management and handling were approved by the Animal Care and Use Committee of the College of Agricultural and Veterinary Sciences at the *Universidade Estadual Paulista "Júlio de Mesquita Filho",* Jaboticabal, São Paulo, Brazil (protocol number 006000/19). The typical climate of the region is subtropical humid type, with dry winters and wet summers. The pastures used were planted with *Brachiaria brizantha* (Hochst ex A. Rich) Stapf Marandu (Marandu grass).

2.2.1. Animals and experimental diets

Twenty Nellore bulls with 353 ± 43 kg (mean ± SD) of initial body weight, at the finishing phase, and raised under at same grazing conditions were used in this study. Animals were randomly allocated in 8 paddocks in the pasture, 4 of these with 3 animals and the rest with 2 animals. Bulls were subjected to a period of adaptation to the dietary treatments and experimental conditions for 14 days. Following the adaptation period, cattle were randomly assigned to one of the following experimental treatments for 106 days: Control – inorganic trace mineral without supplementation of rumen-protected B-vitamin blend (CTRL, n = 11); Supplemented – with supplementation of rumen-protected B-vitamin blend, containing pantothenic acid (B5), pyridoxine (B6), folic acid (B9), biotin (B7), and cyanocobalamin (B12) (Vivalto® - Trouw Nutrition, Italy) and hydroxyl trace minerals, copper and zinc (Intellibond® - Micronutrients Inc., USA) (SUP, n = 9). The basal diet contained the same composition (15.55% NDT, 16.15% CP, and 84.45% TDN on dry matter basis) and it was provided at the same level between treatments (1.75% of BW). The chemical composition of the mineral/vitamin mixture is shown in Table 1.

2.2.2. Liver biopsy

At the end of the finishing period, all animals were subjected to a liver biopsy. The sampling was performed via needle biopsy (Tru-Cut biopsy needle; Care Fusion Corporation, USA), according to the procedure described by Mølgaard [19]. The incision was made between

the 11th and 12th ribs from the right hepatic lobe, and, immediately, the liver samples (30 mg of tissue) were placed in cryotubes, snap-frozen, and stored in liquid nitrogen until processing.

2.2.3. Protein extraction

Liver samples (30mg) were homogenized using turrax (IKA ULTRA-TURRAX T18 digital, Germany) for 10 seconds in a lysis buffer containing 7M urea, 2M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) detergent, 1% *dithiothreitol* (DTT), and 10 ul protease inhibitor. The supernatant was collected after centrifugation at 10,000 x *g* for 30 minutes at 4ºC. The total amount of protein was quantified by the Bradford method (Bio-Rad Laboratories, USA).

2.2.4. Protein digestion

After quantification, 50ug of the sample was transferred to a tube and 2.5 µL of 100 mM DTT was added. The solution was then agitated and allocated in a heat block to 60°C for 30 min. When the tube reached room temperature, it was add 2.5 µL of 300 mM iodoacetamide for cysteine alkylation. This compound is sensitive to light, so after agitation in a vortex, the samples were transferred to the dark, at room temperature for 30 minutes. 10 µL of trypsin solution (Promega) was added to ammonium bicarbonate (Ambic), stirred in a vortex, and digested at (37°C) overnight. After digestion, the samples were dried in "speed vac" and resuspended in 50 µL of 0.1% *trifluoroacetic acid* (TFA) solution prepared in H2O milliQ.

2.2.5. Mass spectrometry of protein samples

Protein samples were concentrated with the microcolumn ZipTip® C18 (Merck Millipore, USA), according to the manufacture protocol. Mass spectrometry analysis was performed at the Chemistry Institute (*Central Analítica*) from USP (São Paulo, Brazil), following their standard protocol, and was carried out on q-ToF maxis 3G Bruker Daltonics (Thermo Scientific, USA) coupled with Easy NanoLC II (Thermo Scientific, USA). The acquired data were analyzed with MaxQuant software version 1.6.10.43 [20] for protein identification searched against the *Bos taurus* database obtained from UniProt (www.uniprot.org). The following parameters were used: trypsin specificity; two missed cleavages; methionine oxidation and acetylation at protein amino-terminal were specified as variable modifications and carbamidomethylation of cysteine as fixed modification (Supplemental Table S1). Peptide and protein false discovery rate (FDR) was set at 1%. Label-

free quantification (LFQ) was performed and only protein ratios calculated from at least two unique peptides ratios (min LFQ ratio count = 2) were considered for calculation of the LFQ protein intensity. A total of 1400 groups of proteins were identified in the bovine liver samples (Supplemental Table S2).

2.2.6. Statistical analysis

Prior to statistical analyses, proteins that were not detected in at least 15% of animals within each treatment were removed from the dataset. These were then subjected to normalization of the library size and subsequent transformation for analyses using a linear model. The protein abundance data were used to obtain normalizing factors using the Trimmed Mean of M-values (TMM) method from the *TMM* package [21] implemented in *R* [22]. Afterward, the relative abundance of the normalized data was obtained and then $\log_2$-transformed. The data were analyzed using the following linear mixed model:

$$Y_{ijk} = \mu + T_i + p_j + b_1(iBW_k - \overline{\imath BW}) + e_{ijk}$$

where Y_{ijk} is the the $\log_2$-transformed normalized relative abundance of the protein being analyzed; μ is the intercept; T_i is the fixed effect of the i^{th} Treatment (CRTL or SUP); p_j is the random effect of paddock, assuming $p_j \sim N(0, \sigma_p^2)$; b_1 is the partial regression coefficient associated in the effect of initial body weight of the animal; iBW_k is the initial body weight of the k^{th} animal; $\overline{\imath BW}$ is the average initial body weight of the data; and e_{ijk} is the residual associated with Y_{ijk}, assuming $e_{ijk} \sim N(0, \sigma_e^2)$. Prior to final analyses, assumptions of homogeneity and normality of the residuals were met. Analyses were performed in SAS 9.4 (Statistical Analysis System Institute, Inc., USA) with the GLIMMIX and UNIVARIATE procedures.

After analyses, FDR [23] was used to adjust the *P*-values (*q*-values) for the effect of treatment due to multiple testing. Significant differentially abundant proteins (DAP) were identified at *q*-value < 0.10. *q*-values were obtained in *R* using the *qvalue* function of the *qvalue* package [24].

2.2.7. Bioinformatic analysis

The protein-protein interaction, and Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathways enrichment analysis were performed with the String software 11.0 (string-db.org), using *Bos taurus* data and minimum confidence score

required of 0.40 [25]. The functional categorization of GO and KEGG pathway were considered enriched at FDR-adjusted *P*-value (P_{FDR}) < 0.05 based on Benjamini-Hochberg's method [26].

2.3. Results

2.3.1. Differentially abundant proteins

There were 37 DAPs (*q*-value < 0.10 and fold change (FC) ± 2.0) between treatment groups, where all proteins were up-regulated in the SUP treatment. Results are presented in Figure 1.

2.3.2. Pathway analysis of differentially abundant proteins

DAPs were categorized according to GO into biological processes, cellular compartment, molecular function, and KEGG. One enriched term for biological process related to protein folding (P_{FDR} = 0.04), for molecular function, most of the DAPs were associated with catalytic activity (P_{FDR} = 0.01), ion (P_{FDR} < 0.01), and protein binding (P_{FDR} = 0.02). We found that DAPs from the liver of supplemented animals were located in the cytoplasm, oxidoreductase complex (P_{FDR} = 0.01), mitochondrial respiratory chain complex IV (P_{FDR} = 0.01), and chaperonin-containing T-complex 2 (P_{FDR} = 0.01).

2.3.3. Protein interaction of differentially abundant proteins

The interaction network between the DAPs was highly significant ($P < 1.43^{-5}$), indicating that the DAPs are at least partially biologically connected (Fig. 3). Only 12 proteins did not present any interactions.

2.4. Discussion

The present study aimed to investigate the changes in the hepatic proteome of beef cattle at the finishing phase after vitamin B and hydroxy trace minerals supplementation. We used a shotgun proteomic approach based on LC-MS/MS to study liver samples of Nellore bulls classified according to their diets. The liver acts as a sensor of nutrient status and regulates its metabolic activity according to nutrient availability, and its imbalance might lead to impaired energy supply [27]. In this sense, despite we did not measure the hepatic level of B vitamins, Cu, and Zn, we were able to report protein variations related to metabolic pathways (bta01100) up-regulated in the liver of beef cattle supplemented with rumen-protected B vitamin blend and

hydroxy trace minerals, which suggests an effect of micronutrient bioavailability on tissue metabolism.

Both UQCRC2 and COX4I1 are important proteins associated with complex III and IV assembly of the electron transport chain (ETC) [28] and were up-regulated in the liver of supplemented animals. In a previous study, adequate levels of vitamin B, iron, Cu, and zinc Zn might directly act on complex IX activation through the heme biosynthetic pathway [29]. Copper deficiency reduces the expression and activity of complex IV, but not other complexes once it contains two molecules of heme bound with Cu centers, all of which are involved in the electron transfer process [30-32]. The higher mitochondrial protein content and complex protein abundance of the respiratory chain might indicate greater metabolic efficiency according to research findings in steers and broilers [33-35].

The up-regulation of these proteins in the liver of supplemented animals and the GO enriched terms mitochondrial respiratory chain complex IV (GO:0005751), electron transfer activity (GO:0009055), and oxidoreductase complex (GO:1990204) indicate higher activity of the oxidative phosphorylation which may be due to the oxidation of, lipids, carbohydrates, and protein to produce energy to meet energy needs for maintenance. Indeed, we found two up-regulated proteins linked to fatty acid oxidation: ECI1 and ETFDH, the latter physically interacting with ETC complex III at the coenzyme Q reduction site [36]. Our results are similar to previous studies, where copper addition reduced the hepatic fat content by enhancing fatty acid oxidation in rabbits [37], and rumen-protected B vitamins and choline supplementation in transition dairy cows diminish liver fat content in the postpartum [38].

Further evidences of altered lipid metabolism between treatments were the up-regulated proteins CYP2C19, CYP3A24, and CYP3A4 in the SUP group. Cytochrome P450 genes are induced by bile acids and oxysterols, coding for liver enzymes involved in major pathways of cholesterol degradation, vitamin D and bile acids metabolism, and maintaining the homeostasis of xenobiotics and other compounds of endogenous decontamination processes [39]. The CYP enzymes, in addition to mitochondrial respiratory chain, are also sources of reactive oxygen species (ROS), once the normal P450 catalytic cycle generates superoxide anion ($O2^{\bullet-}$) and hydrogen peroxide (H2O2) [40].

The accumulation of ROS causes degradation by nonspecifically attacking membranes, proteins, and nucleic acids, leading to impaired energy expenditure [41]. To diminish the oxidative stress caused by ROS, mammalian cells trigger an antioxidant defense system, which consists of antioxidant enzymes, and several non-enzymatic antioxidants such as glutathione (GSH), cysteine, thioredoxin, and vitamins [42]. Changes in GSH content may result in sub-

optimal growth and altered oxidative stress response, once it was observed in studies with obese mice and humans that the level of GSH in skeletal muscle and adipose tissues is decreased [43,44]. The up-regulated glutathione-S-transferases (GSTM1, and GSTM4) are known for conjugate GSH to xenobiotics for detoxification [44], and their increased abundance in the liver of supplemented animals was linked to glutathione metabolism (bta00480), suggesting higher demand for GSH, and greater capacity for detoxification. However, it has been shown that the liver of finished steers, when compared to growing steers, had a bigger expression of GSTM1, but did not present any change in GSH content [45].

Another indication of hepatic cytoprotective effects by rumen-protected B vitamin blend and hydroxy trace minerals in response to ROS production is the level of highly-regulated proteins named “heat shock proteins” (HSPs). The HSPs concentrations in the cell may increase in response to stress signals, such as oxidative stress, inflammatory conditions, toxic stress, and environmental challenges [46], once they are capable of inhibiting pro-inflammatory/apoptotic pathways through the modulation of nuclear factor (NF-kB), activation of caspases and c-Jun NH2-terminal kinase pathway [47]. In accordance, HSPA1A, HSP90AB1, TCP1, and CCT2 were up-regulated in the liver of SUP group, indicating greater oxidative stress status likely due to a higher metabolic rate. Nonetheless, these proteins were associated with enriched terms related to protein folding processes, which suggests enhanced ability of cell defense against cellular oxidative stress toxic effects [48].

2.5. Conclusions

The present study provided the first evidence that protected vitamin B and hydroxy trace minerals supplementation during the finishing phase alters the hepatic proteome in beef cattle. Our data suggest that higher bioavailability of B vitamin, Cu, and Zn acts directly in the abundance of proteins related to the electron transport chain and other oxidation-reduction pathways, boosting the production of reactive oxygen species. Such alteration appears to modulate proteins linked to oxidative damage response in the liver to maintain cellular homeostasis. More research is warranted to better examine the biological mechanism of these micronutrients on metabolic pathways.

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Tables

Table 1. Chemical composition of mineral/vitamin mixture.

	Control	**Supplemented**	***Units***
Ca	140	140	$g\ kg^{-1}$
P	28	28	$g\ kg^{-1}$
Na	75	75	$g\ kg^{-1}$
K	46	46	$g\ kg^{-1}$
Mg	64	64	$g\ kg^{-1}$
S	23	23	$mg\ kg^{-1}$
Zn	1150[1]	1150[2]	$mg\ kg^{-1}$
Cu	312[1]	312[2]	$mg\ kg^{-1}$
F	465	465	$mg\ kg^{-1}$
Mn	1080	1080	$mg\ kg^{-1}$
Co	31	31	$mg\ kg^{-1}$
I	22	22	$mg\ kg^{-1}$
Vitamin A	62,310	62,310	$UI\ kg^{-1}$
Vitamin D3	8,830	8,830	$UI\ kg^{-1}$
Vitamin E	860	860	$UI\ kg^{-1}$
Vitamin B6	-	161	$UI\ kg^{-1}$
Vitamin B12	-	1.934	$ug\ kg^{-1}$
Vitamin B3	-	20.000	$mg\ kg^{-1}$
Vitamin B9	-	2.175	$mg\ kg^{-1}$
Vitamin B7	-	1.615	$mg\ kg^{-1}$
Monensin	600	600	$mg\ kg^{-1}$

[1] Inorganic mineral
[2] Hydroxy mineral

Figure legends

Figure 1. Differentially abundant proteins.

Figure 2. Function categorization of differentially abundant proteins: (**a**) Molecular function; (**b**) Cellular compartment; (**c**) KEGG.

Figure 3. Protein interaction network of differentially abundant proteins (q-value < 0.10) in the liver between control and supplemented animals. Nodes represent the differentially abundant proteins, and the lines represent the connection between proteins.

Figure 1.

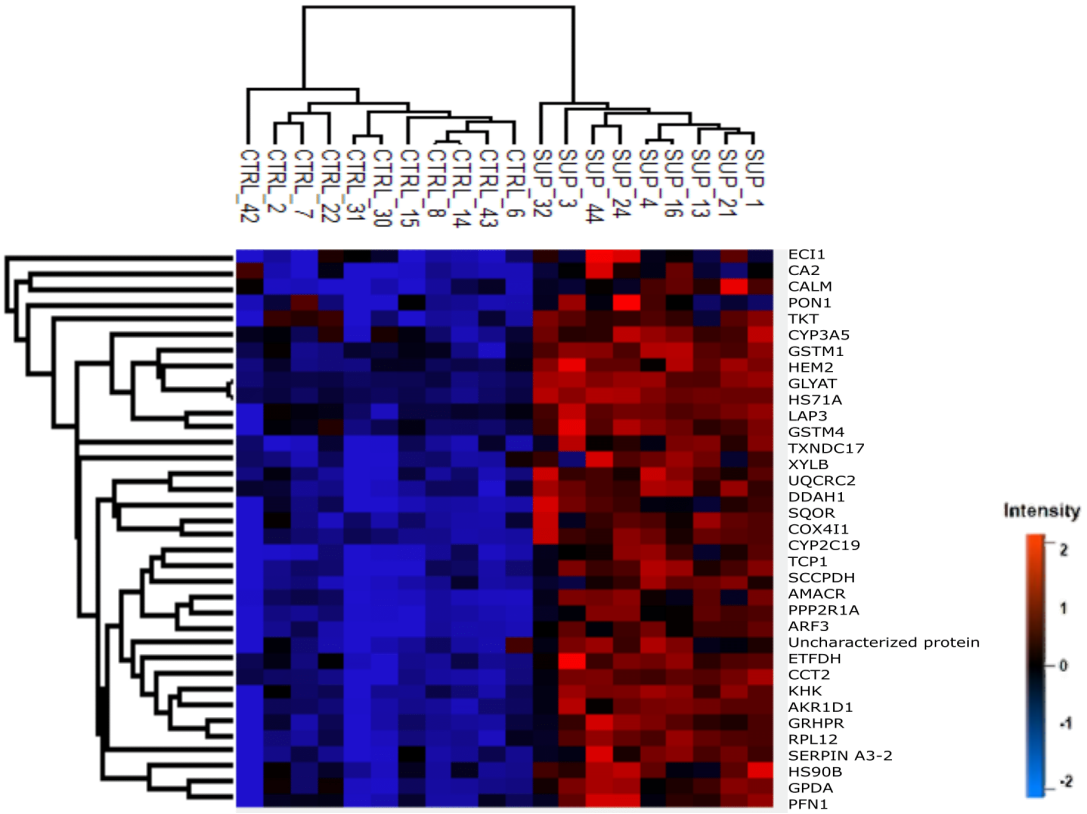


Figure 2.

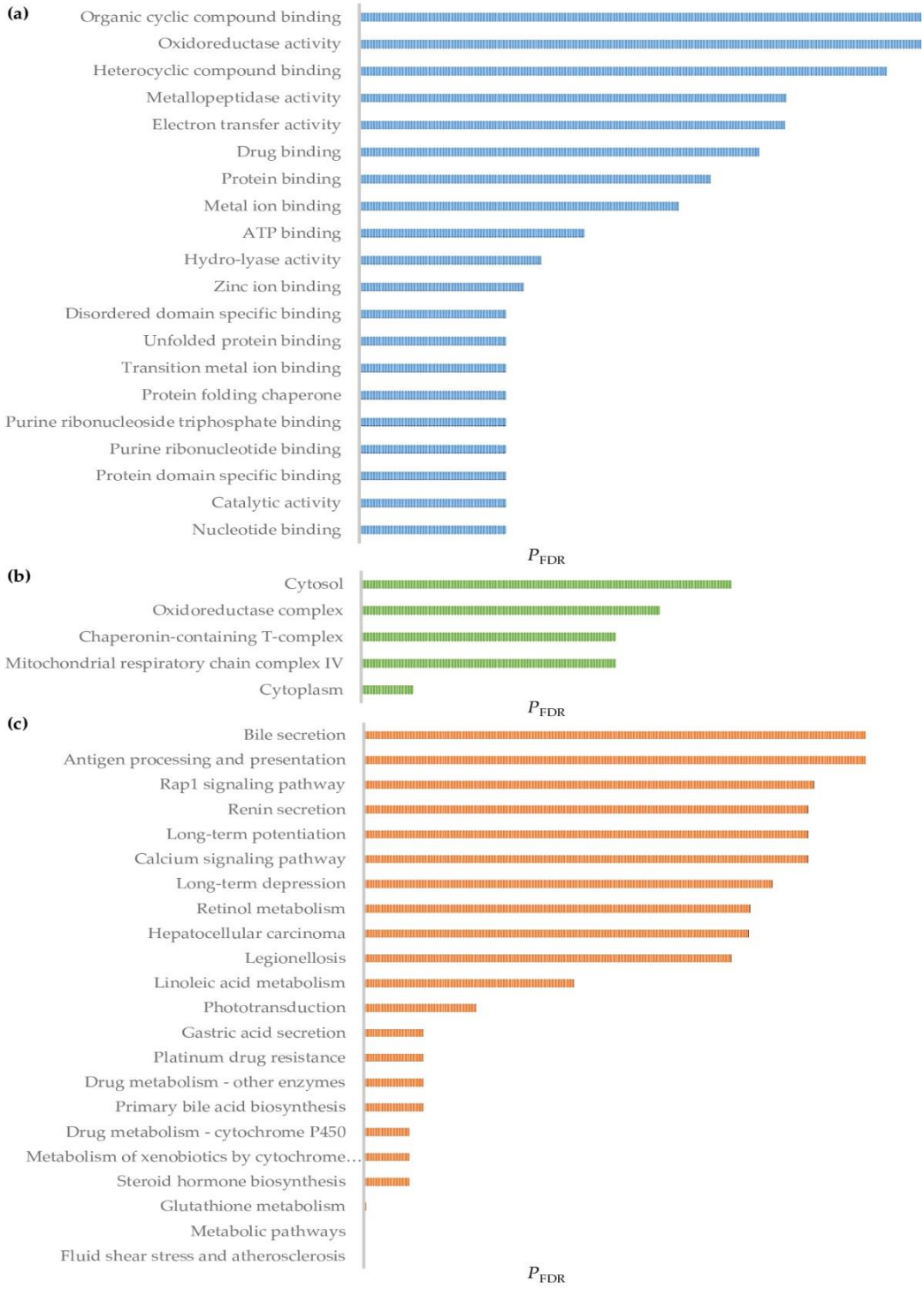
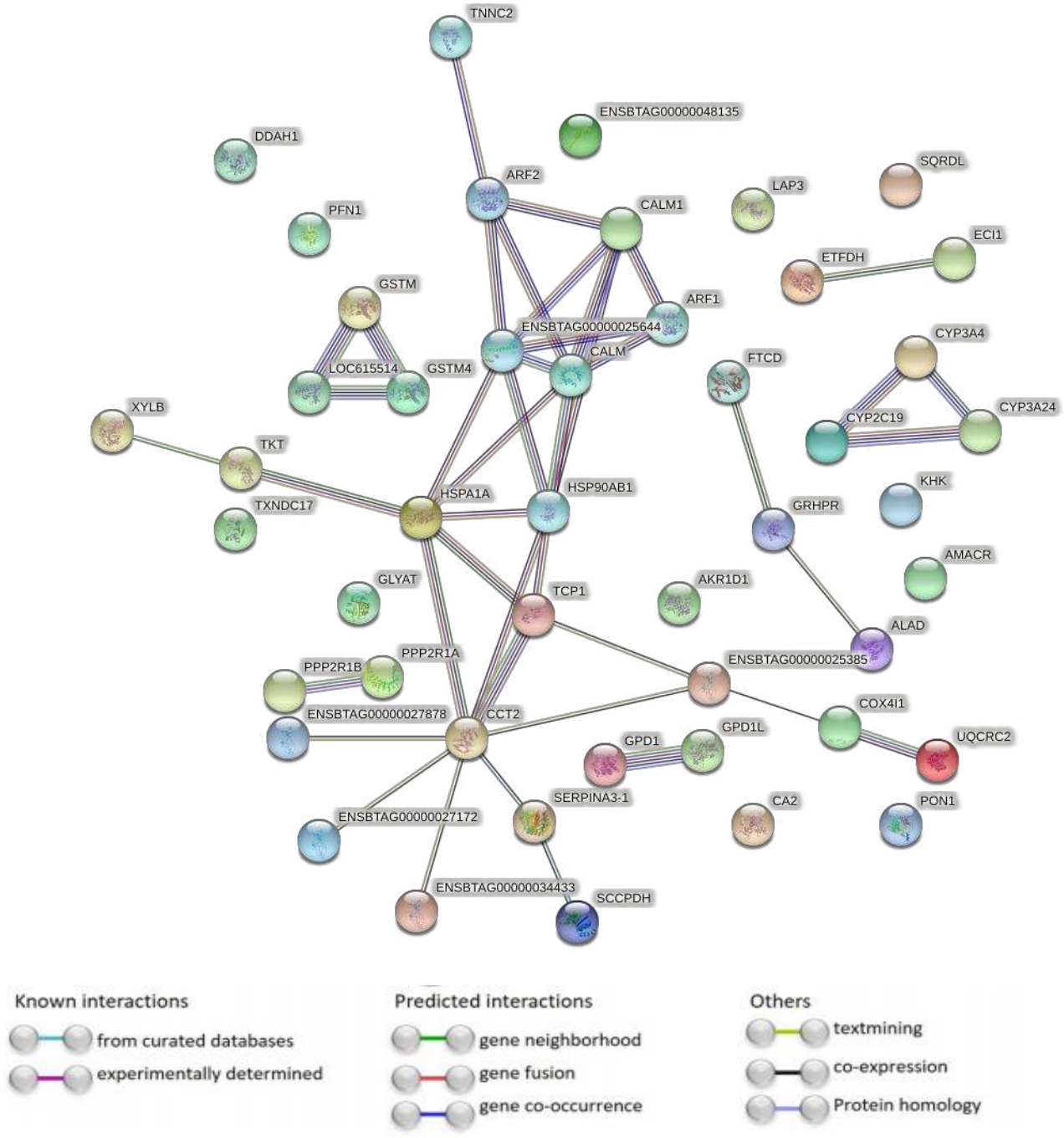

(a)
Organic cyclic compound binding
Oxidoreductase activity
Heterocyclic compound binding
Metallopeptidase activity
Electron transfer activity
Drug binding
Protein binding
Metal ion binding
ATP binding
Hydro-lyase activity
Zinc ion binding
Disordered domain specific binding
Unfolded protein binding
Transition metal ion binding
Protein folding chaperone
Purine ribonucleoside triphosphate binding
Purine ribonucleotide binding
Protein domain specific binding
Catalytic activity
Nucleotide binding
P_{FDR}
(b)
Cytosol
Oxidoreductase complex
Chaperonin-containing T-complex
Mitochondrial respiratory chain complex IV
Cytoplasm
P_{FDR}
(c)
Bile secretion
Antigen processing and presentation
Rap1 signaling pathway
Renin secretion
Long-term potentiation
Calcium signaling pathway
Long-term depression
Retinol metabolism
Hepatocellular carcinoma
Legionellosis
Linoleic acid metabolism
Phototransduction
Gastric acid secretion
Platinum drug resistance
Drug metabolism - other enzymes
Primary bile acid biosynthesis
Drug metabolism - cytochrome P450
Metabolism of xenobiotics by cytochrome…
Steroid hormone biosynthesis
Glutathione metabolism
Metabolic pathways
Fluid shear stress and atherosclerosis
P_{FDR}

Figure 3.


TNNC2
ENSBTAG00000048135
DDAH1
ARF2
SQRDL
LAP3
PFN1
CALM1
ECI1
ETFDH
GSTM
ARF1
ENSBTAG00000025644
CYP3A4
CALM
FTCD
LOC615514
GSTM4
CYP3A24
CYP2C19
XYLB
TKT
KHK
HSPA1A
HSP90AB1
GRHPR
TXNDC17
AMACR
GLYAT
AKR1D1
TCP1
ALAD
PPP2R1A
ENSBTAG00000025385
PPP2R1B
COX4I1
ENSBTAG00000027878
CCT2
GPD1L
GPD1
UQCRC2
SERPINA3-1
CA2
PON1
ENSBTAG00000027172
ENSBTAG00000034433
SCCPDH
Known interactions
from curated databases
experimentally determined
Predicted interactions
gene neighborhood
gene fusion
gene co-occurrence
Others
textmining
co-expression
Protein homology