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Article

Metagenomic and Mineral Content Associations in the Gut of *Bos indicus*

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ABSTRACT: The intestinal microbiota and minerals play vital roles in host metabolism and health, with minerals in the gut potentially influencing microbial activity. This study explored the relationship between gut microbiota and mineral content for understanding the holobiont metabolism. Data were generated from fecal samples collected of 52 Nelore steers (*Bos indicus*) under two dietary groups: conventional and byproduct. An integrative analysis using the DIABLO approach combined metagenomic and mineral data from fecal samples and identified key associations. *Bacillus* correlated positively with calcium which may be used as a probiotic binding mineral. Magnesium was linked to *Paludibacter*, supporting feed efficiency. Phosphorus showed negative correlations with *Listeria*, a pathogenic microorganism, and was negatively correlated with *Roseburia* and the archaeal *Methanocaldococcus*, indicating a role for dietary phosphorus in modulating methane emission. Copper was negatively correlated with *Methanocaldococcus* which also modulating methane emission. Sodium levels influenced aquatic-related bacteria. While this study provides valuable insights, exploring optimal mineral levels in cattle diets can refine strategies for improving health, reducing methane emissions, and maximizing productivity, thus contributing to more sustainable livestock management.

KEYWORDS: *microbiota, minerals, Nelore cattle, methane emission, performance traits*

INTRODUCTION

The gut microbiota is essential for host well-being and is pivotal in diverse metabolic processes. Maintaining a balanced gut microbiota is essential for the host's health, as it prevents the growth of pathogens and produces compounds critical to balance the host's metabolism.[1] In ruminants, the composition of the ruminal microbiome comprises a diversity of microorganisms such as fungi, protozoa, archaea, and bacteria, which are responsible for the fermentation of complex carbohydrates that provide energy for the host.[2,3]

Microorganisms within the microbiota utilize micronutrients for their growth and biological function. Therefore, the dietary intake of micronutrients can influence the compositional and functional structure of the gut microbial community.[4] For example, Lee et al.[5] demonstrated that providing trace minerals to animals positively influenced health and altered bacterial diversity in beef steers.

The intestinal microbiota encloses a diverse microbial community that interacts mutually with its host. This symbiotic interaction aids in their host metabolism, including the production of volatile fatty acids, xenobiotic metabolism, and immune regulation, and shapes their host phenotypes, such as feed efficiency, methane emission and performance traits for animal production.[6,7] Several studies have demonstrated associations between microbiota composition and phenotypes in cattle, such as feed efficiency and methane emission. In some cases, these associations are related to the relative abundance of specific microbial taxa.[2,6,8]

The mineral content of the body primarily depends on its availability, intake, and synergistic or antagonistic effects, such as the presence of chelating compounds in food, the health state of the organism, the environment, and food processing.[1] Knowledge about mineral metabolism in livestock has improved over the years.[9] In beef cattle, during the finishing phase, trace amounts of the nutrients Cu, Co, Se, and I are provided to meet or exceed the requirements for optimal performance.[5] The authors also reported that fecal bacterial diversity was altered by trace mineral supply.[5] The mineral supply required to meet the needs of the ruminal microbiota depends on the energy produced during fermentation.[10] Some studies have investigated the impact of mineral supplementation on microbiota composition.[5,11]

Given the critical roles of both the microbiota and minerals in maintaining homeostasis, exploring their associations could provide valuable insights into their effects on the host's metabolism and phenotype. In this study, we conducted an in-depth investigation into the relationship between minerals and the microbiota in Nelore cattle, focusing on dietary interventions that included both conventional and by-product-based diets. Additionally, we sought to identify biomarkers that could provide valuable information to increase growth and health, predict performance phenotypes and optimize livestock productivity.

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MATERIALS AND METHODS

Sample Collection/Experimental Design. All experimental procedures followed the Animal Welfare and Humane Slaughter guidelines and were approved by the EMBRAPA Livestock Science Ethics Committee on Animal Experimentation, São Carlos, São Paulo, Brazil (Protocol No. 09/2016).

A cohort of 52 Nelore steers (*Bos indicus*) born in 2014 at Embrapa Pecuaria Sudeste (São Carlos, SP, Brazil) were maintained in a feedlot in the same location and were divided into two groups on the basis of the diet provided, conventional or byproduct. The conventional group, $n = 26$, received a diet containing corn silage (72.8%), soybean meal (3.06%), corn grains (21.4%), protected fat (1.19%), urea (0.59%) and a mineral mixture (Confinatto N235 Agroceres Multimix). The byproduct group, $n = 26$, received a diet containing corn silage (57.3%), peanut meal (4.7%), fat corn germ (22.59%), citrus pulp (13.96%), urea (0.30%) and the same mineral mixture (Confinatto N235 Agroceres Multimix). The animals received mineral supplements, active dry yeast, virginiamycin, or monensin in both treatments following Conteville et al.[8] The composition and nutritional levels of both dietary treatments are available in Supplementary Table S1.

In each diet group, the feedlots were divided on the basis of initial body weight (BW), with heavier and lighter animals allocated to each group. An automatic feeding system (GrowSafe Systems Ltd., Airdrie, Alberta, Canada) was used to collect data regarding live weight and daily food consumption. Methane data were obtained via the GreenFeed system (Clock Inc., Rapid City, SD, United States). The animals were slaughtered at 23−24 months of age. Approximately 10 g of stool was collected from each animal 2 weeks before slaughter. The stool samples were kept on ice for approximately 2 h before being stored at −80 °C.

Determination of Mineral Content. The determination of the mineral content in the fecal samples was performed via a model 5110 inductively coupled plasma optical emission spectrometer (ICP−OES, Agilent, Santa Clara, California) with a synchronous vertical dual view (SVDV) system and a model 7800 inductively coupled plasma mass spectrometer (ICP−MS, Agilent, Santa Clara, California) equipped with a collision cell. Argon (>99.99%) from White Martins-Praxair (Sertãozinho, Brazil) was used for both carrier and plasma instrument gas supply, and He (>99.99%, White Martins-Praxair, Sertãozinho, Brazil) was used as collision gas in ICP−MS.

Before analysis, the samples were individually lyophilized (Modelo EC lyophilizer, MicroModulyo, New York, NY) and subjected to cryogenic milling (MA775, Marconi, Piracicaba, Brazil) to decrease the particle size. A sample mass of 100 mg was microwave-assisted digested with 6.0 mL of previously purified 7.0 mol/L nitric acid and 2.0 mL of hydrogen peroxide solution (30% m/v) via the following heating program: (i) ramp of 20 min to 190 °C, (ii) hold for 20 min at 190 °C, and (iii) cool for 10 min. After digestion, the samples were transferred to volumetric vials, and the volume was adjusted to 15 mL with ultrapure water. All the experiments were performed in triplicate.

Analytical blanks were prepared similarly without adding samples to the digestion vessels. Acid digests were diluted 2-fold and analyzed via ICP−OES for the determination of Ca, Fe, K, Mg, Mn, Na, P, S, and Zn via acid-matched external calibration at measuring intervals ranging from 0−1 mg L^{-1} for Fe, Mn and Zn; 0−10 mg L^{-1} for Ca and Mg; and 0−100 mg L^{-1} for K, Na, P, and S. For total mass fraction determination of Cr and Cu, acid digests were diluted 20-fold and analyzed via ICP−MS via acid-matched external calibration at measuring intervals ranging from 0−15 μg L^{-1} for each analyte. Certified reference materials of bovine muscle (SRM 8414, NIST, MD, USA) and bovine liver (SRM 1577c, NIST, MD, USA) were used to evaluate the method's accuracy.

Shotgun Metagenomic Sequencing. The metagenomic data used in this study were previously published under the BioProject ID PRJNA987743.[8] Briefly, total DNA was extracted from 150 mg of homogenized fecal samples using the Quick-DNA Fecal/Soil Microbe Miniprep Kit (ZYMO Research Corp., Irvine, CA), according to the manufacturer's instructions and stored at − 80 °C until sequencing. Metagenomic libraries were constructed with Illumina DNA Prep Kit following the standard protocols. The purified libraries were sequenced on an Illumina NextSeq sequencer platform (ESALQ Genomics Center, Piracicaba, SP, Brazil) using the NextSeq P3 flowcell 300 cycles (Illumina). We obtained an average of ∼51.8 million paired-end reads of 150 bp for fecal samples from the 52 animals.

Data Processing and Normalization. The mineral data were corrected for environmental effects through a linear model, considering a contemporary group composed of weighing group and slaughter date as a fixed effect (Supplementary Table S2). The subsequent analyses used the residual of this model as the new mineral value per sample. The metagenomic reads were trimmed and filtered for quality via Trimmomatic[12] and mapped to the bovine reference genome (ARS-UCD1.2) via Bowtie2.[13] The nonhost reads were extracted and taxonomically classified via Kraken2,[14] with all the bacterial and archaeal genomes in RefSeq from NCBI[15] used as a reference. The resulting genus-level count table for bacteria and archaea was employed for downstream analyses. The residual values of the mineral levels detected in the dietary treatments, conventional and byproduct, and the taxonomic profile are available in Supplementary Tables S3 and S4, respectively.

Integration of Omics Data Sets: Mineral Content and Metagenomic Data. Two omics data sets—taxonomic profiles of bacteria and archaea from metagenomics and mineral content, both from stool samples of Nelore cattle, were assessed through data integration analysis for biomarker discovery via the latent components (DIABLO) approach implemented via the mixOmics R package.[16] DIABLO was used to identify key omics variables (mineral content and metagenomic data) simultaneously during the integration analysis and group classification.

sPLS-DA (sparse-partial least-squares discriminant analysis) was performed to construct the DIABLO model, which, on the basis of a multivariate technique, identifies latent components by maximizing covariance between data sets while selecting a minimal set of highly informative variables via sparse generalized canonical correlation analysis (sGCCA).

The selection of correlated features across both omics data sets was performed with least absolute shrinkage and selection operator (lasso) penalization on the variable coefficient vector defining the linear combinations. sGCCA maximizes the correlation between linear combinations of variables, named as latent components, and projects the data into a smaller space spanned by these components.

Tuning parameters, such as the number of components and the number of variables selected from each data set, were optimized via cross-validation to prevent overfitting and improve model robustness. The performance of the model was evaluated via 10-fold cross-validation repeated 10 times for up to 5 components, and the number of components was given according to the centroid distance and the balanced error rate. The model's efficiency with three components was evaluated in terms of the area under the curve (AUC). The final sPLS-DA model includes tuned parameters to extract the correlations between data sets and discriminate the correlated features.

Differences between Diet Groups. To test whether the selected variables, from the DIABLO approach, contributing to the model's components are capable of separating between diet groups, we performed a partial correlation analysis (PCA) considering two scenarios: all the selected variables and only the four variables with the highest contribution. The results were visualized as a plot via PC1 and PC2.

RESULTS

Discriminating the Groups of Animals Fed by the Byproduct or Conventional Diet. Multivariate analysis via the DIABLO approach was performed to identify potential biomarkers and explore the relationships between two omics data sets, metagenomic data and mineral content data, both of which were extracted from stool samples of Nelore cattle. The animals were divided into two dietary groups, the conventional

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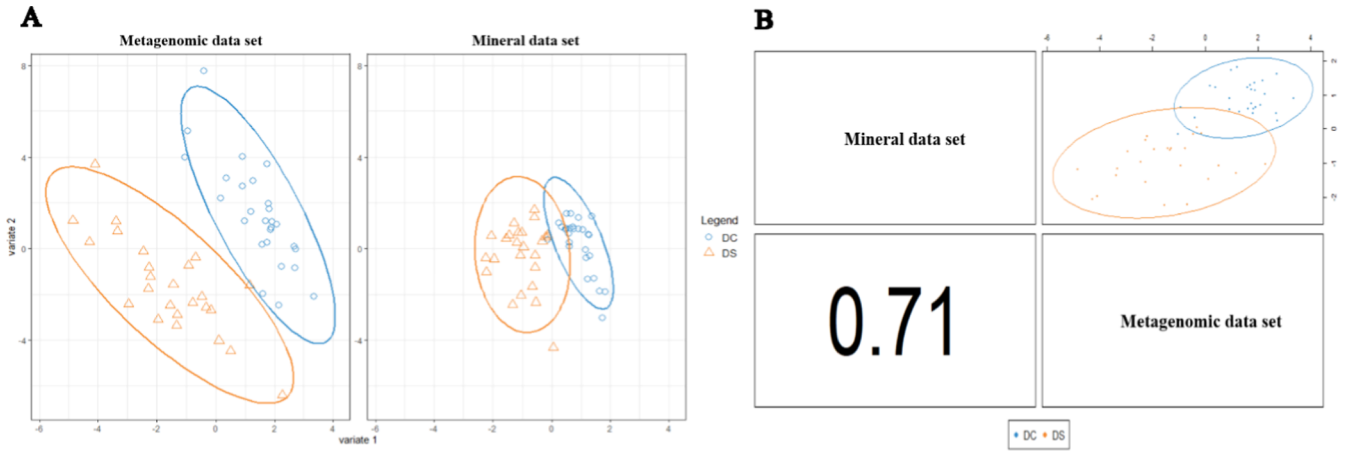

Figure 1. (A)-Sample plot from multiblock sPLS-DA showing the degree of agreement for two interventional diets, DC (conventional) and DS (byproduct diet), and the discriminative ability of each data set, according to their scores on the 1--2 components. On the left are the results obtained from the metagenomic data set; on the right are the results obtained from the mineral data. (B)-Diagnostic plot displaying the correlation coefficient between the first components from each data set.

group and the byproduct group, on the basis of the composition of the diet they received. This method enables the integration of heterogeneous data types while maximizing correlation and ensuring the identification of key variables that contribute to the differentiation of these two groups.[17]

The results revealed segregation between the dietary groups across both data sets. Figure 1A shows this separation via sparse partial least-squares discriminant analysis (sPLS-DA), a supervised method designed to differentiate data into classified groups by selecting the most discriminative features. The model revealed that the first components of the mineral content and metagenomic data sets were well correlated, allowing effective discrimination between the conventional and byproduct dietary groups (Figure 1B).

The DIABLO model demonstrated that the selected features can differentiate the conventional and byproduct dietary groups, as shown in Figure 2. The most important variables are ranked from the bottom to the top, according to the absolute value of their coefficients in each component, with the color indicating the dietary group in which the variable shows the highest level of contribution. For component 1, the loading plot identified calcium (Ca) as the most significant mineral feature for classifying Nelore cattle subjected to a byproduct diet. Additionally, *Caldibacillus* and *Weizmannia* stood out as the most influential microbial genera for the classification of cattle on a byproduct diet (Figure 2A). For the conventional diet, sodium (Na) made the most significant contribution to the first component, whereas chromium (Cr) and copper (Cu) made the most significant contribution to component 2. The methanogenic archaea *Methanocaldococcus and Methanobrevibacter*, as well as the Gram-positive bacterium *Metabacillus*, were identified as the main variables used to discriminate the conventional dietary group, with a high contribution from component 2 (Figure 2B).

Two PCA plots were constructed to further visualize the differences between the investigated dietary groups using either all selected variables contributing to one of the model components or only the features with the highest contribution to each component (Figure 3A,B, respectively). When all the selected variables (PC1 (82.54%) vs PC2 (5.07%, Figure 3A) were used, there was no separation between the dietary groups. However, a separation of the diets can be noted in the PCA plot constructed using only the features with the greatest contribution to each component of the model (PC1 (74.67%) vs PC2 (24.75%), Figure 3B). Therefore, the most discriminative variables included the mineral calcium (Ca) and the bacteria *Caldibacillus*, both of which are features selected in the first component, and the mineral copper (Cu) and the archaea *Methanocaldococcus*, both of which are features selected in the second component. These features could better discriminate the byproduct from conventional dietary groups, as illustrated in Figure 3B.

Correlations between Selected Features. The limited knowledge of the connection between microorganisms and host minerals and how they affect the host's metabolism emphasizes the contribution of our investigation. To this end, the integrative approach implemented in our work aims to identify key molecular features that clarify this relationship and uncover biomarkers relevant to the host's metabolism.

The clustered image map (CIM) (Figure 4A) displays an overview of the classification of the two dietary groups, conventional and byproduct, on the basis of the signatures identified in the first component. For this component, the byproduct group presented a greater abundance of the bacterial genus *Caldibacillus*, whereas sodium (Na) was the most abundant mineral in animals subjected to the conventional diet, and calcium (Ca) was more prevalent in those subjected to the byproduct diet.

The correlations between selected features within data sets were explained by each feature's vector and their respective component, generating positive or negative correlations. As a result, the model generated a correlation network between minerals and microbial taxa (Figure 4B).

Copper (Cu) was negatively correlated with the archaea *Methanocaldococcus* (−0.85) and a set of bacteria: *Listeria* (−0.75), *Fusobacterium* (−0.86), *Pradoshia* (−0.83), *Methanobacterium* (−0.73), *Alkaliphilus* (−0.81), *Pyrococcus* (−0.71), *Gottschalkia* (−0.81), *Thermobacillus* (−0.70), *Metabacillus* (−0.81), *Lentibacillus* (−0.75), *Sccharothrix* (−0.73), *Peptoclostridium* (−0.75), *Thermoanaerobacterium* (−0.79) and *Amycolatopsis* (−0.74). All these genera were more abundant in Nelore cattle fed a conventional diet.

Calcium (Ca), which was present at relatively high levels in the byproduct diet, was positively correlated with the bacteria

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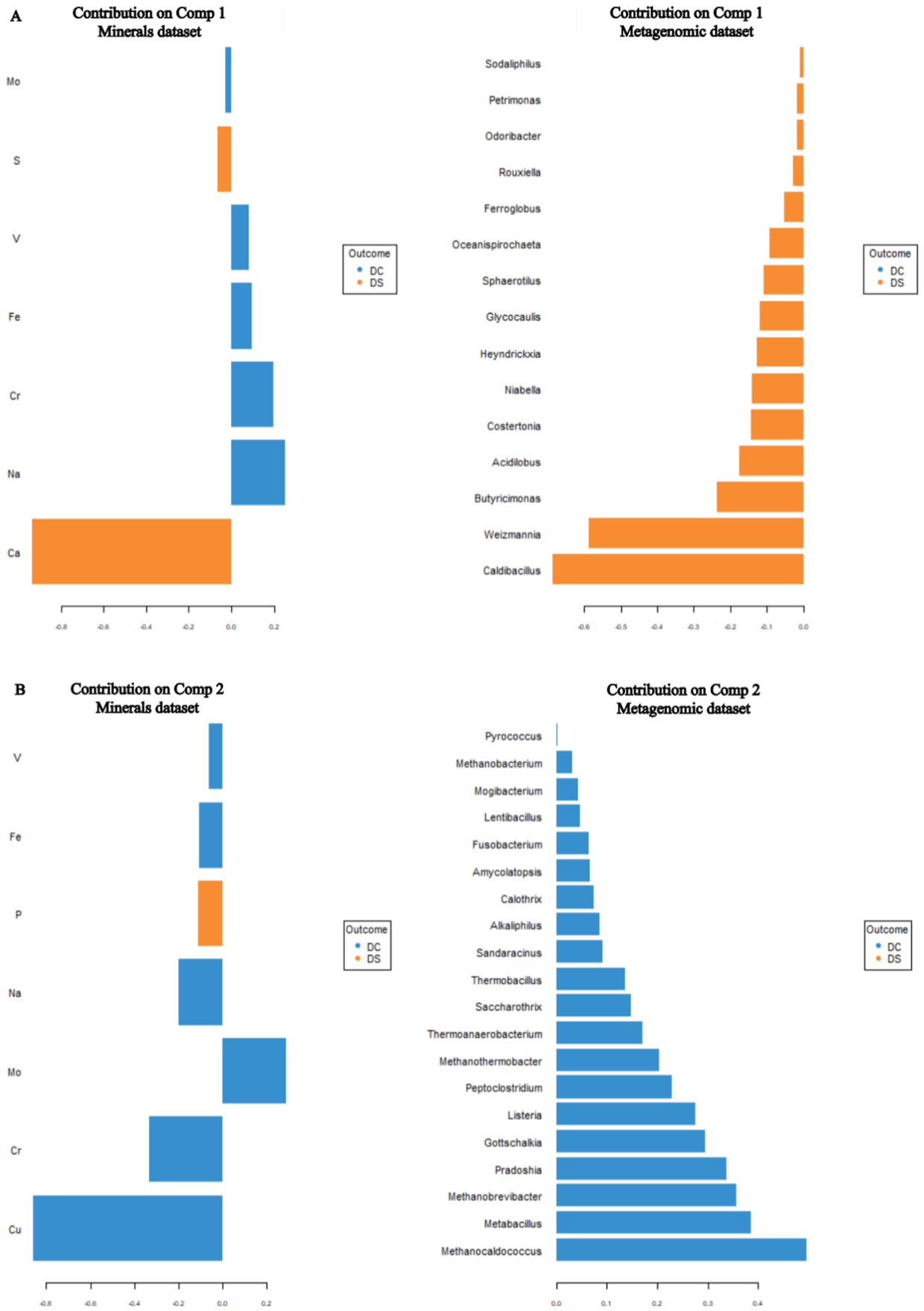

Figure 2. Loading plot for the features selected from the DIABLO model: (A)-component 1; (B)-component 2. The most important variables are ordered from the bottom to the top, according to the absolute value of their coefficients. The colors indicate the class for which the median

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Figure 2. continued

expression value is the highest for each variable, and each panel represents one data set, including mineral and microorganism genus data. DC (conventional) and DS (byproduct diet).

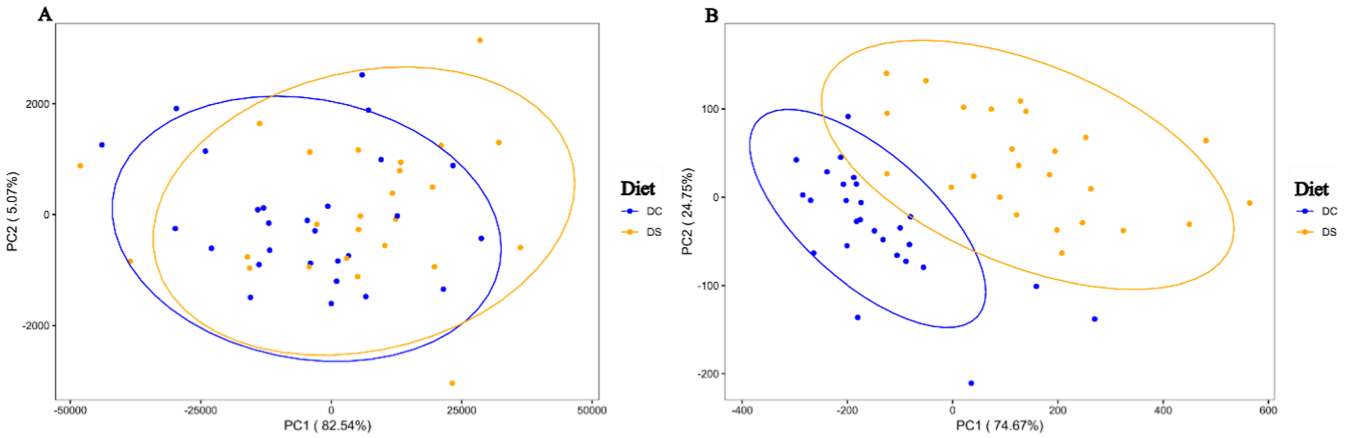

Figure 3. (A)−PCA score plot of the conventional versus byproduct diet (PC1 vs PC2) with all selected variables according to the loading weights in each component of the DIABLO model. (B)-PCA score plot of the conventional versus byproduct diet (PC1 vs PC2) with the variables that presented the greatest contribution to each component of the DIABLO model. DC (conventional) and DS (byproduct diet).

Caldibacillus (0.9), *Weizmannia* (0.86), and *Heydricksxia* (0.76), which were also identified as key contributors to classifying Nelore cattle on a byproduct diet. Magnesium (Mg) and the bacteria *Paludibacter* were positively correlated (0.71), both discriminating the Nelore in the byproduct diet from those in the conventional diet.

In the conventional diet sodium (Na) was negatively correlated with a set of bacteria: *Fluviibacter* (−0.77), *Paludibacter* (−0.74), *Solitalea* (−0.72), *Maribacter* (−0.72), *Aquimarina* (−0.72) and *Cytophaga* (−0.72). Phosphorus (P) was negatively correlated with the archaea *Methanocaldococcus* (−0.70) and the bacteria *Listeria* (−0.7), *Fusobacterium* (−0.76) and *Roseburia* (−0.76).

Performance of the DIABLO Model. The performance of the model was represented by the receiver operating characteristic (ROC) curve and area under the curve (AUC), both of which are derived from the DIABLO model. The ROC curve balances the true positive rate (sensitivity) and the false positive rate (specificity). In contrast, the AUC quantifies the probability that the model can distinguish between groups as a summary of the ROC curve. Therefore, the discriminative ability to predict features between the two interventional diets was given by the area under the curve (AUC) (Figure 5).

The model accuracy for the mineral content data set was 0.997, with a Wilcoxon *t*-test *p* value of 8.016×10^{-6}, indicating a highly significant difference between the predicted components for each group. The metagenomic data set showed an accuracy of 0.9185, with a Wilcoxon *t*-test *p* value of 5.818×10^{-6}, using two components of the multiblock sPLS-DA model.

DISCUSSION

Associations between Selected Features from the Mineral Content and Microbiota. *Calcium.* The correlation network revealed strong positive associations between calcium and three bacteria belonging to the *Bacillus* genus, *Caldibacillus*, *Weizmannia* (*Bacillus coagulans*), and *Heydricksxia*, which are strains classified as Gram-positive, nonpathogenic, rod-shaped, and endospore-producing bacteria, respectively.[18] This positive correlation indicates a beneficial effect on the Ca content in the gut, as *Bacillus* spp. are considered gut health promoters.[18] A previous study with this same Nelore population revealed a significant enrichment of *Bacillus* genera in the microbiome of the byproduct diet, supporting our findings of a positive correlation with Ca.[8]

Bacillus strains have shown the potential not only to produce a variety of digestive enzymes but also to improve the host's digestive enzyme activities during fermentation. The breakdown of polysaccharides promotes the regulation of the intestinal flora through the growth of beneficial microorganisms such as *Lactobacillus* and *Bifidobacterium*, which prevent the proliferation of harmful bacteria and enhance the immune function of the intestine, preventing inflammation.[19,20]

Several studies have reported benefits to the host when an adequate amount of *Bacillus* strains is administered.[20−22] The potential role of probiotics in regulating microbiota composition and activity, enhancing immunity, and alleviating metabolic disorders has been demonstrated. They can promote anaerobic and acidic intestinal conditions that are harmful to pathogenic bacteria but beneficial to the production of short-chain fatty acid-producing bacteria, including the release of acetate, butyrate, and propionate, which are essential for the health and well-being of the host.[20] Specifically, for cattle, *Caldibacillus* strains can potentially improve Japanese black cattle's growth and performance.[23]

Bacillus coagulans have shown the ability to generate a broad spectrum of bioactive peptides with numerous biotechnological and biopharmaceutical applications. One of these factors is the capacity of producing calcium-binding peptides to positively affect host health, preventing the deficiency of this element and promoting the balance of metabolic functions.[21]

Calaca and collaborators[24] reported that Nelore cattle fed a diet supplemented with a *Bacillus* probiotic strain, in spore form, did not improve dry matter intake or nutrient

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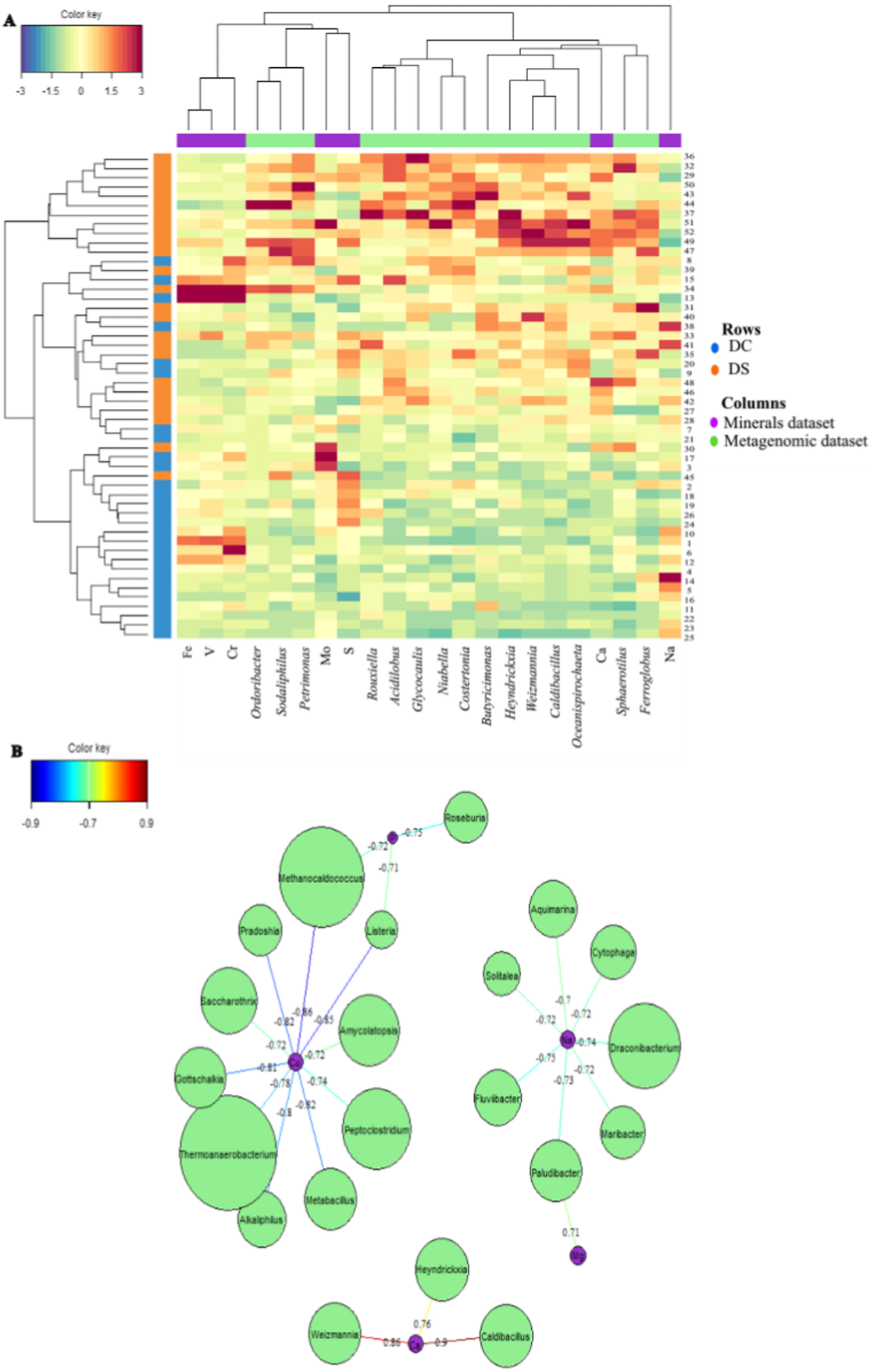

Figure 4. A)– Clustered image map (CIM) represents the samples in rows and selected features in columns and their classification according to the two groups of interventional diets, conventional and byproduct. (B)- Network correlation between minerals (purple circles) and microbiota (green circles); both data sets were obtained from fecal contents. The color of the edges indicates the relationship between latent variables, with a

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Figure 4. continued

cutoff >0.7. DC (conventional) and DS (byproduct diet). The numbering on the right-hand side of the matrix are the individual cattle coded in Supplementary Tables S2, S3 and S4.

digestibility. However, they reported higher isovalerate levels and a significant reduction in carcass fat deposition in Nelore cattle in tropical grass pastures. Isovalerate is a byproduct of the degradation of certain amino acids, such as valine, isoleucine, leucine and proline. It is a crucial nutrient for ruminal cellulolytic bacteria, contributing to a reduction in the acetate/propionate ratio.[25,26] The positive correlation between *Bacillus* strains and Ca quantified in our work suggests that Ca supplementation can affect host health and productivity. However, some authors also suggest either no effect of calcium supplementation in cattle performance.[27−29]

Magnesium. This study revealed a strong positive correlation between Mg and *Paludibacter*, a propionate-producing bacterium. *Succinivibrio*, *Paludibacter* and *Coprobacillus* are bacterial genera abundantly found in animals with relatively high feed efficiency.[7] These genera, which are commonly found in feed-efficient animals, facilitate carbohydrate fermentation, resulting in the complete metabolism of starch. *Paludibacter* plays an important role in metabolism, increasing inositol phosphate production and helping maintain homeostasis.[30] In this context, more efficient animals are more prone to produce short-chain fatty acids via carbohydrate fermentative microorganisms.[7,30] Additionally, *Paludibacter* is reported to be a genus of strongly propionate-producing bacteria and a potential pathway to decrease methane emission by increasing propionate formation.[30]

Magnesium is an essential element for microorganisms in rumen metabolism. It is involved in activating several enzymatic reactions, including phosphohydrolases, phosphotransferases and pathways involving ATP and thiamine pyrophosphatase reactions.[10] These authors reported that magnesium-deficient diets can impair glucose metabolism, resulting in a reduction in volatile fatty acids and in gas production during fermentation.

The depletion of magnesium is known to affect physiological processes in the rumen due to changes in pH, resulting in unbalanced physiological processes. A study conducted with sheep revealed that the withdrawal of magnesium decreased feed consumption in the first days of the experiment and increased the pH significantly, from 6.3 to 7.3 ($P < 0.01$), possibly because of a reduction in microbial digestion, as shown by in vitro and in vivo assays.[31] Additionally, a reduced concentration of volatile fatty acids such as acetic, propionic, and butyric acids was observed, indicating the importance of magnesium in the rumen for maintaining pH and, consequently, cellulolytic activity.[31]

However, a study conducted with dairy cows revealed that elemental Mg supplementation favored the growth of methanogenic bacteria and increased H_2 generation after 2.5 h of feeding. The pulse of H_2 formation promotes H_2 incorporation into succinate, involving the transformation of oxaloacetate to malate followed by the conversion of fumarate to succinate. This process is achieved by increasing the abundance of the *Christensenellaceae* and *Bacteroidales_BS11 groups*, which may favor the growth of methanogens.[32] Nonetheless, succinate is a precursor of propionate and can act as an H_2 sink. The correlation between *Paludibacter* and magnesium levels identified in our study indicates that higher magnesium contents support the growth of propionate-producing bacteria. In support of this hypothesis, we identified *Paludibacter* as one of the sPLS-DA discriminating features against the group of animals fed a byproduct diet, which had a 166% Mg mass fraction compared with the conventional diet, and Malheiros et al.[33] reported greater propionate in both the rumen and feces samples of byproduct-fed animals.

Phosphorus. A negative correlation was found between phosphorus and the bacteria *Listeria*, *Roseburia*, and the archaea *Methanocaldococcus*. *Roseburia intestinalis*, an anaerobic bacterium belonging to the *Lachnospiraceae* family, maintains the homeostasis of the host by producing butyrate via fibers as a source of carbon.[8,34,35] Accordingly, *Roseburia* was previously found to be more abundant in the conventional diet,[8] in which P is 56% more abundant of the content of the byproducts. The same authors demonstrated an association between these butyrate-producing bacteria and elevated methane emission in the conventional diet group. They identified genes encoding specific metalloenzymes ([NiFe]- and [FeFe]-hydrogenases) and isoenzymes (formate hydrogenases and dehydrogenases) involved in fermentation pathways. These enzymes play a role in H_2 catalysis and formate production or consumption, potentially influencing methane dynamics.[36] Thus, our results suggest that increasing P in the diet might decrease methane emissions.

Phosphorus significantly contributes to bacterial growth and plays a critical role in bacterial structure and metabolic processes. Phosphate may be stored as a polyphosphate, which is an energy source for metabolic functions and an essential coenzyme for the activity of fibrolytic enzymes.[37] Thus, the negative correlation observed between *Listeria monocytogenes*, a pathogenic microorganism, and phosphorus needs further investigation since this microorganism can utilize sources of phosphorus, such as inositol-phosphate, as a carbon and energy source to replicate in the gastrointestinal tract.[37] Evidence suggests that sodium polyphosphates can inhibit the growth of *L. monocytogenes*.[38] Although it is unclear whether the phosphorus in our diet was in the form of polyphosphates, the results indicated a negative association between dietary phosphorus and *L. monocytogenes*.

An additional notable discovery supporting our findings of a negative correlation with P was reported in prior studies from our group with the same animals, which identified the genera *Methanocaldococcus* (formerly known as *Methanococcus*) and *Methanobrevibacter*, both methanogenic archaea, as enriched genera in cattle fed a conventional diet (lower P).[6,8] The authors also associated these taxa with methane emission, thus providing evidence for the role of dietary P in indirectly modulating methane mitigation.

Copper. Copper was negatively correlated with the abundances of two archaeal genera, *Methanocaldococcus* and *Pyrococcus*, and 13 bacteria, *Listeria*, *Fusobacterium*, *Pradoshia*, *Methanobacterium*, *Alkaliphilus*, *Gottschalkia*, *Thermobacillus*, *Metabacillus*, *Lentibacillus*, *Sccharothrix*, *Peptoclostridium*, *Thermoanaerobacterium* and *Amycolatopsis*. In the present study, individual differences in Cu content could not be attributed to diet, as both formulations were equivalent for this element.

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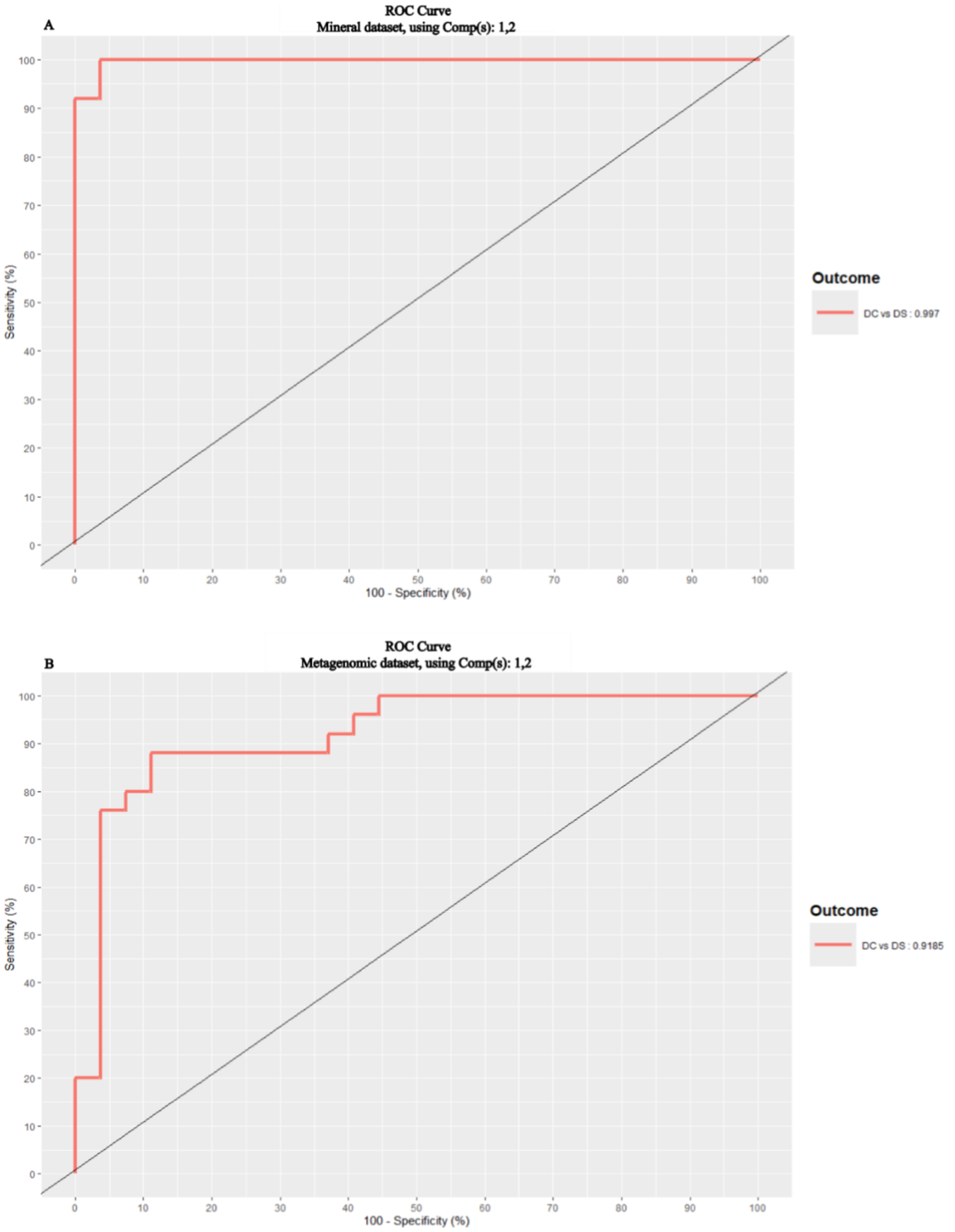

Figure 5. Performance test showing the receiver operating characteristic curve (ROC) and area under the curve (AUC) based on the DIABLO model for the A-mineral content and B-metagenomic data identified in the stool samples of Nelore cattle.

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The copper balance in an organism is critical because it provides a strong redox reaction between the oxidized (Cu^{2+}) and reduced (Cu^{+}) states for many enzymatic reactions essential for biological processes such as melanin formation, respiration, iron uptake, iron transportation, and superoxide detoxification.[39] When in excess, it contributes to the propagation of reactive oxygen species, which may cause damage to the host or its microbiota.[39,40] In contrast, animals deficient in trace elements, such as Cu, are linked to the immune system and enzymatic disturbances due to an increase in susceptibility to bacterial pathogens due to the inefficiency of superoxide dismutase and cytochrome C oxidase enzyme systems.[39−41] Prokaryotic organisms have a limited need for Cu in their biochemistry. However, many bacterial genomes encode Cu exporters and Cu transporter proteins, known as chaperones, to protect against copper toxicity and manage elevated Cu levels as a mechanism to evade the host immune system.[42] A general model for Cu homeostasis in bacterial cells involves three key functions: (1) Cu sensing and activation of transcriptional responses; (2) increasing the production of cuproprotein and Cu efflux pumps; and (3) increasing the synthesis of multiple copper oxidases.[42,43]

The negative correlation between *Pyrococcus* and copper found in our study may be related to the activation of the metalloregulatory protein CopR, which initiates a cellular detoxification process,[44] as suggested by the transcriptome response of the hyperthermophilic archaeon *Pyrococcus furiosus* to increasing Cu levels. The archaeon *Methanococcus*, which was negatively associated with Cu in our study, was found to be significantly associated with higher methane emission and was highly abundant in the conventional diet group in recent studies with the same cohort.[6,8]

A negative correlation was observed between the amount of Cu and the biomass production of the archaeon *Methanococcus maripaludis*,[45] which had a major impact on the metabolism of this taxon. Specifically, Cu can modify the catalytic site of acetyl-CoA synthetase, one of the two pathways employed by *M. maripaludis* to synthesize acetyl-CoA.

Additionally, some authors have demonstrated that the application of copper slightly increased the lag phase of methanogen growth, such as *Methanobacterium*, which resulted in decreased methane production. However, the amount of copper necessary to inhibit methanogens is unknown because Cu may precipitate in the presence of sulfide.[46,47] This interaction might occur in diets with a high content of S, which is naturally found in molasses, a byproduct from the sugar cane industry and is commonly a source of dietary sugars fed to cattle. Additionally, the formation of CuS can originate from interactions with pasture fertilizers, high-S water, and S-containing supplements that reduce Cu bioavailability.[41]

We also found a negative correlation between Cu and the bacteria *Listeria* and *Fusobacterium*, including pathogenic species. Although there is no specific literature providing evidence of this particular relationship, as well as the remaining negatively correlated taxa, Cu has many toxic effects on prokaryotic cells, and mechanisms have evolved to protect them from these effects.[40,43]

In general, Cu toxicity is closely linked to redox properties, which generate reactive oxygen species (ROS) via a Fenton-like reaction under aerobic conditions.[43] However, anoxic mechanisms involve the formation of Cu(I)-thiolate complexes, thus damaging enzymes that depend on free cysteine residues (RSH) or disulfide bonds (RSSR) for their functionality.[40] Therefore, due to their toxicity, cells have evolved mechanisms to maintain Cu homeostasis. Dehkordi et al.[48] reported the significant antibacterial activity of copper oxide (CuO) nanoparticles against *L. monocytogenes*.

For example, under oxidative stress, such as elevated Cu levels, *L. monocytogenes* employs a defense mechanism involving the pleiotropic regulator DegU. DegU modulates global transcriptional profiles, mainly by suppressing carbohydrate uptake through the regulation of genes in the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS), including *ptsH*, *ptsI*, and *hprK*.[49] The authors demonstrated that DegU binds to the promoter regions of these genes. Specifically, in the absence of DegU, the transcription of *ptsI* is significantly upregulated, whereas *hprK* transcripts are markedly downregulated in response to copper ion-induced stress.

Another mechanism, under anaerobic conditions, was demonstrated by the ability of both *Listeria* and *Fusobacterium* to produce copper oxidase shielding against Cu harm. They also contain proteins with metal binding motifs, including Cys-X-Cys sequences, that sequester the copper mineral, generating the less toxic ion, Cu^{2+}, thus reducing potential damage to bacteria.[40]

Sodium. Negative correlations were detected between sodium and *Fluviibacter*, *Paludibacter*, *Solitalea*, *Maribacter*, *Aquimarina*, and *Cytophaga*. Sodium plays an important role in regulating the ion gradient balance between sodium and potassium, which is vital for cellular metabolism. In cattle, the ingestion of balanced sodium improves appetite for nutrient and energy consumption, the production of saliva during digestion, and the absorption of nutrients and other essential elements for bone and muscular health.

Solitalea, *Paludibacter*, *Fluviibacter*, *Maribacter*, *Aquimarina* and *Cytophaga* are Gram-negative bacterial genera commonly isolated from water environments.[50−54] *Solitalea* is described as obligately aerobic or facultative anaerobic and non halotolerant,[53] which corresponds to the negative correlation with sodium in our data.

Considering the sodium levels in both diets of our study, at 0.15% NaCl, the negative correlation observed between Na and the bacteria *Paludibacter*, *Aquimarina*, *Maribacter*, *Fluviibacter*, and *Cytophaga* may be linked to their specific Na^{+} requirements for optimal growth.

Zhang et al.[55] reported that the amount of sodium influences biogas yields and short-chain fatty acid production. Additionally, significant changes in the bacterial and archaeal communities were observed during the anaerobic digestion of seaweed with various concentrations of sodium. *Paludibacter*, a propionate-producing bacterium linked to feed efficiency, is among the microorganisms that play a significant role in acidogenesis and acetogenesis stages of fermentation.[7,55]

Aquimarina and *Maribacter*, both isolated from marine habitats, were demonstrated to require a range of 1−7% NaCl for their optimum growth,[51,56] which is a concentration significantly greater than found in both cattle diets. In contrast, *Fluviibacter*, which are isolated from surface river water in Japan, exhibit low sodium tolerance, with optimal growth observed at 0−0.5% NaCl, which is consistent with our findings.[57]

Cytophaga, a butyrate-producing bacterium,[58] belongs to the *Bacteroidetes* division and can be found in aquatic environments such as hydrothermal vents, lakes, seas and oceans.[59]

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While no direct evidence links this taxon as negatively correlated with sodium, certain members of the *Cytophaga-Flavobacteria* group exhibit halotolerance at higher levels than those observed in our data. For example, *Thermonema rossianum* is a thermophilic bacterium isolated from saline hot springs in Italy that is capable of growing in media containing 1−3% NaCl.[60] Similarly, *Psychroflexus tropicus* sp. nov., an obligately halophilic bacterium from a Hawaiian hypersaline lake, shows optimal growth in media supplemented with 7.5−10% NaCl.[50]

Our results emphasize the potential of supplementation with dietary inorganic elements to optimize the gut microbiota and improve cattle health and productivity. Proper management of minerals such as magnesium, calcium, phosphorus, and copper could refine strategies for enhancing metabolic functions, reducing methane emissions, and promoting sustainability. Further exploration of optimal mineral levels in cattle diets will help achieve a balance between microbiota composition, host health, and environmental impact.

In summary, this study highlights the relationship between mineral content and the microbiota in Nelore cattle, revealing how tailored dietary supplementation can enhance animal health and productivity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.5c00401.

Supplementary Table S1: nutrient composition of conventional and byproduct diets (XLSX)

Supplementary Table S2: contemporary group composed of weighing group and slaughter date as a fixed effect for the linear model. Heavy and light are referred to the average weight of the animal in each dietary group: DC−conventional diet; DS−byproduct diet (XLSX)

Supplementary Table S3: residual values of the mineral levels from the conventional and byproduct diets obtained via inductively coupled plasma optical emission spectrometer (ICP−OES). The mineral levels were corrected for environmental effects through a linear model (XLSX)

Supplementary Table S4: taxonomic profile count for bacteria and archaea at genus-level of the conventional and byproduct diets (XLSX)

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Notes

The authors declare no competing financial interest.

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