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Alterations in the gut microbial composition and diversity associated with diarrhea in neonatal Peruvian alpacas

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ABSTRACT

Diarrhea in alpacas is a clinically significant condition and the primary cause of morbidity in neonatal Peruvian alpacas. This study aimed to correlate early diarrhea in crias of alpaca with changes in the microbiota community. A total of 19 alpacas (aged 1–2 months) were collected, including nine with a health condition and ten healthy ones. Fecal samples were obtained under sterile conditions and their DNA was extracted. Sequencing of the V3-V4 region of the 16S rRNA gene was conducted in the Illumina platform, followed by bioinformatics analysis. Reduced microbial diversity was evident in alpacas afflicted with diarrhea, delineating contrasting microbial compositions in comparison to their healthy counterparts. The study characterized the predominant bacterial classes and phyla within the gut microbiota, with Firmicutes, Verrucomicrobiota, and Bacteroidota collectively constituting approximately 80% of the total bacterial population. Substantial disparities in these microbial compositions were observed between the two groups, a variance that appeared to be influenced by both age and the health status of the alpacas. The bacterial class Verrucomicrobiae exhibited a significant presence within the group of alpacas suffering from illness. Furthermore, specific pathogenic species such as *Clostridium spiroforme*, *Blautia*, and *Bacteroides fragilis* were detected in significantly higher proportions among the afflicted alpacas. The functional diversity across the two groups was also found to be markedly different, a distinction that is graphically represented in a heat map illustrating the fifty principal differential KEGGs. This study provides valuable insights into the role of gut microbiota in alpaca health and may have implications for veterinary care and management.

1. Introduction

Alpacas are a domesticated species of camelids that are native to the Andean region of South America (Rojas et al., 2016). They have a cultural value due to their historical relevance, deep-rooted millennial heritage and their ancestral Peruvian heritage. In addition, they possess unique characteristics resulting from their adaptation to the Andean geographical and climatic environment (Mallma et al., 2020). They constitute a significant genetic resource (Gómez-Quispe et al., 2022) and comprise a vital source of high-value meat, skin, and fiber in Peru (Cordero et al., 2011), delivering a substantial economic boost to communities engaged in alpaca breeding (Vásquez et al., 2015).

Diarrhea represents a clinically significant condition in alpacas, as it constitutes the primary cause of morbidity in crias, affecting approximately 23% of this population (Whitehead, 2009). In neonates alpacas under two months, this pathology can become a lethal condition, as it induces excessive depletion of nutrients and water in the host, resulting in an adverse energy deficit, weakness, and in extreme cases, mortality (Rojas et al., 2016). Neonates, due to their physiological immaturity and limited adaptive capacity, are particularly susceptible to fluid losses associated with diarrhea. As diarrhea causes excessive fluid and electrolyte loss in these neonates, their ability to compensate for these losses

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may vary significantly depending on their age and development (Heller & Chigerwe, 2018).

It is acknowledged that the gut microbiota plays a vital role in the digestive processes of ruminants (Han et al., 2020). This microbiota fulfills crucial functions in maintaining gut homeostasis, host immune responses, and physiological regulation (Delzenne et al., 2019). A symbiotic relationship is established between the host and the gut microbiota, with its significance lying in mucosal immunity regulation and prevention of pathogen colonization. This colonization plays an essential role in the preservation of the general health of the host (Delzenne et al., 2019). Various studies have studied this relationship in alpacas (Carroll et al., 2019), deer (Li et al., 2019) and camels (Hinsu et al., 2021). Therefore, an imbalanced gut microbiota will enhance susceptibility to diseases, including diarrhea.

Recently, advanced technologies based on high-throughput sequencing, such as metagenomics, have been conceived and successfully applied to study the intricate bacterial ecosystem present in the gut tract (Kim and Isaacson, 2015). The 16 S rRNA gene serves as an indispensable molecular marker in rumen microbiome research, furnishing intricate insights into the diversity, structure, and taxonomy of microbial communities (Kim et al., 2017). Through thorough and comparative analysis of the acquired data, a deeper understanding of the underlying mechanisms of pathologies is achieved, enabling the formulation of innovative approaches to address them more efficiently (Cotter et al., 2012).

Despite the significance of the gut microbiota in ruminants (Delzenne et al., 2019), the information regarding the microbial population in alpacas is limited. Given the scarce understanding of the microbiota composition in alpacas, particularly beyond the stomach, and the lack of comprehension regarding its response to dietary disturbances (Henderson et al., 2015), the main objective of this study is to acquire novel insights into the composition of the alpaca gut microbiota and its modulation in response to diarrheal conditions, taking into account the influence of age variability. Additionally, we aim to conduct a comprehensive functional analysis of the microbiome to elucidate the specific biological and metabolic functions affected by diarrheal conditions, providing a more robust and detailed perspective. This approach will not only enrich the understanding of health implications for alpaca crias but will also facilitate the development of effective treatment approaches.

2. Materials and methods

2.1. Animal and sample collection

The collection of samples from Huacaya alpaca specimens was carried out in accordance with Peruvian National Law No. 30407: "Animal Protection and Welfare". A total of 19 alpacas aged between one and two months were collected at La Raya Experimental Center of the Universidad Nacional del Altiplano Puno (UNAP) (Table S1). The alpaca offspring were primarily fed a diet consisting mainly of maternal milk, which they consumed throughout the day. Additionally, they occasionally drank water from the nearby river and grazed *Festuca dolichophila* in an area of 3450 ha (Zarate et al., 2021). This ecosystem is characterized by gently sloping terrain and abundant water availability, especially during the rainy season. They resided in a natural habitat characterized by native grasslands at an altitude of 4160 m, where mothers, healthy, and sick offspring were observed (Oscanoa and Bustinza, 1987; Huanca et al., 2017; Zarate et al., 2021). This ecosystem is characterized by a gently sloping terrain and abundant availability of water, especially during the rainy season. Grazing activities were conducted from 7 am to 5 pm (Oscanoa and Bustinza, 1987; Huanca et al., 2017; Zarate et al., 2021). These samples included nine alpacas affected by a health condition and ten healthy alpacas (n=4–6 replicates per group). The sick alpaca group had a balanced gender distribution, with a 4:5 ratio of males and females. Similarly, in the healthy group. To ensure the integrity of samples and minimize the risk of contamination, alpacas exhibiting diarrhea symptoms and those in optimal health were housed separately in isolated enclosures one day prior to the sampling procedure. Until the moment of sample collection, no supplements or medications were administered to the animals. Sterile instruments were employed to procure individual fecal samples, collected directly from the animals' rectum. These samples were aseptically transferred into sterile 50 ml plastic containers and promptly transported to the laboratory, where they were promptly stored at a temperature of −80°C for subsequent analytical procedures.

2.2. Examination of fecal samples for parasites

The sick alpacas were diagnosed at the Animal Health Laboratory of the Faculty of Veterinary Medicine, National University of San Antonio Abad in Cusco. The methodology employed in this study is consistent with previous research (Sanchez et al., 2024). A direct microscopic analysis was conducted using parasitological lugol staining (Bowman, 2022) to detect protozoan cysts and oocysts in fecal samples from affected alpacas. Subsequently, qualitative flotation concentration methods were applied with saturated sucrose solution (Barriga, 2002), followed by the modified McMaster quantitative method (Cebra and Stang, 2008), specifically tailored for fecal samples from alpacas with pathological conditions. *Eimeria lamae* oocysts were identified in fecal samples from two-month-old alpacas, while *Giardia* sp. cysts were found in samples from one-month-old alpacas. In contrast, no parasites were detected in the control group of alpacas aged 1 and 2 months.*2.3 DNA Extraction and Sequencing*

2.3. DNA Extraction and Sequencing

Microbial genomic DNA from 500 mg of each fecal samples was extracted using the PureLink Microbiome DNA Purification kit following the manufacturer's instructions. Qubit® and 1% agarose DNA were used to assess the DNA quality. This DNA was prepared for sequencing of the V3-V4. The sequence of the 341 F primer and the 806 R primer was: (AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTA-CACGACGCTCTTCCGATCTNNNNCCTACGGGAGGCAGCAG and CAAG CAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCA-GACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT region of the 16 S rRNA gene exactly as described (Kozich et al., 2013). Briefly, the V3-V4 region of the 16 S rRNA gene was individually amplified from each sample using the TruSeq® DNA PCR-free Sample Preparation Kit (Illumina, USA) and primers (Kozich et al., 2013). Library quality was assessed using the Qubit 2.0 fluorometer (Invitrogen, USA) and the Agilent Bioanalyzer 2100 System fragment analyzer. The amplicon libraries were sequenced using the 2 × 250 paired-end protocol on Illumina Novaseq 6000 (San Diego, CA, USA).

2.4. Bioinformatics analysis

The Quantitative Insights into Microbial Ecology analytical platform (Bolyen et al., 2019) was employed for sequencing data preparation and analysis. The DADA2 protocol v.1.18 (Callahan et al., 2016) facilitated the handling of the paired-end fastQ files, generating amplicon sequence variants (ASVs). Initial procedures involved the quality assessment, trimming, and noise reduction of both forward and reverse sequences prior to their integration into ASVs to mitigate the risk of inaccurate ASVs; unique sequences with an overall abundance of fewer than 10 reads across the sample set were excluded. The taxonomic categorization of sequences was achieved utilizing the QIIME2 inbuilt naïve Bayes classifier, which was calibrated on the latest Silva Reference database v.138.1 for bacteria. Unidentified and undesired phyla, such as cyanobacteria/chloroplasts in bacteria, were excised from the ASV tables. High-quality sequences were organized using the comprehensive MAFFT alignment tool (Katoh et al., 2002). Additionally, rooted 16 S

phylogenetic trees were designed employing the QIIME2 phylogenetic component, leveraging the FastTree technique. Effect sizes derived from linear discriminant analysis (LDA) were used to identify variations in predominant bacteria between groups. Finally, we likewise performed the prediction of the metagenome utilizing the latest version of Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) (Douglas et al., 2020) on microbial data.

2.5. Statistical analysis

The rarefaction curve of each sample was generated to assess the depth of sequencing. Gut microbial alpha diversity was calculated according to the relative abundance distribution of OTU in each sample. Statistical analysis of the data was performed with the package phyloseq (McMurdie and Holmes, 2013). in the software R v4.1.1 (R Core Team, 2020). Alpha diversity was calculated with Observe, Pielou, Shannon and Simpson using the library MicrobiotaProcess (Xu et al., 2023) and Kruskal-Wallis tests were used to test the distribution of alpha diversity measures across the tested groups. Beta diversity was computed through the application of unweighted Unifrac distances (Lozupone and Knight, 2005) and by non-metric multidimensional scaling (NMDS) for visualization. Differences in the bacterial communities between groups were further verified using PERMANOVA with 9999 permutations (Anderson, 2017), which employed the package vegan (Oksanen et al., 2019) in R. Principal component analysis (PCA) was performed to analyze the overall dissimilarity of predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs among groups. Also, PERMANOVA was used to perform the multivariate statistical analysis of functional dissimilarity based on Bray–Curtis as a similarity index with 9999 permutations. Values of $p < 0.05$ were considered statistically significant.

3. Results

In this analysis, the presence of *Eimeria lamae* oocysts was detected in fecal samples from two-month-old alpacas, while *Giardia* sp. were found in samples of one-month-old alpacas. In contrast, no parasites were found in the control group alpacas at the ages of one month and two months. Similarly, no oocysts were identified in one-month-old alpacas.

3.1. Composition of the gut microbiota

The compositions and changes of the dominant bacterial class and phyla were characterized. The results indicated the presence, in the gut microbiota of, the 1-month illness, 2-month illness, 1-month control and 2-month control groups, Firmicutes (77%, 72.77%, 66.6% and 58.55%), Verrucomicrobiota (16%, 23.37%, 4.27% and 16.75%). Bacteroidota (6%, 6.59%, 9.49% and 20.08%) and Proteobacteria (1%, 0.2%, 29.89% and 0.72%) in descending order (Fig. 1A). These four dominant phyla accounted for approximately 80% of the total bacterial composition.

The predominance of the Bacteroidota was observed in a 2-month control with 20.08%, the Proteobacteria was also higher in a 1-month control with 29.89%. In addition, the most representative classes (Fig. 1B) were Clostridia (61.91%, 55.46%, 58.58% and 53.57%), Verrucomicrobiae (15.84%, 17.62%, 4.27% and 16.78%), Bacteroidia (5.53%, 6.59%, 9.49% and 20.07%) and Bacilli (15.33%, 17.3%, 7.86% and 4.95%). The most abundant Bacteroidia phylum was found with 20.07% in the 2-month control group, in addition to the Gammaproteobacteria phylum with a representation of 29.89% in the 1-month control group. A significant difference in the abundance of the Phylum Bacteroidota was observed when comparing the one-month control group with the two-month illness group (Figure S2A). Regarding the class, significance was found in Bacteroidia when comparing the one-month and two-month illness groups with the two-month control group (Figure S2B). Additionally, there was a significant difference in Verrucomicrobiae when contrasting the two-month control group with the one-month control group (Figure S2C).

3.2. Alpha diversity of the gut microbiota in the alpaca population

To further explore the variations in gut microbiota communities with respect to age, employment of the rarefaction curve was sought (Figure S1). This curve exhibited a trend towards optimization, indicating that the analyses were representative of the studied communities in the research. As such, the data set proved to be sufficient for further analyses. The results of the alpha diversity index are shown in Fig. 2. A decrease was observed in the group of sick alpacas at one and two months compared to the control groups of the same age. Significant differences were identified between the one-month-old patient group and the two-month-old control group on Pielou index ($p = 0.0087$), Shannon index ($p = 0.017$) and Simpson index. ($p = 0.0087$). The Shannon and Simpson indices facilitate an assessment of the diversity of the gut microbiota. The Pielou index reflects the uniformity of the gut microbiota. Greater richness was observed in the two-month control group.

3.3. Beta diversity of the gut microbiota in the alpaca population

Beta diversity analysis was used to elucidate distinctions within the composition of the gut microbiota. Distance-weighted UniFrac was used to analyze the divergence within gut microbiota diversity between different sample sets derived from two healthy and diseased categories spanning two age groups (1 and 2 months). Results were plotted using an Analysis of non-parametric multidimensional scaling (NMDS) visualization, as illustrated in Fig. 3.

Within the NMDS plot, remarkable convergence was evident among illness subjects, indicating greater homogeneity across the composition. In contrast, the healthy cohort exhibited more extensive distance dispersion, as validated by the PERMANOVA test. This statistical analysis underlined the importance of age (p-value: 0.0264), as visually corroborated in Fig. 3 through age gaps of 1 and 2 months, signifying pronounced age-related variations. In addition, health status exhibited statistical significance (p-value: 0.0113), further emphasizing the discriminatory influence of health status on gut microbiota composition.

3.4. Identification of altered taxonomy composition changes associated with diarrhea

The Linear Discriminant Analysis (LDA) was employed to assess effect size and delineate disparities in bacterial composition between diseased and healthy alpacas at two distinct age groups (1 month and 2 months old). *Erysipelotrichales, Acetitomaculum* and *Kandleria* were observed in the group of illness of two-month-old alpacas (Fig. 4A). In addition, *Erysipelatoclostridium* and *Clostridium spiroforme* were identified in one-month-old illness alpacas (Fig. 4B). Finally, *Blautia, Bacteroides fragilis and Erysipelotrichales* were detected in the illness group (Fig. 4C).

In relation to the two-month control group, taxa such as *Escherichia-Shigella*, belonging to the phylum Proteobacteria, and the Enterobacteriaceae family, classified within the class Gammaproteobacteria, were identified (Fig. 4A).

Additionally, in the one-month control group, the presence of taxa associated with Bacteroidales, classified within the class Bacteroidia and the phylum Bacteroidota, was observed (Fig. 4B). In the Control group, taxa from Bacteroidales and Bacteroidia, as well as taxa from the phylum Proteobacteria, were detected (Fig. 4C).

3.5. Functional diversity

Therefore, a separation between the diseased and healthy groups of alpacas was observed by the Bray-Curtis dissimilarity analysis. This was significantly verified by PERMANOVA with a p-value of 0.023 (Fig. 5A). Subsequently, the top fifty KEGGs exhibiting differential abundance were integrated into a heatmap for visualization (Fig. 5B). Evident

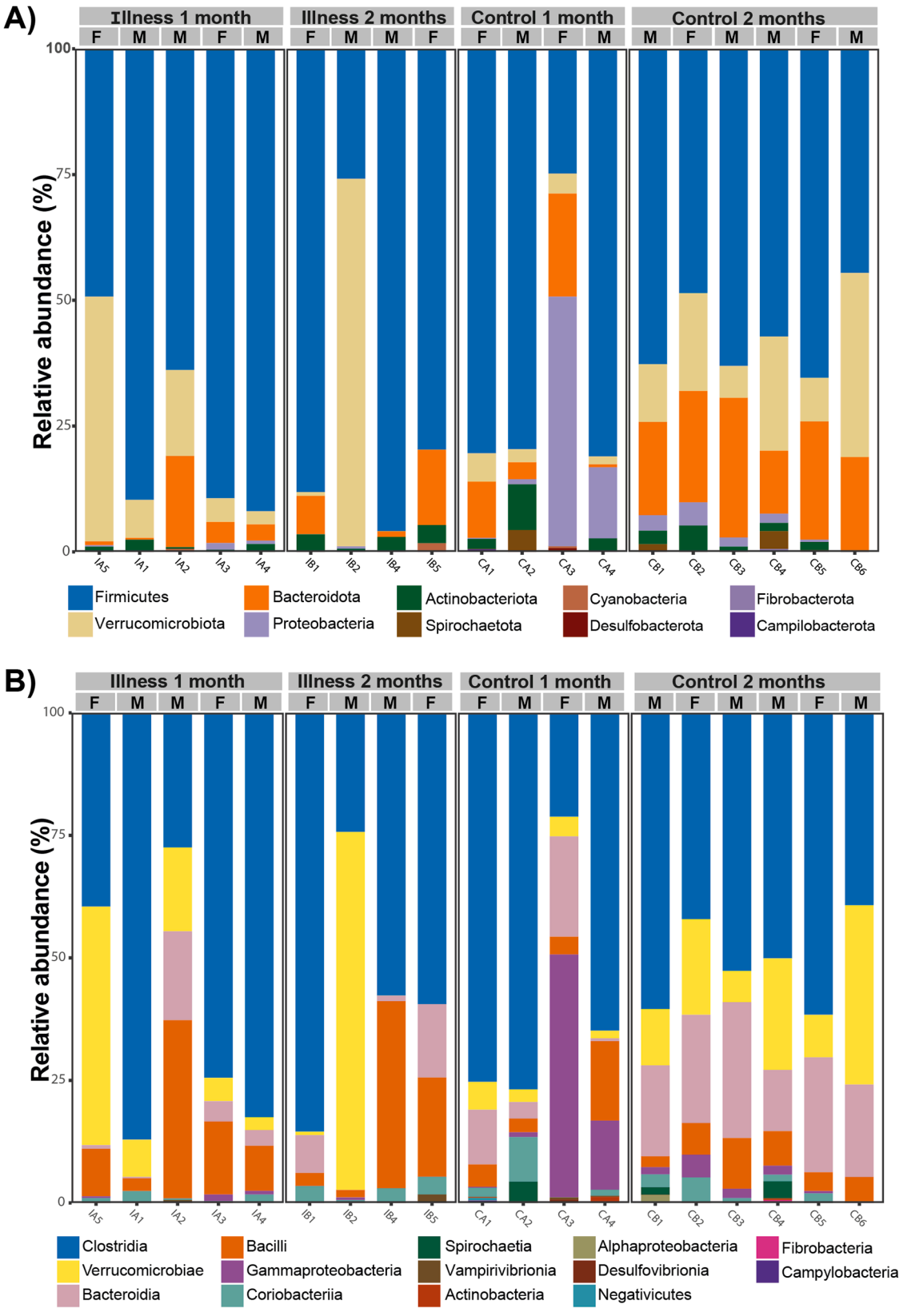

Fig. 1. Relative abundances of phyla (A) and class (B) and observed in the 1-month-old illness and 2-month-old illness groups compared to the 1-month-old control and 2-month-old control groups. Only the most represented taxa are reported. F: female, M: male.

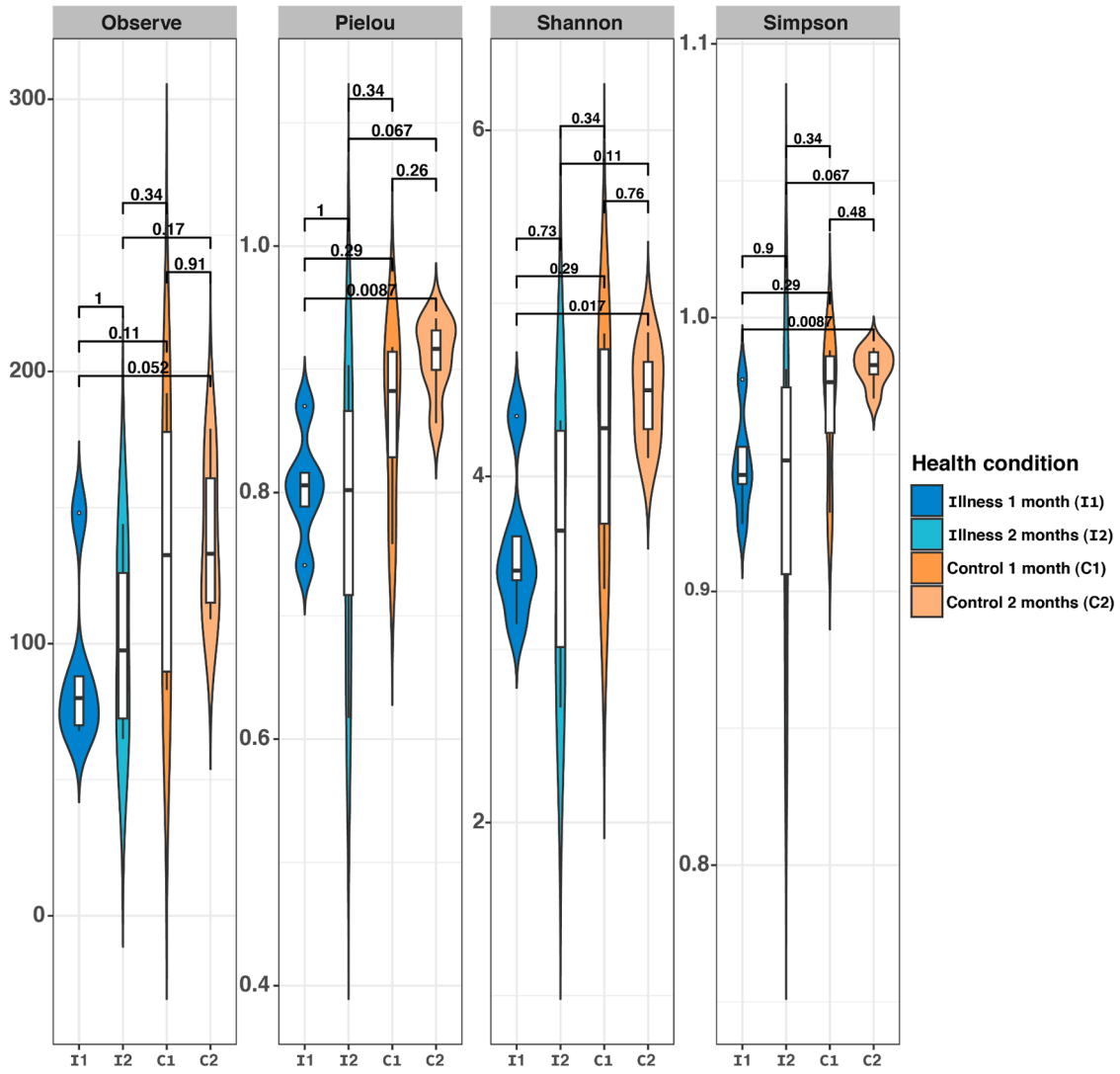

Fig. 2. Alpha diversity (Observe, Pielou, Shannon and Simpson) gut microbiota indices between health conditions with Illness 1 month, Illness 2 months, Control 1 month and Control 2 months.

distinctions in abundance levels were discerned when comparing the sick and healthy groups.

4. Discussion

In this study we conducted an analysis of bacterial diversity and gut abundance in alpacas at different health states and ages. Results revealed that both bacterial abundance and diversity were reduced in diarrheal alpacas compared to their healthy counterparts. Also, the differences in the composition of gut microbiota between diseased and healthy alpacas did exhibit significant statistical significance.

The results exhibited a high degree of concordance with the literature; various investigations have used the analysis of the microbiota to describe the gut microbial composition under conditions of diarrhea in domestic animals, lactating goats (Wang et al., 2023), lambs (Kong et al., 2022) and commercially reared pigs (Xue et al., 2020). Rumen microbiological analysis is ubiquitous in scientific research on various ruminant species (Carroll et al., 2019; Du et al., 2022; O'Donnell et al., 2017). However, it is pertinent to point out that the literature related to the microbial community in alpacas is deficient, and there are no documented records to date that focus on the microbial communities associated with the health status of alpacas.

The predominant taxa distinguished in this study, namely Firmicutes and Bacteroidetes, converge with the dominance found in pre-existing studies focused on alpacas (Bedenice et al., 2022; Carroll et al., 2019). The increased abundance of Firmicutes and Bacteroidetes (Figure S2B) within a gut microbial consortium can contribute substantially to the pronounced daily energy and nutritional requirements of the host (Spence et al., 2006). Also, the phylum Firmicutes, the majority of its members, have been characterized as beneficial bacteria that actively engage in improving the gut environment and countering pathogenic infiltration (Li et al., 2019). Regarding taxonomic class category (Fig. 1B), the most prominent consortium identified in alpaca samples encompasses Clostridia, Bacteriodia and Bacilli (Carroll et al., 2019; Bedenice et al., 2022); instead, a preeminence of Gammaprotebacteria was observed in the one-month control group (Carroll et al., 2019). In the one-month Control group, the presence of the phylum Proteobacteria was reported at 29%. This phylum plays a role in meeting their elevated energy and nutritional needs due to their varied range of metabolic functions (Zhang et al., 2020). Predominance of Verrucomicrobiae was observed in the group of illness aged one month and two months. This phylum has been reported in clinical cases of diarrhea in ruminants (Wang et al., 2023; Liu et al., 2022) and it possesses beneficial properties for the regulation of the immune system (Lindenberg et al., 2019).

We found statistically significant differences in the diversity and

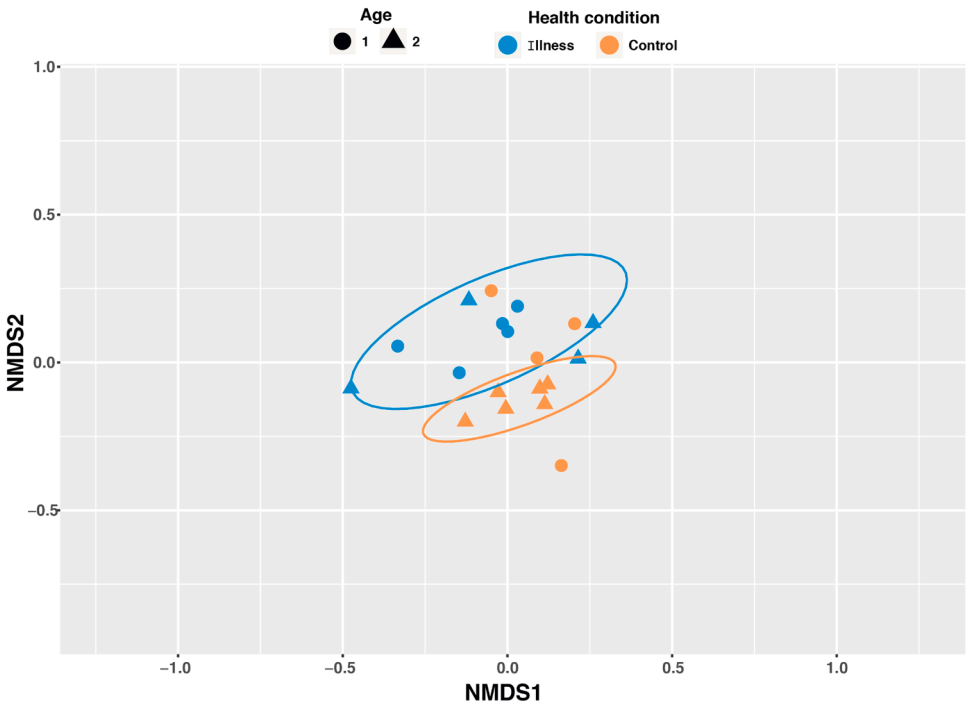

Fig. 3. Analysis of non-parametric multidimensional scaling (NMDS) plot of beta diversity based on weighted UniFrac distance derived from sequencing data. The samples are represented by colors divided into two healthy (orange) and sick (light blue) groups, in addition there is a shape representation for age 1 month (circle) and 2 months (triangle).

richness of the gut microbiota exclusively between the group of illness individuals for one month and the control group for two months. No significant difference was found in the Observed index, suggesting that there is no variation between the number of species present (Wang et al., 2023). The Pielou index presented significant difference, therefore, the uniformity in the distribution of the species could show divergences (Hung et al., 2019). These findings could be positively correlated with gut functions and gut microflora (Barash et al., 2017; Turkyilmaz et al., 2014). That is, an imbalanced gut microbiota can lead to increased gut permeability, resulting in reduced immunity and making the host more susceptible to invasion by pathogenic bacteria (Barash et al., 2017). For this reason, alpacas with diarrhea may also experience similar gut dysfunction, ultimately contributing to impaired gut bacterial diversity.

Through the NMDS analysis using the weighted UniFrac method, it was possible to represent the bacterial composition of the two groups, evidencing a difference in the beta diversity of the gut bacterial communities between these groups. Individuals with diarrhea were grouped separately from the control individuals, as supported by the PERMANOVA test (Table 1), indicating significant bacterial composition differences related to health status and age. Diarrhea is speculated to have played a role in appreciable changes in gut bacterial communities (Wang et al., 2023; Li et al., 2021; Xi et al., 2021).

In the present study, a higher prevalence of Erysipelotrichales and Erysipelotrichaceae has been observed in illness and illness of 1-month-old alpacas. This observation is consistent with previous research, suggesting a potential association between Erysipelotrichales and microbiota imbalances (Stewart et al., 2018; Mizutani et al., 2021). However, it is important to note that the relationship between Erysipelotrichaceae abundance and health is multifaceted and context-dependent (Kaakoush, 2015). While changes in Erysipelotrichaceae abundance have been linked to certain conditions (Stewart et al., 2018; Schären et al., 2017), precise interpretation requires further investigation. Additionally, genetic, environmental, and dietary factors also influence these results (Mizutani et al., 2021).

Similarly, a higher abundance was been observed in sick alpacas aged one month, which could be related to previous research suggesting an association between this genus and lower feed efficiency (F. Kong et al., 2022; McLoughlin et al., 2020). The increased presence of *Acetitomaculum* in sick alpacas may influence reduced efficiency in converting food into energy (McLoughlin et al., 2020). Moreover, as *Acetitomaculum* has been reported to ferment monosaccharides into acetic acid, this fermentative activity could impact the microbial composition and nutrient metabolism in the intestines of sick alpacas (McLoughlin et al., 2020; Guo et al., 2023). However, further research is needed to fully comprehend the specific role of *Acetitomaculum* in the context of health and disease in alpacas.

Regarding the species *Bacteroides fragilis*, a significant increase in its prevalence was observed in the group of sick alpacas. This finding aligns with existing knowledge about *Bacteroides fragilis*, a gram-negative anaerobe that tends to colonize the lower gastrogut tract of mammals (Raabis et al., 2019; Kong et al., 2019). This microorganism demonstrated beneficial properties that can influence the improvement of inflammatory symptoms (Raabis et al., 2019; Kong et al., 2019).

Furthermore, an increased abundance of the *Blautia* genus has been identified in sick alpacas with diarrhea. *Blautia* is known as a carbohydrate-fermenting bacterium (Dias et al., 2018), and its population increase has been linked to various pathological conditions in other animals (Welch et al., 2021), such as diarrhea in cattle (Zeineldin et al., 2018). While circumstances may vary, this finding could suggest a potential role of *Blautia* in the microbial dynamics associated with diarrhea in alpacas.

Likewise, in the context of the present study, the presence of the *Clostridium spiroforme* bacterium has been confirmed in sick alpacas for two months. However, it is crucial to consider that the mere presence of *Clostridium spiroforme* does not necessarily automatically indicate the presence of a disease (Oglesbee and Lord, 2020). While this bacterium can be considered a pathogen in specific circumstances, its excessive proliferation and toxin production may be related to health issues (Oglesbee and Lord, 2020; Solans et al., 2019).

Succinyl-CoA emerges as a central molecule in metabolism, influencing various metabolic pathways and enzyme regulation (Gengenbacher and Kaufmann, 2012). Its role in protein succinylation,

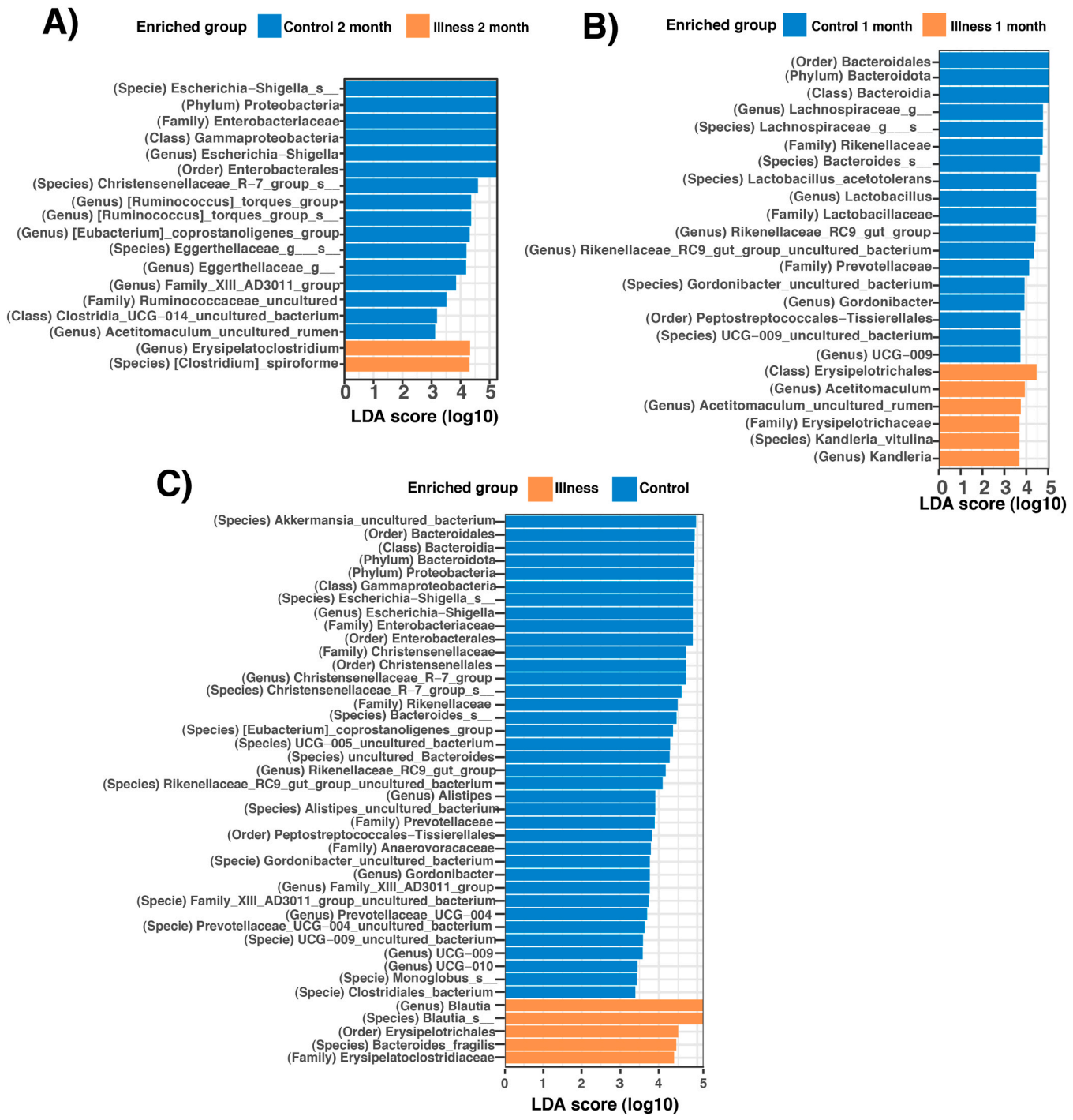

Fig. 4. Differences in gut microbiota among diverse health statuses in alpacas. Bar plot of Linear Discriminant Analysis (LDA) for differentially abundant taxa. (A) Illness at 2 months versus control at 2 months. (B) Illness at 1 month versus control at 1 month. Illness at 2 months versus control at 2 months. (C) Illness versus control alpacas.

modulated by enzymes like SIRT5, can impact the activity of many metabolic enzymes (Mierziak et al., 2021). A higher presence of succinyl-CoA in healthy alpacas suggest it might be associated with more efficient metabolism or specific regulation contributing to the well-being of these alpacas (Ma et al., 2021).

The higher presence of the 2-oxoisovalerate ferredoxin oxidoreductase enzyme in healthy alpacas compared to diseased alpacas could suggest a relevant role of this enzyme in metabolic processes related to compound reduction (Ufarté et al., 2018). This finding raises the possibility that the activity of this enzyme may be linked to the health of alpacas in a metabolic context (Dörner and Boll, 2002). However, it is crucial to note that this association is based on functional similarities and thus requires further research and in-depth analysis to fully comprehend its influence on alpaca health.

A higher predominance of apolipoprotein A-I (apo A-I) was observed in healthy alpacas compared to diseased ones, suggesting a potential relationship between apo A-I levels and the livestock's health status. Apo A-I, a multifunctional protein, is known for its involvement in lipid metabolism regulation and its role in reverse cholesterol transport (Boe-Hansen et al., 2015; Mangaraj et al., 2016). In addition to its effects

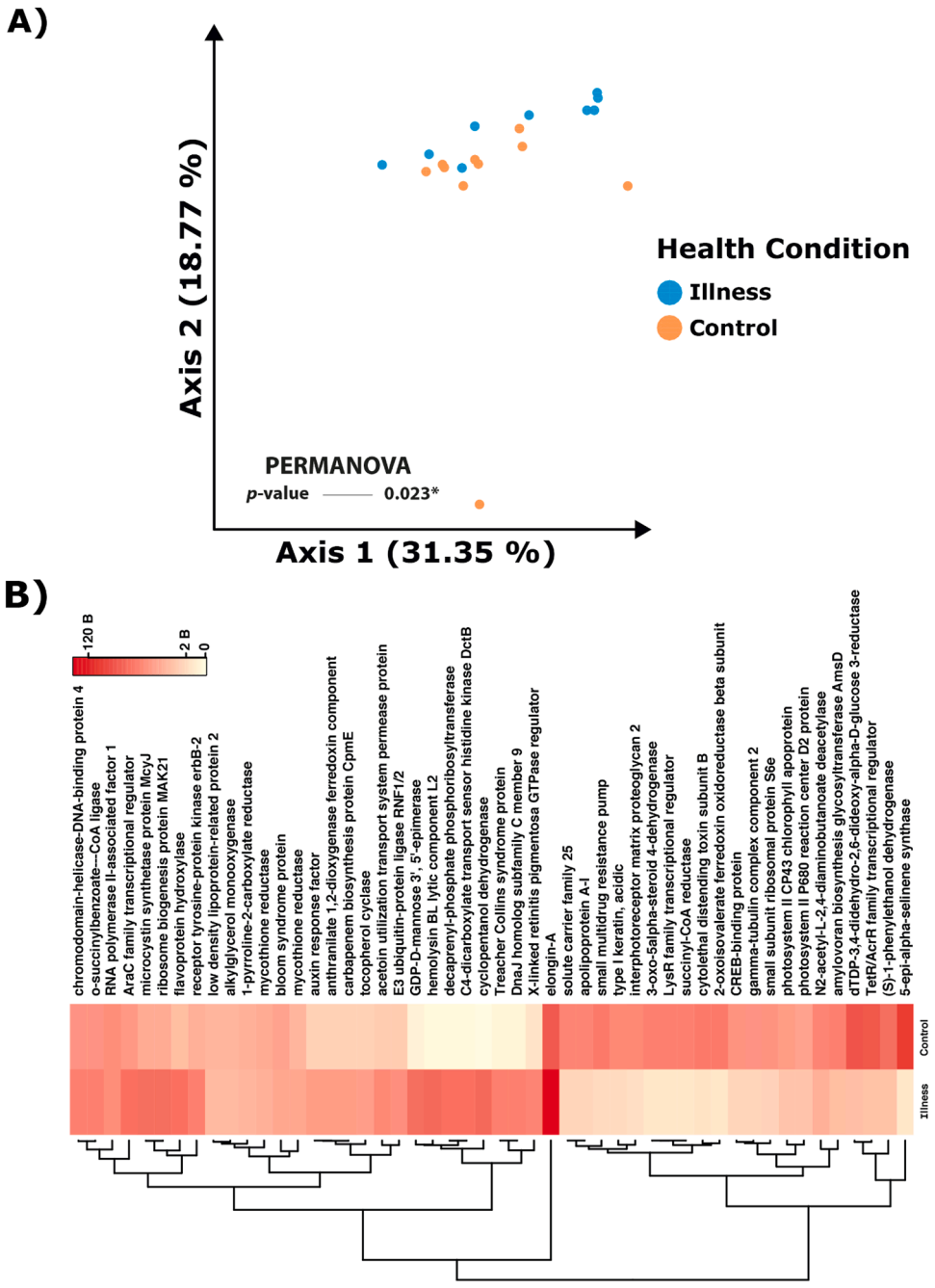

Fig. 5. PICRUSt2 functional prediction. (A) PCoA plot illustrating Bray-Curtis beta diversity matrix calculations. Percentage confidence values for each distance matrix are depicted on the axes in two dimensions. (B) Heatmap illustrates the pathways with differential abundance as predicted by PICRUSt2.

Table 1
Two-way PERMANOVA of the weighted UniFrac distance between the age and health status of the alpacas.

Items	Df	SumOfSqs	R2	F	Pr(>F)
Age	1	0.14240	0.10536	2.2691	0.0264*
Health status	1	0.16373	0.12114	2.6091	0.0113*
Age:Health status	1	0.10413	0.07704	1.6593	0.0987
Residual	15	0.94132	0.69646		
Total	18	1.35137	1		

Significance: p < * 0.05

on the cardiovascular system, its influence on immunological processes and its ability to modulate responses to inflammatory and antitumoral processes in other contexts have been documented (Huang et al., 2022). This result could suggest that elevated levels of apo A-I in healthy alpacas are associated with enhanced immune response and increased disease resistance, potentially contributing to their overall health compared to diseased alpacas. However, further research is crucial to validate this relationship and gain a comprehensive understanding of its implications in livestock health.

Despite the well-documented prevalence of *Clostridium perfringens* (Konieczny and Pomorska-Mól, 2023) and *Escherichia coli* (Carhuapoma et al., 2019) in cases of neonatal diarrheal complex of alpacas, it is interesting to note that these specific pathogens were not detected in our study. Previous studies in the region have identified these

microorganisms as causative agents of diarrhea in alpacas, highlighting the complexity of microbial interactions in the gastrointestinal tract of these animals (Konieczny and Pomorska-Mól, 2023). The absence of *Clostridium perfringens* and *Escherichia coli* in our samples could be attributable to various reasons, such as temporal variability in the prevalence of these pathogens, population-specific factors or even differences in the detection techniques used (Cebra, 2014; Cebra et al., 2003). Therefore, our study contributes to the evolving understanding of alpaca microbiota, yet further detailed research is needed to establish causal relationships or clinical implications.

5. Conclusions

This study investigated bacterial diversity and gut abundance in alpacas across different health states and ages, revealing reduced bacterial abundance and diversity in alpacas with diarrhea compared to healthy counterparts. The composition of gut microbiota exhibited significant differences, emphasizing the impact of imbalanced gut microbiota on alpacaś health. Notably, distinct microbial taxa, including Erysipelotrichales, *Acetitomaculum*, and *Bacteroides fragilis*, showed significant associations with health status and age, providing insights into potential microbial imbalances linked to diarrhea in alpacas. However, it is crucial to acknowledge certain limitations in the study, given that it was conducted in the same geographical area where no significant differences in diet and climatic variables were observed. Therefore, it is recommended that future investigations address these factors to achieve a more comprehensive understanding. Furthermore, Illumina sequencing may introduce distortions in capturing microbial diversity, suggesting that future studies incorporating PacBio-HiFi sequencing could provide a more thorough evaluation.

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CRediT authorship contribution statement

Oscar Oros: Writing – original draft, Visualization, Project administration, Investigation. **Richard Estrada:** Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Jorge L. Maicelo:** Writing – review & editing, Validation, Supervision, Resources, Funding acquisition. **Diana Sánchez:** Writing – original draft, Visualization, Supervision, Resources, Investigation. **Pedro Coila:** Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization. **Carlos I. Arbizu:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Funding acquisition. **Celso Zapata:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Supplementary Materials

Figure S1: Rarefaction curves of species richness show the sequencing depth of 16 S data obtained from gut samples; Figure S2: Wilcoxon test in the Class and Phylum with respect to health condition (* $p < 0.05$, ** $p < 0.01$); Table S1: Weight of alpacas in relation to their health condition.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.smallrumres.2024.107273.

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