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Original Article

The effects of *Cryptosporidium* infection on gut fungi and enzyme abundance in *Sus domesticus*

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Abstract

Pigs are known to be natural hosts of *Cryptosporidiosis*, which poses a serious threat to public health. According to the World Health Organization (WHO), *Cryptosporidiosis* is the major cause of severe diarrhea which causing death in infants. Many researchers suggest that gut fungi have an indispensable function in host metabolism and immunity. However, few studies have been performed to understand how *Cryptosporidium* infection induces alterations in the intestinal fungal communities of Tibetan pigs. Therefore, fecal samples from *Cryptosporidium* infected and Healthy Tibetan pigs were examined by internal transcribed spacing (ITS) gene amplification sequencing. Results showed that a total of 2 phyla, 5 classes, 9 orders, 10 family, 13 genera of fungi were detected from both the H (Healthy Tibetan pigs) and INF (*Cryptosporidium* infection pigs) groups. The results manifested that proportion and profile of the total fungal population obviously changed under the *Cryptosporidium* infection, marked by a reduction in the abundance of beneficial fungi, i.e., *Leotiomycetes* ($p < 0.05$), *Aspergillaceae* ($p < 0.05$), *Penicillium* ($p < 0.05$), *Xenoacremonium* ($p < 0.05$) and *Eurotiales* ($p < 0.05$) and an increase in the abundance of disease-causing fungi that threaten health, i.e. *Colletotrichum* ($p < 0.05$) and *Clarireedia* ($p < 0.05$). In addition to these changes, some enzymes including *Arabinose-5-phosphate isomerase*, *Quercetin 3-0-methyltransferase* and *Amylosucrase* were found to be significantly altered ($p < 0.01$) after *Cryptosporidium* infection using fungal function prediction analysis. This study also focused on the *Cryptosporidium* infection in Tibetan pigs in terms of gut fungal diversity, composition, and the abundance of enzyme. It is providing a better understanding of *Cryptosporidium* infection in Tibetan pigs and insights for further development of therapeutics against *Cryptosporidium* from the gut fungal perspective.

Keywords: *Sus domesticus*, Tibetan pig, *Cryptosporidium*, Gut fungi, Enzyme, Microbiota

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Introduction

Tibetan pigs are inhabitant of the cold Tibetan plateau in China, which is approximately 3,000 meters above sea level (Li et al., 2016; Wang et al., 2021). These indigenous pigs are economically important to farmers because they have flavorsome, high-quality meat with more protein and a higher percentage of lean meat in the carcass (Li et al., 2018; Guo et al., 2022). However, Tibetan pigs are susceptible to *Cryptosporidium* due to malnutrition, diet management and hostile environments. In addition, similar cases of *Cryptosporidium* infection in Tibetan pigs and yaks have been reported, as evidenced by epidemiological results (Chen et al., 2023). Therefore, it is necessary to better understand *Cryptosporidium* from new points of view.

Cryptosporidium are one of the main causative agents of diarrhea and life-threatening diseases in infants and elderly animals, which further complicating public health protection (Striepen, 2013; Ijaz et al., 2021). Unfortunately, there is no effective vaccine to prevent this disease in animals. At younger age, *Cryptosporidium* is the second leading cause of severe diarrhea and death in animals (Thathaisong et al., 2020). Pigs are reservoir hosts of *Cryptosporidium* while *Cryptosporidium suis* and *Cryptosporidium scrofarum* are abundant in pigs and humans (Wang et al., 2022; Lan et al., 2021). The clinical course usually ranges from diarrhea to death, associated with growth retardation. This suggests that *Cryptosporidium* infection not only causes economic losses, but also poses a public health threat (Qi et al., 2020; Lv et al., 2022). Therefore, a safe and effective strategy to prevent and control *Cryptosporidium* infection is urgently needed.

The gut is the primary organ of elimination and ingestion of nutrients, and is home to trillions of microbes (Chen et al., 2021; Chen, 2022; Wolff et al., 2023). Alteration of the gut microbiota can always be associated with diseases such as diarrhea, affecting both proximal and distal tissues (Cryan and Dinan, 2012; Dinan and Cryan, 2015). Previous studies have shown the relation between gastrointestinal disorders and intestinal microbiota (Singh et al., 2015; Jiang et al., 2017; He et al., 2022). However, the most of studies have shown the importance of intestinal bacteria, but the link between host health and intestinal fungi has rarely been explored.

Fungi, which help to regulate immunity and improve gut microbiota balance, may be altered in diversity and composition by comorbidity (Coker et al., 2019). There has been significant disruption of gut fungal species in patients with a range of diseases, including diarrhea and colorectal cancer (Sokol et al., 2017; Arfken et al., 2020). The intestinal tract is colonized by fungi in humans and animals immediately after birth (Bliss et al., 2008) and fungi can be acquired vertically, horizontally or in the environment (Liggenstoffer et al., 2010; Ward et al., 2018). In short, fungi form a stable ecosystem in the digestive tract that contributes to disease prevention, metabolism and intestinal homeostasis (Zhan et al., 2020). Therefore, we hypothesize that cryptosporidium infection in Tibetan pigs may be related to changes in gut fungi.

To our knowledge, there are no studies on the relationship between gut fungi and *Cryptosporidium* infection in Tibetan pigs. Therefore, it is necessary to study the connection between the gut fungi and *Cryptosporidium.* The aim of this study was to assess the devastating effects of *Cryptosporidium* on Tibetan pigs living in mountainous areas, which can help uncover the link between gut fungi and *Cryptosporidium* infection.

Material and Methods

Sample collection

All experimental samples were collected from pigs living on the Tibetan plateau (altitude >2000m) and detailed records of sex, age and feeding records were maintained. In our previous molecular epidemiological study of *Cryptosporidium* in Tibetan pigs, we collected fecal samples from both diarrheal and healthy Tibetan pigs. A total of 100 samples were collected from a farm in Nyingchi, Tibet, including 50 diarrhea samples and 50 healthy pig's samples, followed by assessment and diagnosis of the condition of Tibetan pigs by a professional veterinarian (Li et al., 2017). Referring to the Felice et al (De Felice et al., 2020), we discovered and analyzed diarrhea samples in the feces of Tibetan pigs and found that some of the samples were infected with *Cryptosporidium*. Additionally, *Cryptosporidium* positive and negative samples were stored at -80°C for further analysis. Four positive and four healthy *Cryptosporidium* feces were selected from samples from earlier studies.

ITS gene amplification and sequencing

Fungal genomic DNA from feces of Tibetan Pigs was extracted using the QIAamp DNA Mini kit (QIAGEN, Germany) according to the manufacturer's recommendations, followed by DNA concentration detection by Namedrop, USA and agarose gel electrophoresis (0.8%). The ITS2 region was amplified by PCR, and ITS gene primers were synthesized according to the method described by Li et al. (2021). PCR target fragments were evaluated and recovered using 2% agarose gel electrophoresis and AxyPrep DNA kit (KkuozhePrep, USA). Subsequently, the PCR-recovered products were subjected to fluorescence quantification using Quant-iT PicoGreen dsDNA Assay Kit and the Microplate reader (BioTek, FLx800). Based on the fluorescence quantification results, the acceptable recovered PCR products were blended to the corresponding ratio based on the sequencing volume requirements of each sample. The obtained certified PCR products are then used to create sequencing libraries using the NEB Next Ultra DNA Library (Illumina, USA) preparation kit in accordance with the manufacturer's recommendations. Prior to sequencing, the libraries were quality-checked on Agilent Bioanalyzer using the Agilent High Sensitivity DNA Kit, with eligible libraries having a single peak and being free of junctions. Finally, eligible libraries were sequenced on the Illumina NovaSeq platform after being diluted and mixed with a corresponding ratio, as required by the sequencing standard.

Fungal bioinformatics analysis

The raw sequence data were screened to provide reliable data for bioinformatics analysis. The query sequences contained chimeras, mismatched primers and ineligible sequences (<200 bp) were discarded by the QIIME software. The operational taxonomic units (OTUs) were classified and clustered according to the 97% similarity of the sequence. Meanwhile, the representative sequences were taxonomically identified and phylogenetic analyzed. In addition, fungal diversity was inferred from alpha analysis and differences in fungal composition between groups were inferred from beta analysis. Alpha analysis metrics, including the Shannon diversity index, ACE diversity index, and the Chao1 richness estimator, were calculated based on the OTU distribution in each sample. Beta analysis was performed by calculating weighted and unweighted UniFrac distances to assess differences and similarities between samples. To further detect the change in the gut fungal composition, other beta analysis indices including (nonmetric multidimensional scaling (NMDS) and Principal Component Analysis (PCA) were performed to reveal the fungal variation.

The differences in species abundance composition between groups and the search for marker species were further determined by clustering, and modeling tools combined with DESeq2 analysis. PLS-DA (Partial least squares discriminant analysis) is a recognized and effective tool for revealing relationships between microbial communities and samples. Co-occurrence analysis was performed by calculating Spearman rank correlations between dominant taxa, and correlations between groups were plotted using network maps. Additionally, the KEGG Ortholog (KO) were predicted using the PICRUSt annotation of the ENZYME database. The parameters used in the biochemical analysis were all set as standard conditions.

Statistical analysis

We used the SPSS (v 23.0) and GraphPad Prism (V 9.0) software to perform data analyses and plot images, respectively. A p-value < 0.05 indicated a significant difference.

Results

Data collection and analysis

A total of 1042 fungal OTUs were identified based on a 97% nucleotide sequence similarity after filtering and taxonomic assignment. The number of core OTUs of H and INF groups was 678 and 416 respectively (Figure 1), of which 52 were common OTUs between the two groups (Figure 2D). Furthermore, the scarcity curves were saturated and broad, indicating that all fungal species were identified in the (Figure 2A-C).

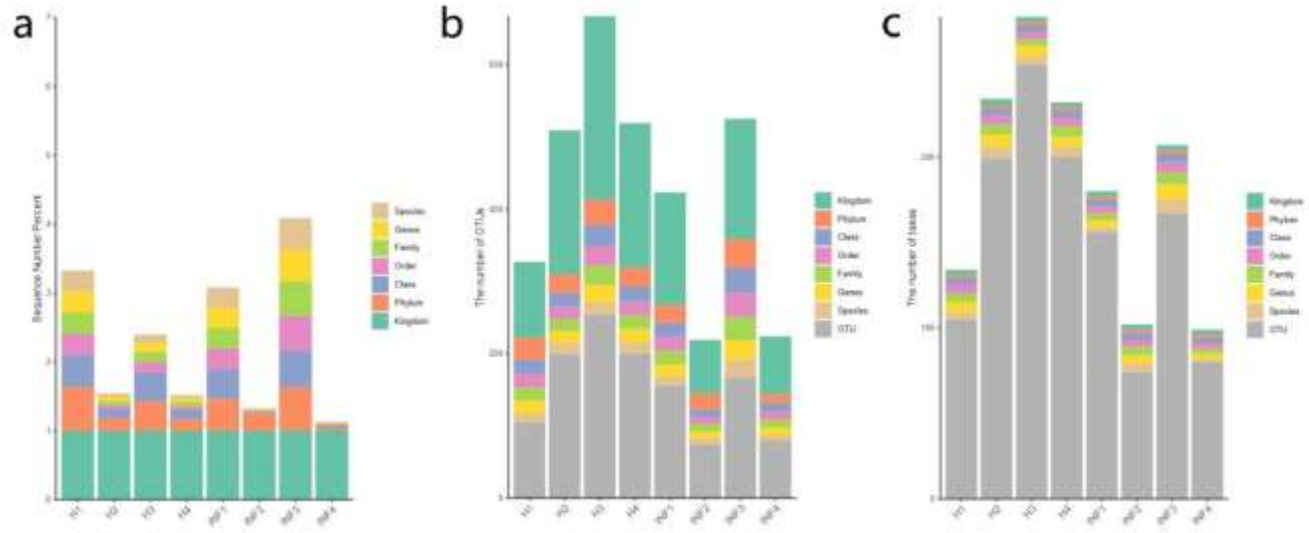

Figure-1. Analysis of OTUs composition and distribution. H1, H2, H3 and H4 belong to the H group; INF1, INF2, INF3 and INF4 belong to the INF group. (a) Sequence number percent of samples at different levels i.e., Kingdom, phylum, class, order, family, genus, species; (b) The number of OTUs of samples at different levels, i.e., Kingdom, phylum, class, order, family, genus, species; (c)The number of taxas of samples at different levels, i.e., Kingdom, phylum, class, order, family, genus, species.

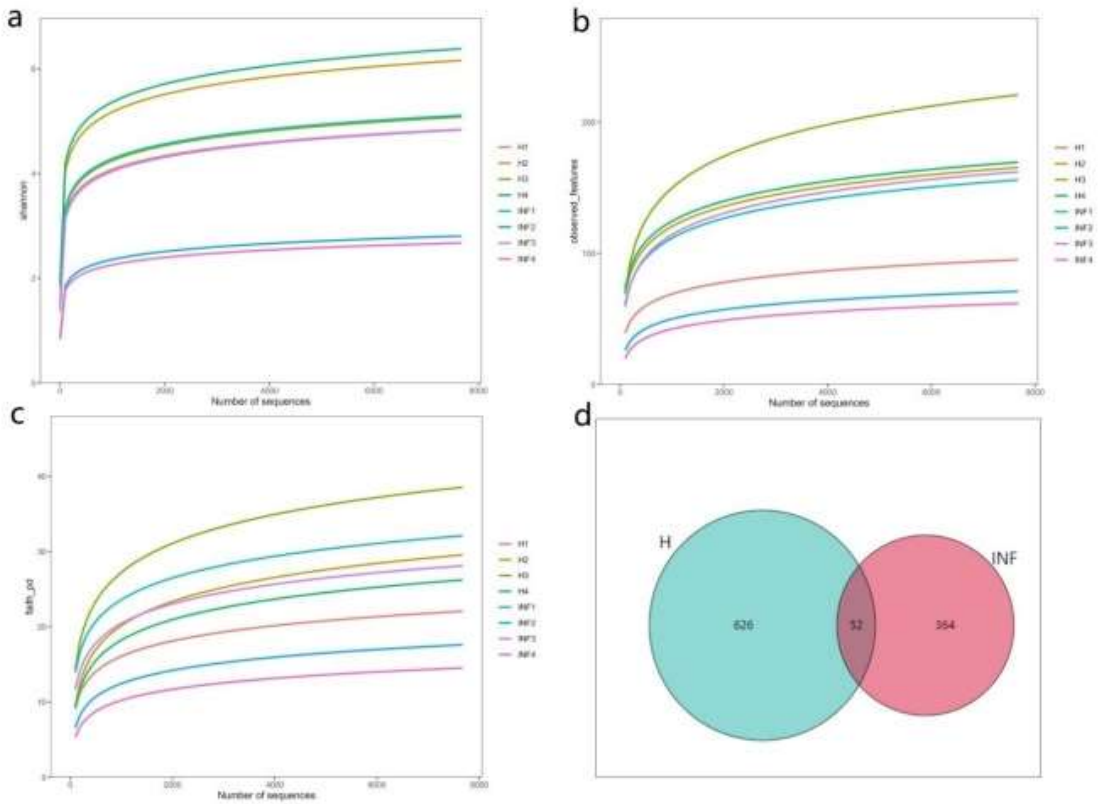

Figure-2. The rarefaction and rank abundance curves. H1, H2, H3 and H4 belong to the H group; INF1, INF2, INF3 and INF4 belong to the INF group. (a) The rank abundance curve was used to describe the sequencing evenness; (b) The observed features were reflected by the sequencing curve; (c) The fungal rarefaction curve was used to assess the sequencing depth; (d) Venn diagrams were used to reflect the gut fungal OTU distribution.

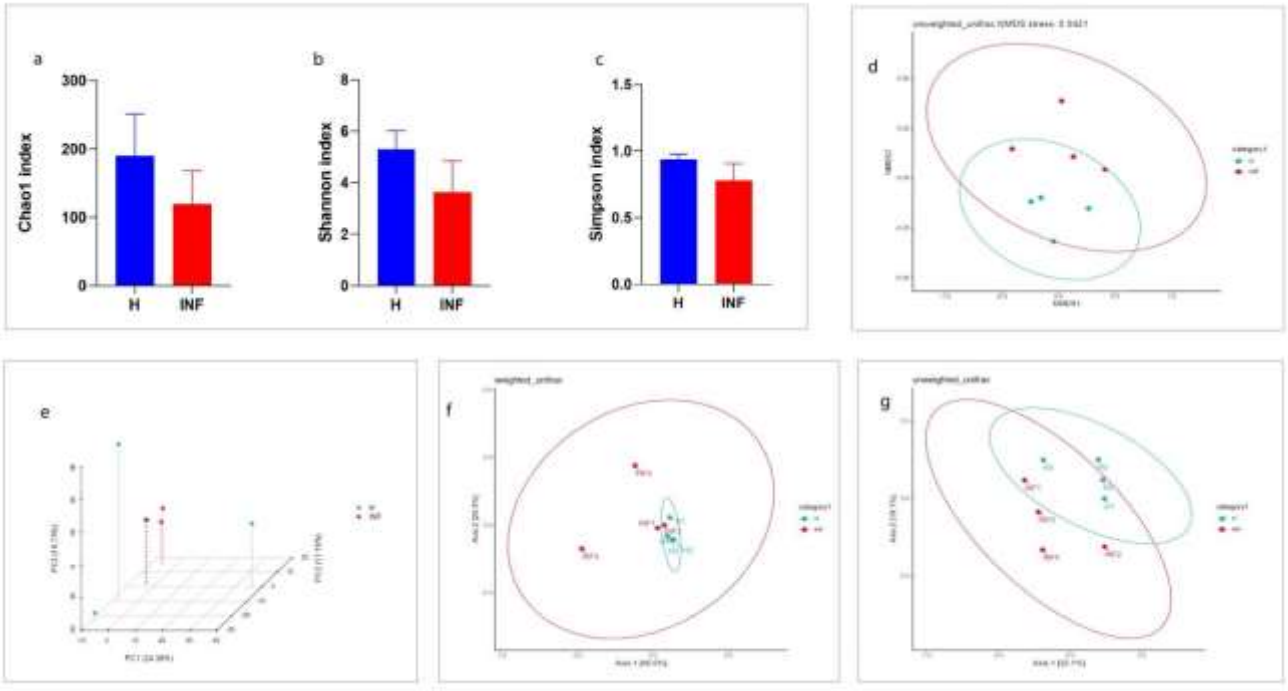

Figure-3. (a-b)The alpha analysis indices indicated the diversity and richness of gut fungi. (a) The Chao 1 was used to reflect the gut fungal diversity; (b) The Shannon index was used to characterize the gut fungal diversity; (c) The Simpson index was used to characterize the gut fungal diversity; (d-g)The Beta analysis includes NMDS, PCA and PCoA. (d) The NMDS analysis; (e) The PCA analysis; (f) The weighted unifrac Principal coordinate (PCoA) analysis; (g) The unweighted unifrac PCoA analysis.

The effect of *Cryptosporidium* infection on the gut fungal diversity

To investigate the adverse effects of *Cryptosporidium* infection on Tibetan pigs, we compared and analyzed the diversity and composition of gut fungi in groups H and INF using ITS gene sequencing. Alpha indices including ACE, Simpson and Shannon were used to characterize intestinal fungal diversity. The results showed that there was no significant difference in intestinal fungal diversity between groups INF and H ($p > 0.05$) (Figure 3A-C). Moreover, the PCoA based on weighted Unifrac distance analysis generated scattered data points in the map (Figure 3F), of which the data points in the H group showed a similar tendency of partially overlap with the INF group. In turn, the specimens in group IFN were clearly separated from those in group H by unweighted Unifrac distance analysis, indicating the significant difference in gut fungal profile between the groups (Figure 3G). Similar results were found in the NMDA (Figure 3D) and PCA analyses (Figure 3E), with individuals in the H group being closer together but far apart from those in the INF group. The above results all indicate that the composition and fungal profile of the intestine were significantly affected by *Cryptosporidium* infection in Tibetan pigs.

Taxonomic composition of gut fungal community

A total of 2 phyla, 5 classes, 9 orders, 10 families, and 13 genera were observed in groups H and INF (Figure 4). At the phylum level, *Anthophyta* (H=22.52%, INF=25.31%) and *Ascomycota* (H=12.64%, INF=9.4%) were the most abundant phyla in groups H and INF, accounting for more than 34% fungi taxonomic (Figure 4A). At the class level, the H group of *Pseudomycetes* (H=15.36%, INF=5.05%), *Sordariomycetes* (H=10%, INF=8.94%) and *Eurotiomycetes* (H=2.64%, INF=0.70%) had higher abundance compared to the INF group (Figure 4B). At the order level, *Nectriaceae* (H=9.94%, INF=8.86%), *Poaceae* (H=1.14%, INF=0.35%), *Aspergillaceae* (H=2.64%, INF=0.50%) and *Achariaceae* (H=0.17%, INF=0.28%) were the main orders in two groups (Figure 4C). Equally important, at the family level, *Nectriaceae* (H=9.94%, INF=8.86%), *Poaceae*

(H=1.14%, INF=0.35%), and *Aspergillaceae* (H=2.64%, INF=0.48%) were represented with higher abundance in H group compared with INF group (Figure 4D). Besides above-mentioned variants, at the genus level, *Ilyonectria* (H=9.33%, INF=8.24%) and *Festuca* (H=1.0%, INF=10.10%) were dominant in two groups. Compared with group INF, other fungal genera such as *Penicillium* (H=2.64%, INF=0.48%), *Xenoacremonium* (H=22%, INF=0), *Trichosanthes* (H=0.07%, INF=0) and *Syspastospora* (H=0.04%, INF=0.02%) were represented with a higher abundance in H group related to health status (Figure 4E).

Significantly, we found similar alterations in the grouping heat map. At the phylum level, *Anthophyta* and *Ascomycota* were enriched in the H and INF groups, while *Ascomycota* was higher in the H group compared to the INF group (Figure 5A). At the class level, *Eurotiomycetes*, *Eudicotyledonae*, and *Sordariomycetes* were identified in high abundance in Group H, while *Leotiomycetes* and *Monocotyledonae* were the most dominant fungi in the INF group (Figure 5B). At the level of order, strong abundance of *Brassicales*, *Cucurbitales*, *Fabales* and *Eurotiales* were detected in group H.

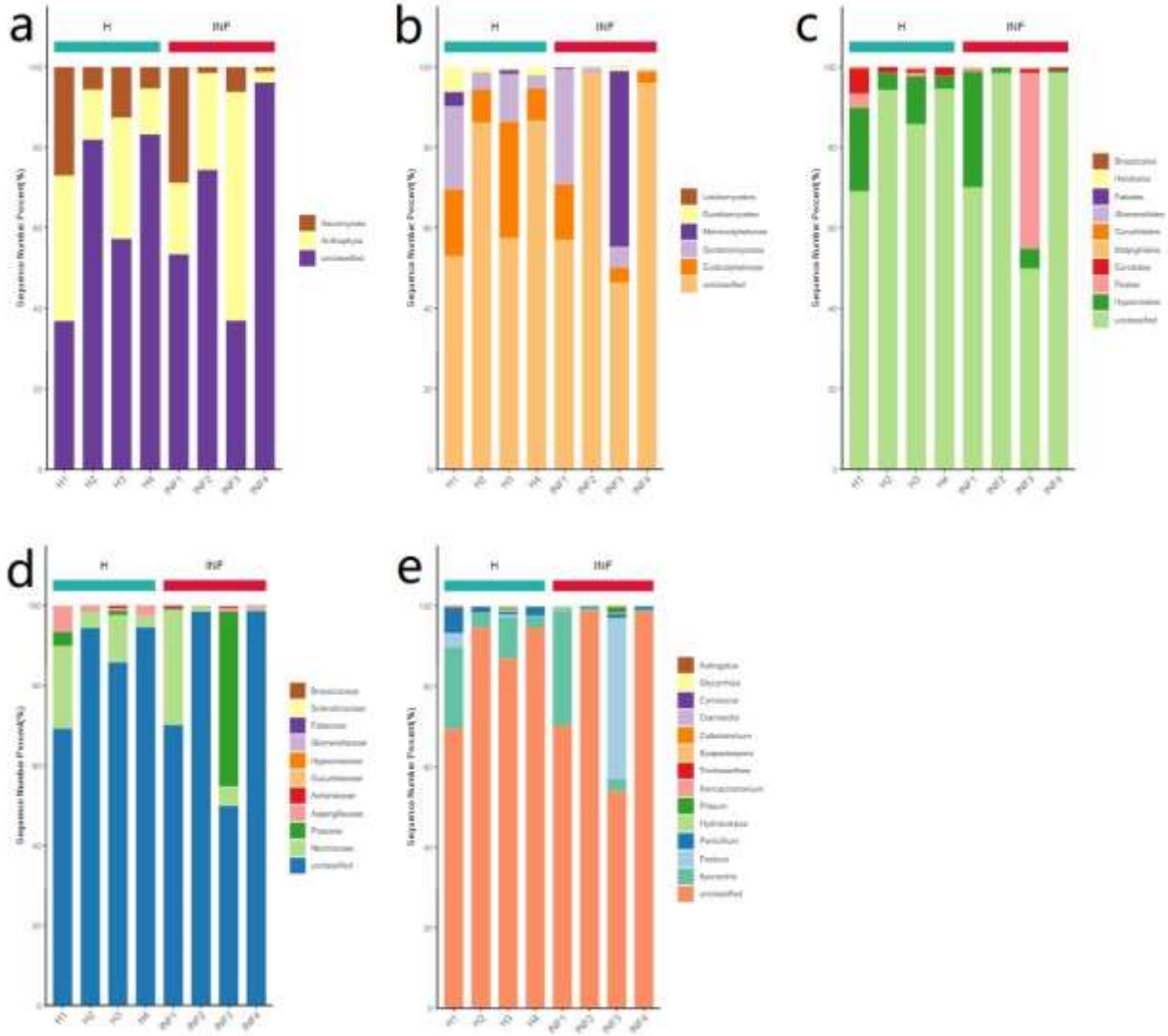

Figure-4. The gut fungal abundances for different levels of groups are shown in Grouping percentage pile bar chart. (a) The abundance and community of gut fungi at the phylum level; (b) The abundance and community of gut fungi at the class level; (c) The abundance and community of gut fungi at the order level; (d) The abundance and community of gut fungi at the family level;(e) The abundance and community of gut fungi at the genus level.

In the INF group, on the other hand, *Glomerellales*, *Poales*, *Malpighiales*, and *Helotiales* were predominant (Figure 5C). At the level of family, *Brassicaceae*, *Cucurbitaceae*, *Fabaceae*, and *Aspergillaceae* were more abundant in the H group. In the INF group, however, we did not even find the *Cucurbitaceae* and *Brassicaceae*, while the *Glomerellaceae*, *Poaceae*, *Achariaceae* and *Sclerotiniaceae* were well represented in the INF group with high abundances. (Figure 5D). At the genus level, the most abundant genera of *Syspastospora*, *Ilyonectria*, *Glycyrrhiza*, *Penicillium*, *Trichosanthes* and *Xenoacremonium* were detected in the H group. Conversely, in the INF group, *Xenoacremonium* and *Trichosanthes* were absent, while *Colletotrichum*, *Festuca*, *Astragalus*, *Cynosurus*, *Hydnocarpus*, *Phleum*, and *Clarireedia* were the dominant genera (Figure 5E).

Variation in the composition of intestinal fungi

Apparent variations in gut fungi at different levels are shown in Figure 6. At the class level (Figure 6A), *Leotiomycetes* was represented by a lower abundance in the H group compared to the INF group ($p < 0.05$). At the order level (Figure 6C), the abundance of *Eurotiales* was significantly higher in H group than in the INF group ($p < 0.05$). At the family level (Figure 6B), it was found that the abundance of *Aspergillaceae* in the H group differed significantly from that in the INF group, of which *Aspergillaceae* decreased significantly under the the effect of *Crytosporidium* infection ($p < 0.05$). Similarly, a comparison between the INF and H groups at the genus level (Figure 6E), revealed a significant decrease in the abundance of *Penicillium* ($p < 0.05$) and *Xenoacremonium* ($p < 0.05$). In addition, we found other significant differences between individual results. *Trichosanthes* and *Glycyrrhiza* were present at low abundances in the H group, but were absent in the INF group (Figure 6D).

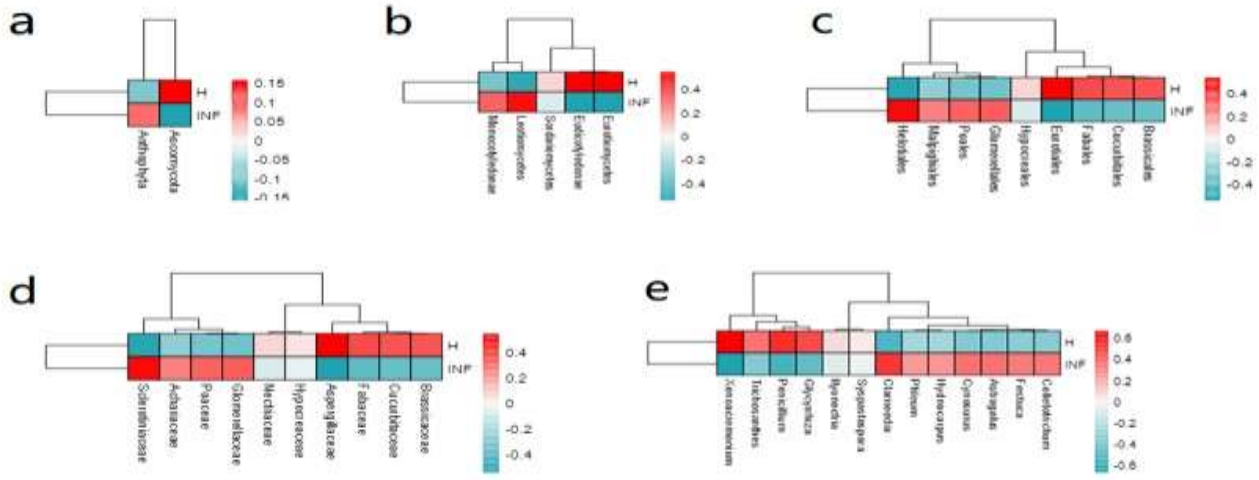

Figure-5. The main fungi in groups H and INF are shown in the heat map of Group clustering at different levels. (a) At the phylum level; (b) At the class level; (c)At the order level; (d) At the family level; (e) At the genus level.

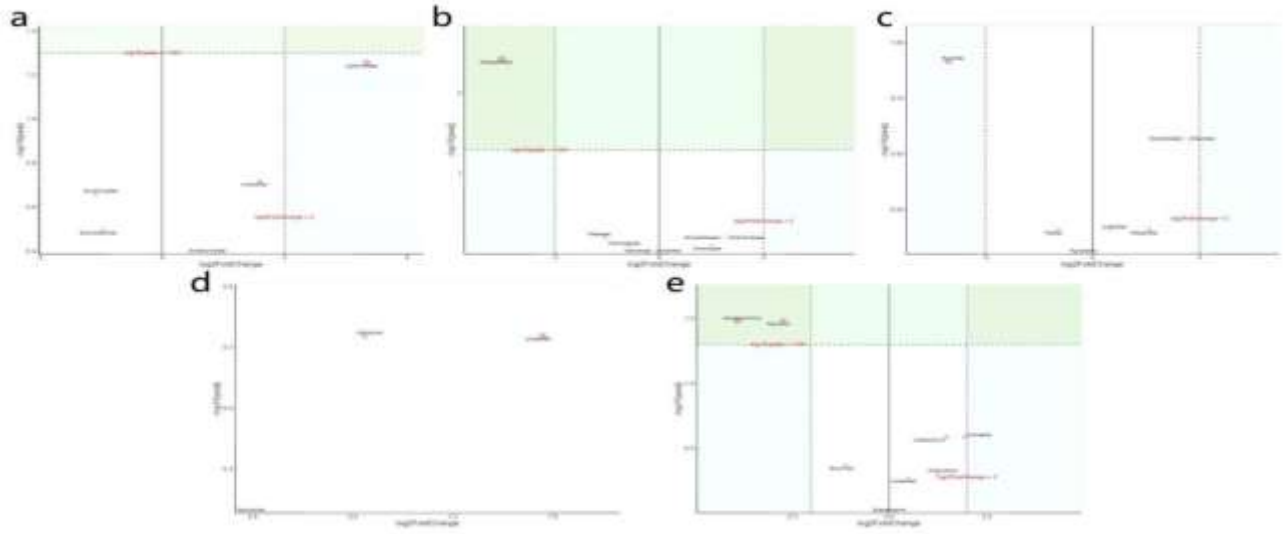

Figure-6. Evident changes of gut fungi under the Cryptosporidium infection in Tibetan pigs. Moreover, based on the DESeq2 analysis, there are some species that are significantly different between groups H and INF at different levels. (a) the class level; (b) the family level; (c) the order level; (d) the phylum level; (e)the genus level.

Correlation network analysis

The correlation between the abundance of different fungal genera in the intestine of Tibetan pigs was determined using Spearman correlation matrix. 5 nodes and 3 edges were found in the fungal network (Figure 7A). The results of the Spearman correlation analysis showed that *Penicillium* was positively associated with *Cynosurus* (0.08) and *Colletotrichum* (0.233), *Cynosurus* was positively associated with *Penicillium* (0.08) and *Colletotrichum* (0.76), *Astragalus* was positively associated with *Clarireedia* (0.76).

The metabolism effect of *Cryptosporidium* infection on Tibetan pigs

To further investigate the association between *Cryptosporidium* infection and intestinal fungi, we compared and determined the metabolic difference using fungal function prediction, which identified 856 enzymes. Some enzymes including *Arabinose-5-phosphate isomerase*, *Quercetin 3-0-methyltransferase* and *Amylosucrase* were significantly altered after *Cryptosporidium* infection in Tibetan pigs (Figure 7B). The enzyme pathways showed increased enzyme activity in individuals in the INF group compared to those in the H group, indicating significant changes in enzyme activity and abundance under the *Cryptosporidium infection*.

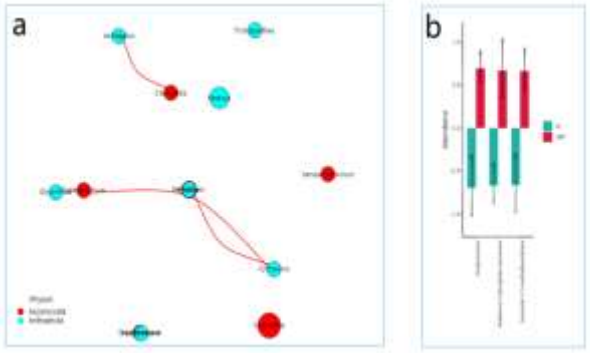

Figure- 7. (a) Correlation Network Analysis indicate the relationship between fungal species; (b)The abundance of enzymes in the H and INF group.

Discussion

Diarrhea is a common clinical condition and alteration in the microbiota population has been identified as the main cause affecting both humans and animals (Xi et al., 2021) . The gut microflora consists of trillions of different fungi, bacteria and viruses. All of these worked together to significantly benefit the host in terms of digestion, metabolism, development and immunological regulations (Bisgaard et al., 2014; Round and Palm, 2018; Ruff et al., 2020). In addition, dysfunctional microbiota balance is commonly observed in a variety of

disorders including diarrhea, obesity, and diabetes (Bharti and Grimm, 2021; Li et al., 2021; Peterson et al., 2021). High throughput sequencing is a technology with a high level of efficiency in using the microbiota, and is widely used to accurately identify different microbial species. Intestinal fungi are essential to host health and growth, contributing to the host's immune system and improving the intestinal barrier. The combination of ITS amplification and DESeqs analysis made it possible to clearly investigate the connection between *Cryptosporidium* and gut fungi. Previous studies have always focused on the relationship between gut bacteria and *Cryptosporidium* infection, and more importantly, the animal studies have always focused mice (Aboelsoued et al., 2022) and cattle (Gong et al., 2017; Tarekegn et al., 2021). Information on fungi associated with *Cryptosporidium* infection in Tibetan pigs is very limited. In this study, ITS sequencing was able to characterize the changes in intestinal fungal spectrum, composition and properties after *Cryptosporidium* infection in Tibetan pigs. Furthermore, analysis of Spearman's correlation matrix and prediction of fungal function revealed differences in fungal correlation and enzymatic activity, respectively. The research showed the relation between *Cryptosporidium* infection and intestinal fungi in Tibetan pigs from a new perspective.

It was found that the alpha diversity indices in the H and INF groups are not significantly different. In Li's report (Li et al., 2021), gut fungal diversity in diarrheal giraffes was similar to that in healthy giraffes, consistent with our findings. It is interesting to note that *Cryptosporidium* infection has significantly reduced bacterial diversity in intestine. In Chen's report (Chen et al., 2023), alpha diversity indices (including Chao1, Faith-pd and observed traits) were lower in *Cryptosporidium*-infected pigs then in healthy pigs. This may be due to that there were so many types of bacteria that were altered by diarrhea.

Although there was no significant change in intestinal fungal diversity between groups H and INF, the proportion of fungi did change. Results of PCoA, NMDS and PCA showed that individuals in the H group apparently separated from those in the INF group, suggesting that *Cryptosporidium* infection altered the composition and profile of gut fungi in Tibetan pigs. The composition of gut fungi varied between taxa at all levels. Groups H and INF contained a high relative abundance of *Anthophyta* and *Ascomycota*. *Anthophyta* originate in the environment and are essential for gut health. *Ascomycota* is the largest fungal phyla and contains healthy species that contribute to the environment and the economy, such as mutualists (Samson et al., 2014). In addition, we found significant changes in the abundance of *Leotiomycetes*, *Aspergillaceae*, *Eurotiales*, *Penicillium* and *Xenoacremonium* between the H and INF groups. We found a lower abundance of *Aspergillus*, *Xenoacremonium*, and *Penicillium* in *Cryptosporidium* infected pigs compared to healthy pigs, suggesting that *Cryptosporidium* infection causes an imbalance of gut fungi by reducing the abundance of main beneficial fungi. *Aspergillus* encompasses a variety of species with metabolic, physiological and phylogenetic characteristics closely related to human or animal life (Park et al., 2019). It has been used in the food fermentation industry for more than two thousand years. At the same time, the *Aspergillaceae* were often considered cell factories with excellent properties for producing organic acids and other secondary metabolites (Guevara-Suarez et al., 2020). Equally important, *Penicillium* is among the most important fungal genera that have contributed to natural ecosystems, agriculture and biotechnology. *Penicillium* is crucial for the ripening process, and is considered a safe additive in the food industry (Weber, 2004). The best-known secondary metabolite of penicillin is produced by *Penicillium*, which saved countless lives during World War II and continues to play an irreplaceable role in medicine (Malekinejad and Fink-Gremmels, 2020). Similarly, the secondary metabolites of *Xenoacremonium* have shown inhibitory effects on tumor cells and have shown promising therapeutic effects in clinical practice. At the order level, we found a lower abundance of *Eurotiales* in the INF group compared to the H group. Galanopoulou et al. (2014) reported that *Eurotiales* play an important role in environmental processes, many of which are of commercial and health importance, such as the production of *penicillin* and citric acid. Other variants have also shown adverse effects of *Cryptosporidium* infection. *Glycyrrhiza* has been used in medicine for thousands of years and exhibits strong anti-inflammatory and allergic activities (Feng et al., 2021). *Glycyrrhiza* has proven successful in treating ulcers (Martin et al., 2008). In addition, five conjugated linolenic acids (CLNA) have been identified in Trichosans,

contributing to health promotion and having potential for clinical application. In turn, no Glycyrrhiza and Trichosans were found in the INF group, which we suspect is the most likely due to *Cryptosporidium* infection causing an imbalance in the gut fungal profile and a reduction in beneficial fungi.

It is significant and interesting that both *Colletotrichum* and *Clarireedia* were very abundant in the INF group. *Colletotrichum* which includes more than 200 species, is considered one of the ten most pathogenic fungi, causes huge financial damage to crops. Due to its virulence and health hazard, *Colletotrichum* is considered a model pathogen for genetic and biochemical physiology (Casas et al., 2021). In addition, *Clarireedia* form a diverse group of species. *Clarireedia* spp, which is considered one of the most destructive and economically destructive fungi, is the pathogen of dollar spots, causing large economic losses and health risks (Liu et al., 2023).

The intestinal microbiota forms a stable ecosystem and plays an important role in enzyme activity, disease prevention and intestinal homeostasis (Gagliardi et al., 2018; Alvarez-Mercado and Plaza-Diaz, 2022). *Cryptosporidium* infection not only altered the gut fungal profile and proportions, but also fungal function in Tibetan pigs. *Amylosucrase* ($p < 0.05$), *Arabinose-5-phosphate isomerase* ($p < 0.05$) and *Quercetin 3-O-methyltransferase* ($p < 0.05$) were significantly different between groups H and INF. *Amylosuctase* own the ability to utilize abundant biomasses to provide energy. We found a higher abundance of *Amylosucrase* in the INF group, which is the most likely due to the fact that *Cryptosporidium* also requires the energy provided by the *Amylosucrase* function. In the study, the level of *Arabinose - 5-phosphate isomere* was higher in the INF group than in the H group. *Arabinose-5-phosphate isomerase* has long been recognized as an anti-bacterial design target, which could explain the reduced diversity of gut bacteria in the *Cryptosporidium* infection (Chen et al., 2023). In view of the phenomenon of change in enzyme activity, we speculate that gut fungi may play an essential role in metabolism of Tibetan pigs.

Conclusion

In summary, a total of 2 phyla, 5 classes, 9 orders, 10 families and 13 genera were detected in the H and INF groups. *Cryptosporidium* has induced significant changes in the proportions and metabolism of intestinal fungi in Tibetan pigs. The profile of fungi varied between taxa at the phylum, class, order, family and genus levels, in which the abundance of *Leotiomycetes* ($p < 0.05$), *Aspergillaceae* ($p < 0.05$), *Penicillium* ($p < 0.05$), *Xenoacremonium* ($p < 0.05$) and *Eurotiales* ($p < 0.05$) were significantly reduced under *Cryptosporidium* infection. The above mentioned fungi were beneficial to the health of animals, human beings or environmental processes. Conversely, the highly abundant genera of *Colletotrichum* and *Clarireedia* were enriched in the INF group. Both *Colletotrichum* and *Clarireedia* are considered as destructive pathogenic fungi, causing significant economic losses and health threats. The results showed significant variability in the composition and distribution of fungi in Tibetan pigs infected with *Cryptosporidium*, characterized by a significant decrease in the abundance of beneficial fungi and an increase in the abundance of disease-causing fungi that threaten health. In addition to changes in fungal composition of *Cryptosporidium* infected Tibetan pigs, the enzyme activity of *Cryptosporidium* infection in Tibetan pigs was also significantly different, such as increased abundance of *Amylosucrase* ($p < 0.05$), *Arabinose-5-phosphate isomerase* ($p < 0.05$) and *Quercetin 3-O-methyltransferase* ($p < 0.05$). Simultaneously, Spearman correlation analysis indicted that *Penicillium* was positively associated with *Cynosurus* (0.08) and *Colletotrichum* (0.233), *Cynosurus* was positively related to *Penicillium* (0.08) and *Colletotrichum* (0.76), *Astragalus* was positively with *Clarireedia* (0.76), which provided another perspective for understanding the associations among taxa and samples. Our findings shed light on the alteration of the fungi in both healthy and *Cryptosporidium*-infected pigs, as well as providing a new perspective in the identification of *Cryptosporidium* and involving in details for further development of therapeutics against *Cryptosporidium* in pigs.

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The data in the research are available from the corresponding author on reasonable request.

References

Aboelsoued D, Abdullah H, Megeed K, Hassan SE and Toaleb NI, 2022. Evaluation of a vaccine candidate isolated from Cryptosporidium parvum oocyst in mice. Vet World. 15(12): 2772-2784. doi: 10,14202/vetworld,2022,2772-2784

Alvarez-Mercado AI and Plaza-Diaz J, 2022. Dietary Polysaccharides and Gut Microbiota Ecosystem. Nutrients. 14(20). doi: 10,3390/nu14204285

Arfken AM, Frey JF and Summers KL, 2020. Temporal Dynamics of the Gut Bacteriome and Mycobiome in the Weanling Pig. Microorganisms. 8(6). doi: 10,3390/microorganisms8060868

Bharti R and Grimm DG, 2021. Current challenges and best-practice protocols for microbiome analysis. Brief Bioinform. 22(1): 178-193. doi: 10,1093/bib/bbz155

Bisgaard H, Bonnelykke K and Stokholm J, 2014. Immune-mediated diseases and microbial exposure in early life. Clin. Exp. Allergy. 44(4): 475-481. doi: 10,1111/cea,12291

Bliss JM, Basavegowda KP, Watson WJ, Sheikh AU and Ryan RM, 2008. Vertical and horizontal transmission of Candida albicans in very low birth weight infants using DNA fingerprinting techniques. Pediatr. Infect. Dis. J. 27(3): 231-235. doi: 10,1097/INF,0b013e31815bb69d

Casas LL, Azevedo JL, Almeida LN, Costa-Neto PQ, Bianco RA and Pereira JO, 2021. Mycoviruses infecting Colletotrichum spp,: A comprehensive review. Braz. J. Biol. 83: e248975. doi: 10,1590/1519-6984,248975

Chen D, Zhong G, Su H, Rahman MAU, Chen K, Tang J and Li F, 2021. Physiological variation in ruminal microbiota under altered energy levels in starter ration of suckling Angus calves. Pak. Vet. J. 41(3): 409-413. http://dx.doi.org/10.29261/pakvetj/2021.018

Chen X, Zhao X, Zhao C, Ashfaq H, Fakhar-E-Alam KM, Bhutta ZA and Li K, 2023. Cryptosporidium infection induced the dropping of SCFAS and dysbiosis in intestinal microbiome of Tibetan pigs. Microb. Pathog. 17 (4): 105922. doi: 10,1016/j,micpath,2022,105922

Chen X, 2022. Molecular Epidemiological Investigation of Cryptosporidium sp., Giardia duodenalis, Enterocytozoon bieneusi and Blastocystis sp. Infection in Free-ranged Yaks and Tibetan Pigs on the Plateau. Pak. Vet. J. 42: 533-539. http://dx.doi.org/10.29261/pakvetj/2022.060

Coker OO, Nakatsu G, Dai RZ, Wu W, Wong SH, Ng SC and Yu J, 2019. Enteric fungal microbiota dysbiosis and ecological alterations in colorectal cancer. Gut. 68(4): 654-662. doi: 10,1136/gutjnl-2018-317178

Cryan JF and Dinan TG, 2012. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. Nat. Rev. Neurosci. 13(10): 701-712. doi: 10,1038/nrn3346

De Felice LA, More G, Cappuccio J, Venturini MC and Unzaga JM, 2020. Molecular characterization of Cryptosporidium spp, from domestic pigs in Argentina. Vet. Parasitol. Reg. Stud. Reports. 22: e100473. doi: 10,1016/j,vprsr,2020,100473

Dinan TG and Cryan JF, 2015, The impact of gut microbiota on brain and behaviour: implications for psychiatry. Curr. Opin. Clin. Nutr. Metab. Care. 18(6): 552-558. doi: 10,1097/MCO,0000000000000221

Feng Y, Mei L, Wang M, Huang Q and Huang R, 2021. Anti-inflammatory and Pro-apoptotic Effects of 18beta-Glycyrrhetinic Acid In Vitro and In Vivo Models of Rheumatoid Arthritis. Front. Pharmacol. 12: e681525. doi: 10,3389/fphar,2021,681525

Gagliardi A, Totino V, Cacciotti F, Iebba V, Neroni B, Bonfiglio G and Schippa S, 2018. Rebuilding the Gut Microbiota Ecosystem. Int. J. Environ. Res. Public Health. 15(8). doi: 10,3390/ijerph15081679

Galanopoulou K, Scazzocchio C, Galinou ME, Liu W, Borbolis F, Karachaliou M and Amillis S, 2014. Purine utilization proteins in the

Eurotiales: cellular compartmentalization phylogenetic conservation and divergence. Fungal Genet. Biol. 69: 96-108. doi: 10,1016/j,fgb,2014,06,005

Gong C, Cao XF, Deng L, Li W, Huang XM, Lan JC and Peng GN, 2017. Epidemiology of Cryptosporidium infection in cattle in China: a review. Parasite. 24: 1. doi: 10,1051/parasite/2017001

Guevara-Suarez M, Garcia D, Cano-Lira JF, Guarro J and Gene J, 2020. Species diversity in Penicillium and Talaromyces from herbivore dung and the proposal of two new genera of penicillium-like fungi in Aspergillaceae. Fungal Syst. Evol. 5: 39-75. doi: 10,3114/fuse,2020,05,03

Guo Y, Li R, Sun X, Zhang Z, Zheng H, Han L, Cui Y, Zhang D and Liu M, 2022. In vitro antibiotic susceptibility, virulence genes profiles and integrons of streptococcus suis isolates from pig herds in Liaoning Province of China. Pak. Vet. J. 42(1): 117-121. http://dx.doi.org/10.29261/pakvetj/2021.074

He Z, Ma Y, Yang S, Zhang S, Liu S, Xiao J and Cao Z, 2022. Gut microbiota-derived ursodeoxycholic acid from neonatal dairy calves improves intestinal homeostasis and colitis to attenuate extended-spectrum beta-lactamase-producing enteroaggregative Escherichia coli infection. Microbiome. 10(1): 79. doi: 10,1186/s40168-022-01269-0

Ijaz M, Manzoor A, Mohy-ud-Din MT, Hassan F, Mohy-ud-Din Z, Ans M, Saleem MI, Khan HH and Khanum F, 2021. An economical non-antibiotic alternative to antibiotic therapy for subclinical mastitis in cows. Pak. Vet. J. 41(4): 475-480. http://dx.doi.org/10.29261/pakvetj/2021.059

Jiang TT, Shao TY, Ang W, Kinder JM, Turner LH, Pham G and Way SS, 2017. Commensal Fungi Recapitulate the Protective Benefits of Intestinal Bacteria. Cell Host Microbe. 22(6): 809-816. doi: 10,1016/j,chom,2017,10,013

Lan Y, Li K, Mehmood K and Qudratullah, 2021. Molecular investigation of important protozoal infections in yaks. Pak. Vet. J. 41(4): 557-561. http://dx.doi.org/10.29261/pakvetj/2020.048

Li A, Liu B, Li F, He Y, Wang L, Fakhar-E-Alam KM and Jiang X, 2021. Integrated Bacterial and Fungal Diversity Analysis Reveals the Gut Microbial Alterations in Diarrheic Giraffes Front. Microbiol. 12: e712092. doi: 10,3389/fmicb,2021,712092

Li K, Luo H, Zhang H, Lan Y, Han Z, Shahzad M and Li J, 2016. First report of Metastrongylus pudendotectus by the genetic characterization of mitochondria genome of cox1 in pigs from Tibet China. Vet. Parasitol. 223: 91-95. doi: 10,1016/j,vetpar,2016,04,036

Li K, Shahzad M, Zhang H, Mehmood K, Jiang X, Luo H and Li J, 2018. Characterization of the complete mitochondrial genome of Metastrongylus salmi (M. salmi) derived from Tibetan pigs in Tibet China. Acta Parasitol. 63(2): 280-286. doi: 10,1515/ap-2018-0032.

Li K, Zhang L, Zhang H, Lei Z, Luo H, Mehmood K and Li J, 2017. Epidemiological investigation and risk factors of Echinococcus granulosus in yaks (Bos grunniens) Tibetan pigs and Tibetans on Qinghai Tibetan plateau. Acta Trop. 173: 147-152. doi: 10,1016/j,actatropica,2017,06,019

Liggenstoffer AS, Youssef NH, Couger MB and Elshahed MS, 2010. Phylogenetic diversity and community structure of anaerobic gut fungi (phylum Neocallimastigomycota) in ruminant and non-ruminant herbivores. ISME J. 4(10): 1225-1235. doi: 10,1038/ismej,2010,49

Liu J, Wu J, Jin P, Hu J, Lamour K and Yang Z, 2023. Activity of a succinate dehydrogenase inhibitor fungicide benzovindiflupyr against Clarireedia spp. Plant Dis. https://doi.org/10.1094/PDIS-02-23-0201-RE

Lv C, Li C, Wang J and Qian W, 2022. Detection and molecular characterization of Cryptosporidium spp, in pet hairless uinea pigs (Cavia Porcellus) from China. Parasitol. Res. 121(9): 2739-2745. doi: 10,1007/s00436-022-07603-7

Malekinejad H and Fink-Gremmels J, 2020. Mycotoxicoses in veterinary medicine: Aspergillosis and penicilliosis. Vet. Res. Forum. 11(2): 97-103. doi: 10,30466/vrf,2020,112820,2686

Martin MD, Sherman J, van der Ven P and Burgess J, 2008. A controlled trial of a dissolving oral patch concerning glycyrrhiza (licorice) herbal extract for the treatment of aphthous ulcers. Gen. Dent. 56(2): 206-210. doi: 10,30466/vrf,2023,211-212 224

Park MS, Oh SY, Fong JJ, Houbraken J and Lim YW, 2019. The diversity and ecological roles of Penicillium in intertidal zones. Sci. Rep. 9(1): 13540. doi: 10,1038/s41598-019-49966-5

Peterson D, Li T, Calvo AM and Yin Y, 2021. Categorization of Orthologous Gene Clusters in 92 Ascomycota Genomes Reveals Functions Important for Phytopathogenicity. J. Fungi (Basel). 7(5): 337. https://doi.org/10.3390/jof7050337

Qi M, Zhang Q, Xu C, Zhang Y, Xing J, Tao D and Zhang L, 2020. Prevalence and molecular characterization of Cryptosporidium spp, in pigs in Xinjiang China. Acta Trop. 209: e105551. doi: 10,1016/j,actatropica,2020,105551

Round JL and Palm NW, 2018. Causal effects of the microbiota on immune-mediated diseases. Sci. Immunol. 3(20). doi: 10,1126/sciimmunol,aao1603

Ruff WE, Greiling TM and Kriegel MA, 2020. Host-microbiota interactions in immune-mediated diseases. Nat. Rev. Microbiol. 18(9): 521-538. doi: 10,1038/s41579-020-0367-2

Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH and Frisvad JC, 2014. Phylogeny identification and nomenclature of the genus Aspergillus. Stud. Mycol. 78: 141-173. doi: 10,1016/j,simyco,2014,07,004

Singh P, Teal TK, Marsh TL, Tiedje JM, Mosci R, Jernigan K and Manning SD, 2015. Intestinal microbial communities associated with acute enteric infections and disease recovery. Microbiome. 3: 45. https://doi.org/10.1186/s40168-015-0109-2

Sokol H, Leducq V, Aschard H, Pham H, P, Jegou S, Landman C and Beaugerie L, 2017. Fungal microbiota dysbiosis in IBD. Gut. 66(6): 1039-1048. doi: 10,1136/gutjnl-2015-310746

Striepen B, 2013, Parasitic infections: Time to tackle cryptosporidiosis. Nature. 503(7475): 189-191. doi: 10,1038/503189a

Tarekegn ZS, Tigabu Y and Dejene H, 2021. Cryptosporidium infection in cattle and humans in Ethiopia: A systematic review and meta-analysis. Parasite Epidemiol. Control. 14: e219. doi: 10,1016/j,parepi,2021,e00219

Thathaisong U, Siripattanapipong S, Inpankaew T, Leelayoova S and Mungthin M, 2020. High prevalence of Cryptosporidium infection caused by C, scrofarum and C, suis among pigs in Thailand. Parasitol. Int. 77: e102122. doi: 10,1016/j,parint,2020,102122

Wang P, Li S, Zou Y, Du ZC, Song DP, Wang P and Chen XQ, 2022. The infection and molecular characterization of Cryptosporidium spp, in diarrheic pigs in southern China. Microb. Pathog. 165: e105459. doi: 10,1016/j,micpath,2022,105459

Wang DB, Yan-Bin Z, Zhan-Dui P, Dun-Zhu L, Yang-Ji C, Yang-La D, Guang-Ming S and Guang-Xin E, 2021. Exploration of the exogenous male yak introduction breeding model and its effects on Tibetan small-sized family farms. Pak. Vet. J. 41(1): 137-141. http://dx.doi.org/10.29261/pakvetj/2020.096

Ward TL, Dominguez-Bello MG, Heisel T, Al-Ghalith G, Knights D and Gale CA, 2018. Development of the Human Mycobiome over the First Month of Life and across Body Sites. mSystems. 3(3). doi: 10,1128/mSystems,00140-17

Weber RW, 2004. Penicillium. Ann. Allergy Asthma Immunol. 93(4): A6. doi: 10,1016/s1081-1206(10)61386-5

Wolff R, Shoemaker W and Garud N, 2023. Ecological Stability Emerges at the Level of Strains in the Human Gut Microbiome. mBio. 14(2): e250222. doi: 10,1128/mbio,02502-22

Xi L, Song Y, Han J and Qin X, 2021. Microbiome analysis reveals the significant changes in gut microbiota of diarrheic Baer's Pochards (Aythya baeri). Microb. Pathog. 157: e105015. doi: 10,1016/j,micpath,2021,105015

Zhan K, Zheng H, Li J, Wu H, Qin S, Luo L and Huang S, 2020. Gut Microbiota-Bile Acid Crosstalk in Diarrhea-Irri table Bowel Syndrome. Biomed. Res. Int. 2020: e3828249. doi: 10,1155/2020/3828249

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Qinghui K: Conceived idea, supervised study and secured funding for research