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Article

Livestock, Humans, and the Environment as Reservoirs of *Cryptosporidium* and *Giardia duodenalis*: Evidence from a One Health Study in Türkiye

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Abstract

Cryptosporidium spp. and *Giardia duodenalis* are globally important intestinal protozoa causing diarrheal disease in humans and animals, with significant zoonotic potential. This study aimed to investigate the prevalence, molecular diversity, and potential transmission dynamics of these parasites in humans, livestock, and environmental samples from a rural community in Türkiye using a One Health approach, and to assess their associations with gut microbiome composition. Faecal samples were collected from 124 humans, 305 livestock (cattle, sheep, and goats), and 40 environmental samples (water and mud). Parasites were detected using qPCR and nested PCR, with positive samples genotyped by sequencing. Microbiome profiling was performed using 16S rRNA gene amplicon sequencing. *Giardia duodenalis* was detected in 12.1% of humans, 17.5% of livestock, and 2.5% of environmental samples, with assemblages A, B, and E identified, including the first detection of assemblage E in a human in Türkiye. *Cryptosporidium* spp. were found in 8.9% of humans, 19.3% of livestock, and 55% of environmental samples, with *C. parvum* as the dominant zoonotic species. Microbiome analysis revealed no significant differences in overall diversity. This study provides the first One Health assessment of *Cryptosporidium* spp. and *Giardia duodenalis* in Türkiye, emphasizing zoonotic transmission risks linked to livestock and the environment.



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Keywords: *Cryptosporidium* spp.; *Giardia duodenalis*; One Health; zoonotic transmission; livestock; gut microbiome; molecular epidemiology; environmental contamination

1. Introduction

Intestinal protozoa continue to pose a significant global public health challenge, contributing to gastrointestinal diseases that affect both human and animal health. Among these, *Cryptosporidium* spp. and *Giardia duodenalis* (*G. duodenalis*) are two of the most prevalent waterborne protozoan parasites, causing diarrheal diseases in humans and animals alike [1]. These infections can lead to severe dehydration, malnutrition, and chronic

gastrointestinal complications, particularly in children and immunocompromised individuals [2,3]. Beyond their immediate health effects, these parasites also impose economic burdens, reducing productivity due to illness and affecting livestock growth and yield in agricultural settings [4].

The epidemiology of *Cryptosporidium* spp. and *G. duodenalis* is complex, with multiple transmission pathways, including direct human-to-human and animal-to-human transmission, as well as indirect environmental exposure through contaminated water, food, or soil [5]. While these parasites have long been recognised for their role in gastrointestinal disease, emerging research suggests that they may also influence the composition and function of the gut microbiome [6,7]. The gut microbiome plays a crucial role in immune function, metabolism, and maintaining gut homeostasis, and disruptions caused by enteric infections can have far-reaching consequences for health [8,9].

Studies have shown that *Cryptosporidium* spp. and *G. duodenalis* infections can lead to dysbiosis, an imbalance in the gut microbial community, which may exacerbate disease severity and prolong symptoms [10,11]. *Cryptosporidium* spp. infections, for example, have been associated with reduced bacterial diversity and increased inflammatory taxa. In contrast, *G. duodenalis* infections have been linked to altered carbohydrate metabolism and microbial shifts that may predispose individuals to long-term gastrointestinal disorders [12,13]. Understanding these interactions is essential for developing comprehensive strategies to mitigate the impact of infection and improve patient outcomes.

Given the zoonotic nature and environmental persistence of these parasites, a holistic One Health approach that integrates human, animal, and environmental health is essential for effective surveillance, prevention, and control strategies. One Health acknowledges that human health is interconnected with the health of animals and the environment, and therefore, a collaborative, transdisciplinary effort is necessary to tackle these infections effectively [14].

Rural areas, where close interactions between humans, livestock, and natural water sources are shared, represent critical zones for studying transmission dynamics and potential intervention strategies [15,16]. By understanding the shared risks and developing cross-sectoral collaborations, the One Health approach enables a more robust response to controlling protozoan infections, reducing disease burden in both humans and animals while safeguarding environmental health [17–19].

This study hypothesises that *Cryptosporidium* spp. and *G. duodenalis* infections in rural Türkiye are influenced by environmental factors and interspecies transmission, leading to alterations in the gut microbiome that may exacerbate disease outcomes. To test these hypotheses, we aim to determine the presence of *Cryptosporidium* spp. and *G. duodenalis* in humans, livestock, and environmental samples from Kırıklı Village in the Adana Province, Türkiye. This can give us a simple idea about Mediterranean countries in general, due to the similarity of food and social cultures to some extent. Through molecular characterisation and microbiome analysis, we will assess the genetic diversity of the parasites, evaluate their zoonotic potential, and investigate potential associations between infection and gut microbiome composition. This research will provide valuable insights into the transmission dynamics of these parasites and inform targeted intervention strategies to reduce their burden on public health.

2. Materials and Methods

2.1. Study Area

The study was conducted in Kırıklı Village of the Adana Province (Figure 1), with a population of 582, in the Mediterranean climate zone of Türkiye (37°10' N, 35°14' E). The village, which relies on farming and animal husbandry, is 35 km from the city centre

and the Karaisalı district. The Seyhan Dam, constructed 70 years ago, is 12 km away from the village. The lake's branches recede during November and December, making it accessible for recreational activities like picnics and camping. The area also serves as pasture for grazing animals. In April and May, the area fills with water once more as a result of snowmelt.



Figure 1. The lower right picture shows Türkiye map. The left picture shows the districts of Adana province (the location of the village is marked with a pin). The diagram shows households where both human and animal samples were collected (red circles) and those where only human samples were collected (black circles). This figure was made in BioRender, Akdur Öztürk, E. (2025) <https://app.biorender.com/illustrations/69b91b741c5aba4a201da83f>.

2.2. Sample Collection

Considering the minimum sample size calculated with the G-Power program (version 3.1.9.7), as many human, animal and environmental samples as possible were included in the study. The required sample size was calculated using a single-proportion formula with finite population correction at a 95% confidence level ($p = 0.5$). In total, 124 human, 305 livestock, and 40 environmental samples were collected once from each host between October and November 2023. The samples were stored in DNA/RNA Shield™ (Zymo Research, Freiburg, Germany) at a 1:2 ratio for further examination. In this survey, we used DNA samples of human, animal, and environmental origin from a previous study conducted by our research group, which investigated the transmission dynamics of *Blastocystis* within a One Health framework [20].

2.2.1. Human Faecal Samples

A “purposive sampling method” was used to determine the number of human samples [21]. A total of 64 households, comprising 90 individuals engaged in animal husbandry and 34 individuals who declared that they had not had animals for the past six months, were randomly selected for the study, regardless of any gastrointestinal symptoms. A total of 124 individuals were enrolled in the study, corresponding to approximately 21% of the village's population. Each participant was given a labelled, sterile faeces collection container. The necessary study permission was obtained from the Çukurova University Ethics Committee for human sample (approval number 136-49) and microbiome analysis (approval number 39/147).

2.2.2. Animal Faecal Samples

The number of each animal group (89 cattle, 151 sheep, and 65 goats) was calculated using the “proportional stratified sampling” method, and animals were selected from animal holdings by the “simple random sampling” method [21]. One fresh faecal sample was obtained from each individual animal, and thus the number of faecal samples corresponded to the total number of animals included in the study. To minimise environmental contamination, the samples were obtained during defecation or immediately after defecation and stored in pre-labelled containers.

2.2.3. Environmental Samples

Twenty-four water samples from the Seyhan Dam lake (five limpid, 10 slightly turbid, and nine highly turbid) and 16 mud samples were collected from the nearest locations where human activities (picnics, camping, fishing, etc.) and animal grazing took place, as well as from areas utilised for livestock irrigation and agricultural purposes. Dam water samples (one litre each) were left overnight, drained, and then transferred to tubes for centrifugation (Hettich EBA 25 centrifuge, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) [22]. The sediment and mud samples were preserved in a DNA/RNA shield for further analysis.

2.3. Genomic DNA Extraction

A PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, Carlsbad, CA, USA) was used for DNA isolation according to the manufacturer's protocol using 200 µL of the well-vortexed DNA/RNA shielding sample mixture.

2.4. Screening and Molecular Genotyping of *Cryptosporidium* spp. and *Giardia duodenalis*

The initial stage in identifying *G. duodenalis* involved the quantitative Polymerase Chain Reaction (qPCR) method (Supplementary Table S1), which amplified a 62 bp fragment of the rRNA (*ssu RNA*) of the parasite [23]. To determine the genotype of *Giardia* sp., the qPCR-positive samples were analysed by nested Polymerase Chain Reaction (nPCR). Genotype-specific primers were used to amplify a 511 bp fragment of β -giardin (β g) [24] and a 530 bp fragment of triosephosphate isomerase (*tpi*) [25] in all positive samples [26].

The presence of *Cryptosporidium* spp. was investigated by amplifying the 578 bp *ssu rRNA* gene of this parasite using nPCR methods [27]. To analyse the intraspecific genetic diversity of *Cryptosporidium* spp., nPCR was applied to amplify the 60 kDa glycoprotein (*gp60*) gene (~820–864 bp) (Supplementary Table S1). A negative control (nuclease-free water) and a positive control (genomic DNA of targeted parasites) were used in both qPCR and nPCR runs. The PCR settings and oligonucleotide sequences used for the molecular characterisation and genotyping analysis of all specified parasites are presented in Table S1 of the Supplementary Materials.

For each positive secondary PCR product of both *Cryptosporidium* spp. and *G. duodenalis*, DNA sequencing was performed using internal PCR primers (listed in the Supplementary Materials). To determine species-level identifications, the obtained sequences were used as queries in a BLAST comparison against gene sequences in the National Center for Biotechnology Information (NCBI) database, accessed through the BLAST tool (v. 2.17.0) using the core nucleotide database. The nucleotide sequences generated from this study have been deposited in GenBank under the following accession numbers: PZ117843-PZ117877 for the *Cryptosporidium18s rRNA* gene and PZ133881-PZ133911 for the *Giardia* β g and *tpi* genes.

2.5. 16S rRNA Gene Amplicon Sequencing

High-throughput amplicon sequencing was outsourced to Novogene, (Novogene Company Limited, Cambridge, UK) following a modified protocol based on Caporaso et al. [28]. One nanogram of extracted DNA was used, fragmented, and prepared for paired-end sequencing. Amplification was performed using the primer pair 515f (GT-GCCAGCMGCCGCGGTAA) and 907r (CCGTCAATTCCTTTGAGTTT), which targets the hypervariable region. Sequencing was carried out on the Illumina NovaSeq platform.

Raw sequencing reads were processed using the Lotus2 software [29]. The workflow included chimera detection and the removal via Minimap2 [30]. Minimap2 was also employed to identify off-target reads containing human DNA by conducting a BLAST search against the Genome Reference Consortium Human Build 38.p14. Trimmed reads from the

V3-V4 region were clustered into Amplicon Sequence Variants (ASVs) using the Divisive Amplicon Denoising Algorithm 2 [26], with a dissimilarity threshold of ≤ 1 nucleotide. Taxonomic classification of ASVs (to the species level) was performed via BLAST against the GreenGenes2 (GG2) database [31]. GG2 was selected for its robustness, as it is a unified database applicable to 16S data, ensuring reproducibility.

2.6. Statistical Analysis

The parasite sequencing data collected from the research were examined using IBM SPSS version 29 software for statistical evaluation. Comparative assessments were conducted using the chi-square test, with a p -value less than 0.05 considered to indicate statistical significance.

Statistical analysis and data visualisation of the 16S microbiome sequencing data were conducted using R Studio 4.2.3. To account for variations in sequencing depth, sequencing data were rarefied before analysis. A decision was reached after plotting and consulting rarefaction/species discovery curves (Supplementary Figure S1). A cutoff of 60,000 reads was considered suitable to maximise the number of species discovered while limiting the effect of vastly larger read counts in a handful of samples (>250,000 reads). Diversity indices, including Shannon, Chao1, Simpson, and observed taxa, were computed using the Phyloseq package. These diversity metrics were analysed for statistical differences between the sample groups (see paragraph below). The sample groups consisted of two sets of positive and negative samples, determined by the infection status of *Giardia* or *Cryptosporidium* (both had a positive and negative group).

To determine the correct statistical tests for comparing diversity index scores between infection groups, Shapiro–Wilk tests were performed. Normally distributed data were analysed using ANOVA, followed by the Tukey HSD test for pairwise comparisons. Non-normally distributed data were assessed using the Kruskal–Wallis test, with pairwise comparisons conducted using the Dunn test (Bonferroni p -adjusted).

Microbiome composition was visualised using compositional plots, which highlighted taxa comprising more than 1% of the total read counts. Principal coordinate analysis (PCoA) was used to evaluate differences in overall microbiome composition between infection groups. Microbiome sample content was plotted according to each sample's Bray–Curtis dissimilarity matrix values. Finally, statistical significance of group clustering was assessed using PERMANOVA [32] to determine whether the centroids of the different infection statuses for both parasites were significantly distant from each other.

To identify taxa whose presence/abundance was significantly correlated with one or the other of the infection status groups, linear discriminant effect size (LEfSe) analysis [33] was performed. LEfSe analysis is used to detect taxa whose presence/absence best explains differences between *Cryptosporidium*/*Giardia*-positive and *Cryptosporidium*/*Giardia*-negative groups. No multiple-testing correction was applied.

3. Results

3.1. Occurrence of *Giardia duodenalis* and *Cryptosporidium* spp.

A total of 469 samples were investigated for the presence of *G. duodenalis* by qPCR and *Cryptosporidium* spp. by nested PCR. Of the 124 human samples analysed, 15 (12.1%; 95% CI: 6.1–17.9) showed positivity for *G. duodenalis*. The overall *G. duodenalis* positivity was 17.5% (52/305; 95% CI: 12.1–22) in domestic animals. Among these, the highest positivity rate was found in sheep with 23.8% (36/151), followed by goats with 15.4% (10/65), and cattle with 6.7% (6/89). Only one of 40 environmental samples (2.5%; 95% CI: 0.06–13.2) was determined to be positive. While the *Cryptosporidium* spp. positivity rate of human samples was detected as 8.9% (11/124; 95% CI: 3.8–14), overall positivity was 19.3% (59/305;

95% CI: 11.8–20.3) in domestic animals, with 47.2% (42/89) in cattle, 9.9% (15/151) in sheep, 3% in goat (2/65), and 55% (22/40; 95% CI: 39.7–70.3) in environmental samples. The occurrence of *G. duodenalis* and *Cryptosporidium* spp. is presented in Table 1.

Table 1. Descriptive prevalence of *Giardia duodenalis* and *Cryptosporidium* spp. in human, animal and environmental samples.

Sample Type	Total Samples (n)	No. of Positive Samples <i>Giardia duodenalis</i> * (%) ** (95% CI)	No. of Positive Samples <i>Cryptosporidium</i> spp. * (%) (95% CI)
Human	124	15 (12.1%, 6.1–17.9)	11 (8.9%, 3.8–14)
Total animals	305	52 (17.5%, 12.1–22)	59 (19.3%, 11.8–20.3)
Cattle	89	6 (6.7%, 3.1–13.9)	42(47.2%, 37.2–57.5)
Sheep	151	36 (23.8%, 17.7–31.2)	15 (9.9%, 6.1–15.7)
Goat	65	10 (15.4%, 8.6–26.1)	2 (3%, 0.8–10.5)
Environmental Samples	40	1 (2.5%, 0.06–13.2)	22 (55%, 39.7–70.3)

Each sample represents one individual human or animal; therefore, the number of samples corresponds exactly to the number of individuals sampled. * The percentages in each row in the table show the positivity rates within the relevant sample group; ** CI; Confidence Interval.

The overall investigated intestinal parasite positivity rate (*G. duodenalis* and/or *Cryptosporidium* spp.) in humans was found to be 20.2% (25/124). There was only one case of co-infection with *G. duodenalis* and *Cryptosporidium* spp. The relationship between demographic factors, such as age, gender, and livestock, and intestinal parasite positivity was statistically examined, revealing a significant correlation ($p < 0.05$) only between gender and intestinal parasite positivity. Of the participants in the positive samples, 28% (7/25) were female and 72% (18/25) were male.

3.2. Molecular Diversity of *Giardia duodenalis* and *Cryptosporidium* spp.

Of the 68 *G. duodenalis*-qPCR positive samples, 25 were genotyped with at least one of the *bg* and *tpi* loci by multilocus genotyping (MLG)-nested PCR. Overall, three *G. duodenalis* assemblages were identified, including zoonotic assemblage A (16%, 4/25) in two humans and two sheep, and assemblage B (8%, 2/25) in two humans. The ungulate-adapted assemblage E (76%, 19/25) was found in all host types, including one human, thirteen sheep, one cow and four goats.

Additionally, the assemblage A samples detected in humans and sheep belonged to AII and AI, respectively. Only one qPCR-positive environmental sample could not be genotyped.

In this study, 35 out of 80 *Cryptosporidium* spp. nPCR-positive samples were successfully sequenced by 18S *rRNA* analysis, revealing the presence of five *Cryptosporidium* species circulating in human, cattle, sheep and environmental samples, including *C. parvum* (54.3%, 19/35), *C. andersoni* (37.2%, 13/35), *C. ryanae* (2.6%, 1/35), *C. bovis* (2.6%, 1/35) and *C. ubiquitum* (2.6%, 1/35). In human samples, only *C. parvum* (n: 5) was identified. In cattle isolates, *C. parvum* (n: 11) was detected as the most common species, followed by *C. andersoni* (n: 10), *C. ryanae* (n: 1), *C. ubiquitum* (n: 1) and *C. bovis* (n: 1), while in sheep isolates, *C. parvum* (n: 2) and *C. andersoni* (n: 1) were detected. *C. parvum* (n: 1) and *C. andersoni* (n: 2), which were also identified in other hosts, were detected in environmental samples. The distribution of *G. duodenalis* assemblages and *Cryptosporidium* subtypes among different hosts is shown in Figure 2. Also, 24 samples tested positive for *Cryptosporidium* spp. The *ssu* gene was further amplified or re-amplified using nPCR targeting the *gp60* gene, which is commonly used to identify subspecies of *C. parvum* and *C. hominis*. Unfortunately, we could not successfully amplify these genes (see Discussion).

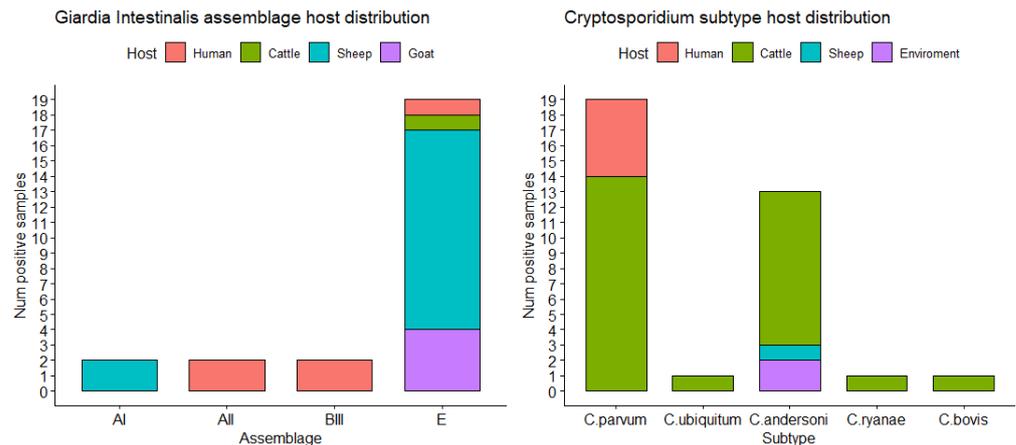


Figure 2. Distribution of *Giardia duodenalis* assemblages and *Cryptosporidium* subtypes among different hosts. The stacked bar chart shows the host-specific occurrence of *Giardia* assemblages AI-II (zoonotic), BIII, and E (ungulate-adapted), and *Cryptosporidium* subtypes *C. parvum*, *C. ubiquitum*, *C. andersoni*, *C. ryanae* and *C. bovis*.

Additionally, co-infection with *G. duodenalis* and *Cryptosporidium* spp. was detected in only one human, three cattle, and two sheep stool samples.

3.3. Transmission Dynamics of *Giardia duodenalis* and *Cryptosporidium* spp. in Humans and Animals at the Household Level

A total of 64 households were examined for the presence of *Giardia duodenalis* and *Cryptosporidium* spp. in human and/or farm animal samples. Regardless of the host, 75% (48/64) of these households were positive for *G. duodenalis* and/or *Cryptosporidium* spp. in at least one sample type, while polyparasitism was detected in 56.25% (27/64). Additionally, only *Cryptosporidium* spp. was found in 17.2% (11/64) of the households, while only *G. duodenalis* was detected in 15.6% (10/64). In only nine households was it seen that a human and at least one farm animal sharing a common living area were infected with the same parasite. The occurrence of *G. duodenalis* and *Cryptosporidium* spp. at the household level is presented in Supplementary Table S2.

3.4. The Effect of *Giardia duodenalis* and *Cryptosporidium* spp. Infection on Human Microbiota Dynamics

3.4.1. Analysis of Changes in Alpha and Beta Microbiome Diversity

Samples were grouped into infection status categories for *G. duodenalis* and *Cryptosporidium* spp. infections (*Giardia*-positive/negative and *Cryptosporidium*-positive/negative). Comparisons between the microbiota diversity of these groups were done using various diversity indices. Metrics for microbial evenness (Shannon), dominance (Simpson) and richness (Chao1/observed taxa (true richness)) were used. Diversity scores of the two infection status groups for each parasite were analysed (Supplementary Figure S2). Statistical analysis confirms that none of the differences observed between *Cryptosporidium*-positive and -negative samples are statistically significant. (p -values 0.95, 0.98, 0.85 and 0.94, respectively). In the comparison of samples grouped by *Giardia* infection status, statistical analysis confirmed a similar lack of significance in diversity scores between the groups, with all pairwise comparisons showing a p -value above 0.05 (p -values 0.36, 0.14, 0.41 and 0.36, respectively).

Visualisation of the most abundant taxa within each group was done to observe which groups had changed in relative abundance. Shown in Supplementary Figure S3 are the averaged relative abundances from both the *Cryptosporidium* (Supplementary Figure S3A,B) and *Giardia* (Supplementary Figure S3C,D) infection status groups (plots showing individual sample relative abundances are shown in Supplementary Figure S3). Shown in

Supplementary Figure S4B, *Cryptosporidium*-positive samples showed a reduction in the relative abundance of the phyla Firmicutes_D and Bacteroidota, and increases in the phylum Actinobacteria, Firmicutes_A and Proteobacteria. Supplementary Figure S4A shows that at the genus-level aggregation of the sample, the five most abundant genera are *Faecalibacterium*, *Prevotella*, *Blautia_a*, *Agathobacter* and *Bifidobacterium*. In *Cryptosporidium*-positive samples, an increase in the genera *Agathobacter* and *Bifidobacterium*, and a decrease in the genera *Prevotella* and an unclassified member of the family Actinobacteriaceae is observed. When samples are grouped by *Giardia* infection status, an inverse trend appears, with Supplementary Figure S4D showing an increase in the relative abundance of phyla Bacteroidota and Firmicutes_D, and a reduction in the relative abundance of Actinobacteriota. At the genus level (Supplementary Figure S4C), similar taxa make up the most abundant, with an unclassified member of the family Lactnospiraceae being more abundant than *Agathobacter*. When comparing changes between *Giardia* infection status, a similar inverse trend to *Cryptosporidium* infection is seen. The genus *Prevotella* makes up a visibly larger portion of the relative abundance within *Giardia*-positive samples, with a corresponding reduction in *Agathobacter*, *Bifidobacterium* and the unclassified *Lactnospiraceae* taxa.

Visualisation of the changes occurring between groups of differing parasite infection status was also done using principal coordinate analysis (PCoA), factoring in changes in the abundance of every taxon, rather than the most abundant. Shown in Supplementary Figure S5 are Bray–Curtis dissimilarity matrix PCoA plots of samples grouped by *Cryptosporidium* infection status (Supplementary Figure S5A) and *Giardia* infection status (Supplementary Figure S5B). Permutational multivariate analysis of variance (PERMANOVA) is then used to determine significance. Supplementary Figure S5A shows the PCoA plot comparing the *Cryptosporidium* infection status groups; no apparent clustering of the *Cryptosporidium*-positive samples is seen. Statistical analysis of the centroids resulted in a *p*-value of 0.147, indicating non-significance.

In comparison to the positive samples for *Cryptosporidium*, the samples positive for *Giardia* are seen to be more clustered together. Despite the more clustered appearance, PERMANOVA revealed a *p*-value of 0.103, indicating no significant difference between the centroids of the two groups. Overall, this indicates that there are no significant differences between the microbiome content of samples positive for *Giardia*/*Cryptosporidium* and those negative. However, it should be noted that PERMANOVA is prone to type II errors, especially in scenarios where the design of the test results in one group being significantly larger or smaller than the other. Due to the small number of samples within the positive group in the case of both parasites, the PERMANOVA test for variance should be considered strong but not infallible.

3.4.2. Identification of Linearly Discriminant Microbial Taxa Associated with *Giardia duodenalis* and *Cryptosporidium* spp. Infection

Analysis was performed to identify differentially abundant taxa whose presence/absence correlated strongly with a sample's presence in the positive or negative infection group. To do this, linear discriminant effect size (LEfSe) analysis was employed. Shown in Figure 3 are the LEfSe plots containing the genus-level taxa with an LDA score of $>2/\leq -2$ for either the positive or negative groups for *Cryptosporidium* (Figure 3A) and *Giardia* (Figure 3B) infection. Figure 3A shows that there were 14 taxa whose presence/abundance was significantly correlated with *Cryptosporidium*-positive samples, and two taxa correlated with the *Cryptosporidium*-negative condition. The taxa correlated with *Cryptosporidium* infection were as follows: SFM01 (of the family Aristaellaceae), UBA1254 (of the class Alphaproteobacterium), Onthenecus (proposed order Eubacteriales incertae sedis), Barnesiella, a member of the order RFP12 (class Kiritimatiellia), CAG-710 (of class Bacilli), BICA1-8 (of the order Peptococcales), Oxalobacter, CAG-977 (class Alphaproteobacteria), an unclassified

member of the family Gemmatimonadaceae, QAKW01 (of the order Oscillospirales), and Xylanivirga. The two taxa that significantly correlated with *Cryptosporidium*-negative samples were Ellagibacter and an unclassified member of the family Eggerthellaceae. Figure 3B shows the taxa significantly correlated with either of the *Giardia* infection statuses, with 11 taxa correlated with *Giardia* infection and four taxa correlated with the negative group. The taxa correlated with *Giardia* infection were the genera: *Prevotella*, *Lachnospira*, *Ruminococcus*, *Eubacterium*, *Slackia*, *Sodaliophilus*, AM51-8 (also known as the genus *Pararoseburia*), *Coprococcus*, UBA2279 (family Anaerosomataceae), *Clostridium* and *Baileyella*. The taxa correlated with *Giardia*-negative samples were: *Coprobacter*, *Merdibacter*, UMGS1071 (family *Actualibacteraceae*) and *Enterococcus*. The prevalence of LEfSe-identified taxa across varying relative abundance thresholds (0–100%) is shown in Supplementary Figure S7.

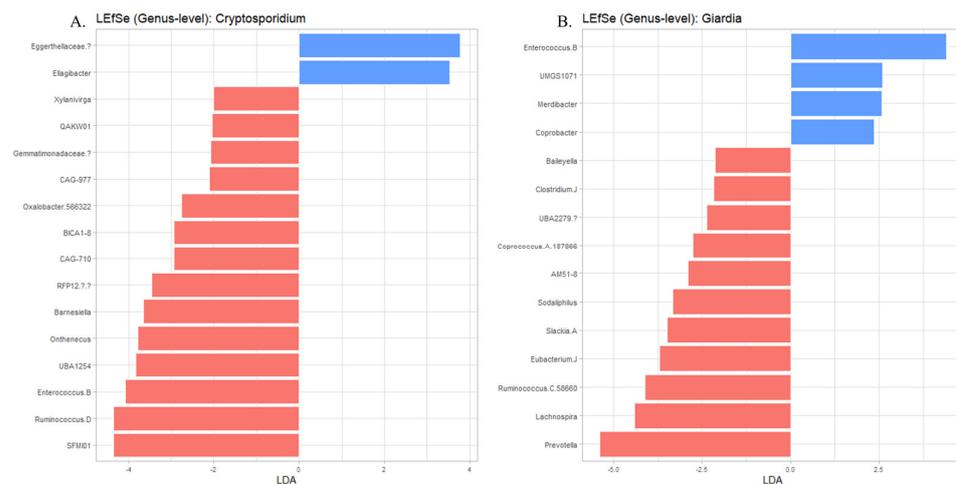


Figure 3. Linear discriminant effect size (LEfSe) analysis comparing the linear discriminant ability of taxa to discriminate between *Cryptosporidium* or *Giardia* positive (red) and negative (blue) status. (A) LEfSe plot for *Cryptosporidium*. (B) LEfSe plot for *Giardia*.

Samples with a higher LDA score have a stronger discriminant effect size, and all LDA scores $>2/\leq -2$ are considered significant.

4. Discussion

Our cross-sectional One Health investigation in rural Türkiye reveals that *Cryptosporidium* spp. and *G. duodenalis* are widely circulating among humans, livestock, and the local environment. Three findings are most noticeable for veterinary and public health practice. First, cattle showed the highest *Cryptosporidium* positivity (47.2%) and yielded zoonotic *C. parvum* in this dataset, consistent with livestock-dense settings. Second, environmental matrices, especially mud and slightly turbid water, harboured high *Cryptosporidium* positivity, which may indicate the accumulation and re-suspension of (oo)cysts in low-flow microhabitats, based on the observed positivity patterns. Third, although 75% of households were positive for at least one parasite in at least one sample type, concordant detections of the same parasite in humans and their animals were uncommon, pointing to multiple routes of exposure with a strong environmental component [34,35].

The predominance of *C. parvum* across hosts underscores a tangible zoonotic interface [4]. Calf management and manure handling are therefore central to mitigation. Practical measures include maintaining hygiene in calving areas, promptly removing or composting manure, improving drainage around watering points, and providing and keeping troughs off stream to reduce the direct use of surface waters [36,37]. For households that use dam margins for recreation, targeted risk communication (hand hygiene after animal contact and avoiding stagnant, shallow pools) is warranted, particularly for children

and immunocompromised individuals [38–40]. The environmental findings align with hydrological drivers of contamination. Annual shoreline retreat creates shared spaces for people and animals; rainfall then re-suspends settled material and transports (oo)cysts towards low-flow inlets. The very high positivity observed in mud likely reflects concentration by evaporation and sedimentation. Feasible landscape-scale mitigations include strategic fencing of small inlets with heavy animal use, rotational access to pastures adjacent to the shoreline, and maintenance of clean water points to reduce reliance on surface waters [41,42].

Giardia multilocus genotyping identified assemblages A, B and E, with assemblage E predominating in livestock and detected once in a human. Although assemblage E is classically considered ungulate-adapted, human detections are increasingly reported. Our finding, to our knowledge, is the first documented in Türkiye, and it adds to the evidence that host ranges can be permeable in livestock-dense communities, supporting continued molecular surveillance at the animal–human interface.

Our previous project, which investigated the molecular epidemiology of *Blastocystis* from the same region and samples, found limited data suggesting zoonotic transmission. Despite high positivity rates in both humans and animals, only one sheep detected the ST4 subtype, which is common in humans. Furthermore, despite a high positivity rate of 76% in humans, no animal-specific subtype was detected; only the ST1–ST4 subtypes commonly reported in humans were detected [20]. This setting suggests that the village’s geographical location, living conditions, and livestock practices create limited exposure patterns that restrict the spread of the pathogen among host species, while infection may also originate from environmental sources; detailed assessments are needed to confirm this hypothesis.

Microbiome analyses did not reveal significant differences in alpha or beta diversity by infection status, likely due to interindividual variability and the modest number of positive cases. Nonetheless, consistent, parasite-specific taxon shifts (e.g., *Prevotella* enrichment with *Giardia*; enrichment of several anaerobic fermenters with *Cryptosporidium*; and the strongest associated taxa with *Cryptosporidium* is Enterococcus B, which is one of the strongest negative correlations with *Giardia*) suggest biologically plausible ecological effects, such as altered carbohydrate metabolism or mucosal niche utilisation, which merit hypothesis-driven follow-up in larger cohorts. Prevalence analysis showed that while several LEfSe-identified taxa occurred in relatively few samples, *Prevotella* was the most consistently prevalent taxon across samples and Enterococcus B was also widely prevalent, albeit at low relative abundance (Figure S6).

Taken together, the data suggest that veterinary interventions targeting young stock and water access, combined with environmental management of high-risk microhabitats, are likely to yield the most significant immediate benefits. Embedding these measures within coordinated human–animal–environmental surveillance [43] will enable the earlier detection of transmission changes and a better attribution of risk pathways in similar rural Mediterranean settings.

This study has several limitations. It was cross-sectional and conducted in a single rural community; temporal dynamics and causality cannot be inferred, and generalisability may be limited. The number of PCR-positive samples that yielded high-quality sequences, particularly those from environmental matrices, was modest, which reduced the resolution for source attribution. For microbiome analyses, the *Giardia*- and *Cryptosporidium*-positive human groups were relatively small, which limits statistical power and increases the risk of type II error in PERMANOVA and alpha-diversity tests. Rarefaction to 60,000 reads, while standardising depth, also excluded one sample and may have attenuated subtle differences. Finally, we did not collect detailed clinical metadata (e.g., stool consistency and recent antimicrobials) that could influence microbiome composition.

A specific limitation concerns the subtyping of *Cryptosporidium* at the gp60 locus (Supplementary Figure S6). Although nPCR produced visible amplicons in a subset, most sequences were of insufficient quality for reliable subtype calls. Several factors likely contributed: (i) gp60 is a single-copy target, inherently less sensitive than multicopy ssu rRNA at low parasite loads; (ii) stool-derived inhibitors and partial DNA degradation—more likely in field-collected and environmentally exposed samples—impair amplification and sequencing; (iii) primer–template mismatches due to gp60 genetic diversity reduce binding efficiency across circulating subtypes; and (iv) low and fragmented target DNA in environmental samples further limits yield [44]. Future work should consider inhibitor-removal steps, increased template input, nested or semi-nested assays with broadened primer sets, and complementary multilocus approaches (e.g., *cp47* and *actin*) or targeted amplicon sequencing to improve subtyping success.

This study presents the first One Health assessment of *Cryptosporidium* spp. and *G. duodenalis* in Türkiye, revealing clear connections between livestock reservoirs, environmental contamination, and human exposure. By integrating molecular epidemiology and microbiome profiling, we offer new insights into parasite ecology at the human–animal–environment interface. The findings may have potential implications for veterinary management and rural public health, particularly in livestock-dense Mediterranean regions. Future work should build on this cross-sectional baseline by undertaking longitudinal and seasonal studies to capture temporal variation in infection dynamics, parasite shedding, and environmental persistence. Such studies will be essential for identifying high-risk periods, evaluating intervention strategies, and ultimately reducing the burden of these protozoan infections under a One Health umbrella.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app16062899/s1>, Figure S1: Rarefaction curves showing ASV discovery rates in amplicon samples. Total sample read count is shown on the X axis, and number of ASVs identified within that read range is shown on the Y axis. Figure S2: Statistical analysis comparing Shannon (A,E), Simpson (B,F), Chao1 (C,G) and observed taxa (D,H) diversity index values between samples positive (red) or negative (blue) for *Cryptosporidium* presence (A,D) and *Giardia* presence (E,H). Statistical test (Kruskal–Wallis/ANOVA) depends on the normality of the data. Figure S3: Compositional plots showing the most abundant taxa in samples positive or negative for *Cryptosporidium* or *Giardia* within each individual sample. Plots have been aggregated to phylum (A,B) and genus (C,D) taxonomic levels, and samples have been grouped by *Cryptosporidium* infection status (A,C) and *Giardia* infection status (B,D). Taxa shown each make up more than 1% of the total reads within each sample. Figure S4: Compositional plots showing the most abundant taxa in samples positive or negative for *Cryptosporidium* and *Giardia*. Plots have been aggregated by genus (A,C) and phylum (B,D) to show infection statuses of *Cryptosporidium* (A,B) and *Giardia* (C,D), and sample content has been averaged by *Cryptosporidium*/*Giardia* infection status. Each taxon shown comprises more than 1% of the total reads within its respective sample. Figure S5: PCoA plot comparing Bray–Curtis dissimilarity matrix values of each sample. Statistical analysis comparing positions of samples based on *Cryptosporidium* infection status (A), positive (red) or negative (blue), or *Giardia* infection status (B), where groups that had significantly different centroids are highlighted in purple/yellow. Statistical analysis between group centroid positions was performed using PERMANOVA. A *p*-value below 0.05 indicates significance in the positions of the groups. Figure S6: Representative gel images of the nPCR amplification of the *Cryptosporidium* spp. gp60 gene in the samples. (A–C) Screening for gp60 via nPCR using primers F1 (AL3531) + R1 (AL3535) in the first reaction and F2 (AL3532) + R2 (AL3534) in the second reaction. (D) Troubleshooting nPCR amplification using a 1:10 diluted DNA template and high-fidelity polymerase. Figure S7: Heatmap showing the prevalence of taxa identified by LEfSe. Colour indicates what level of prevalence each taxa was at each relative abundance threshold (X-axis) (0–100%). Table S1: Detailed information of the primers used in this study. Table S2: Presence of *Giardia duodenalis* and *Cryptosporidium* spp. in humans and animals at household level.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Çukurova University (protocol code: 136-49, date of approval: 1 September 2023). Ethical review and approval were waived for the animal component of this study in accordance with the guidelines of the Local Animal Experiments Ethics Committee (HADYEK), as only non-invasive faecal sampling was performed without any experimental procedures. Permission for sample collection was obtained from the animal owners.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. For child participants, informed consent was obtained from their parents or legal guardians.

Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy and ethical restrictions related to the participants.

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