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Prevalence and genetic diversity of *Giardia duodenalis* in pet dogs from Zhengzhou, central China and the association between gut microbiota and fecal characteristics during infection

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ABSTRACT

As a common zoonotic intestinal parasite, *Giardia duodenalis* could infect humans and various mammals worldwide, including pet dogs, leading to giardiasis. This study detected the infection of *G. duodenalis* in asymptomatic pet dogs in Zhengzhou, and evaluated the possibility of zoonosis and the relationship between gut microbiota and fecal characteristics. We randomly collected 448 fresh fecal samples from Zhengzhou, and *G. duodenalis* was screened based on the beta-giardin (*bg*), glutamate dehydrogenase (*gdh*), and triose phosphate isomerase (*tpi*) genes. The difference of gut microbiota between five *G. duodenalis*-positive and five *G. duodenalis*-negative samples was investigated by 16S rRNA gene sequencing. The overall prevalence of *G. duodenalis* was 7.1% (32/448) based on *bg*, *gdh*, and *tpi* locus, two *G. duodenalis* assemblages (C = 13, D = 14) and five (15.6%) mixed infection (C + D) were identified. Moreover, compared with the *G. duodenalis*-negative group, the diversity of gut microbiota increased in *G. duodenalis*-positive group. The decrease of *Lactobacillus* spp. and considerable increase of *Prevotella* spp. were associated with the fecal characteristics. These results show that the transmission of zoonotic giardiasis between humans and pet dogs is rare in Zhengzhou, central China, and support the use of *Lactobacillus* spp. as a potential probiotic agent to improve intestinal health in dogs, or even humans, by treating *G. duodenalis*. Therefore, the public health significance of *G. duodenalis* to humans, companion animals, and the environment should be further evaluated from One Health perspective.

1. Introduction

Giardia duodenalis is a foodborne and waterborne parasite, which predominantly parasitizes the duodenum of humans and various mammals with zoonosis potential [1]. The simple life cycle consists of trophozoites that cause symptoms and infective cysts that accompany host fecal shedding [2]. Susceptible hosts ingest cysts through contaminated food or water, or via the fecal-oral route, and then the fresh cysts are discharged from the host with the feces and can infect other susceptible hosts through contaminating food or water, or via the fecal-oral route [3].

Eight *G. duodenalis* assemblages have been named and classified to date. Infections in humans and other mammals are associated with assemblages A and B, and dogs and other canines often report being infected with assemblages C and D [3]. Sporadic reports of assemblages C, D, E and F in humans have also impacted the concept that assemblages C—H is widely considered to be host specific to a certain extent [4–7]. *G. duodenalis*, a global parasite, is estimated to infect more than 280 million people worldwide [8]. At the same time, its clinical manifestations range from asymptomatic to acute or chronic diarrhea with abdominal pain and nausea [9]. However, there is still no effective and safe vaccine to prevent *G. duodenalis* infection, and synthetic drugs have some limitations [10].

The gut microbiota plays a key role in the homeostasis and overall health of the gut and is frequently found to be altered during parasitic infection [11]. For example, the destruction of the microbial biofilm

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structure, the change of virulence, abundance, and diversity of species frequently occur in the course of *G. duodenalis* infection [12–14]. Conversely, the disrupted microbiota can also affect the pathogenesis of *G. duodenalis*, including colonization resistance, immune response, and brush edge defects [14].

Approximately 17% of Chinese households own at least one companion animal, among which dogs are the most popular [15]. Owing to close contact with humans, dogs play an increasingly important role in the study of zoonotic pathogens transmission [16]. In addition, dogs are increasingly regarded as an ideal model system for human gut microbiota research since they often share the environment with humans, eat a similar omnivorous diet, and are often infected with intestinal parasites in early life [11].

G. duodenalis is widely detected in humans, non-human primates (NHPs), ruminants, companion animals, livestock, and wild animals and even in the environment in China [17]. The estimated prevalence of *G. duodenalis* among dogs in China was 9.3% and 14.3% by microscopy and serology, respectively [18]. Molecular analysis revealed that *G. duodenalis* was detected in approximately 12.3% of dogs in China, including aggregates A, B, C, D, E and F [18]. However, limited information about pet dogs infected with *G. duodenalis* is available in central China [19,20]. It has been reported that fecal characteristics are an important indicator of intestinal health [21]. The damage caused by *G. duodenalis* to the host intestine has also attracted substantial attention; however, the relationship between fecal characteristics and gut microbiota during infection remains unclear. Hence, this study focused on investigating the prevalence of *G. duodenalis*, and evaluating its genetic diversity and zoonosis potential of pet dogs in Zhengzhou, central China. The relationship between fecal characteristics and gut microbiota during infection was also investigated.

2. Materials and methods

2.1. Fecal collection and DNA extraction

From September 2017 to March 2019, 448 fresh fecal samples of asymptomatic pet dogs mainly living indoors were randomly collected in pet hospitals and pet shops in Zhengzhou, central China, and the owner's questionnaire was also collected during the collection of fecal samples. Each specimen (30–50 g) was collected immediately after being defecated and placed separately in sterile gloves marked with sample information, including ID number, date, origin, age, sex, scores of consistency and shape of feces (scored by an objective third party among six experimenters). Fecal consistency was scored on a 5-point scale proposed by Moxham [22]. Through the Zieger method [23], fecal shape was scored using a 4-point scale. After being stored in containers with ice, the specimens were immediately sent to laboratory. Fresh fecal samples were stored at −80°C after collection, and genomic DNA was extracted within one week with the E.Z.N.A. ® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA).

2.2. PCR amplification

PCR amplification targeting the *bg, gdh,* and *tpi* genes was performed by the nested-PCR method. The resulting fragments were of 511 bp, 520 bp, and 530 bp in length, respectively (Table S1) [19,24–26]. The nested-PCR results of each target loci (*bg, gdh*, and *tpi*) were detected by electrophoresis with 1% agarose gel. Staining screening was performed by SYBR green (TIANDZ Inc., Beijing, China).

PCR amplification of the microbiota 16 S rRNA genes V3–V4 region of 10 fecal samples (Five were identified as *G. duodenalis* assemblage D only by the *bg* locus, while the other were negative) was performed by forward primer 338F and reverse primer 806R (*Cryptosporidium* and *Enterocytozoon bieneusi* were not detected in the 10 fecal samples) (Table S1). According to the owner questionnaire, all 10 dogs were asymptomatic males (≥12 months old), dewormed and vaccinated, lived in the house with their owners and fed commercial dog food.

2.3. Sequencing and the sequence analysis

The commercial sequencing company (SinoGenoMax, Beijing, China) conducted bidirectional sequencing for the positive PCR amplification products of the each target loci (*bg, gdh,* and *tpi*). Chromas Pro, version 2.182 was used for assemble the sequence, and resultant sequences was aligned in GenBank (http://blast.ncbi.nlm.nih.gov) by Clustal X 2.1 (http://www.clustal.org/). The nucleotide sequences of *G. duodenalis* have been submitted to the GenBank database (GenBank accession No. ON168743-ON168749 and ON243609).

Illumina sequencing was completed by Shanghai personal Biotechnology Co., Ltd. (Shanghai, China). QIIME2-DADA2 pipeline or Vsearch software was used to process and analyze the sequencing data. The primer fragments and mismatched primer sequences were removed using the cutadapt tool. Quality control, denoising, splicing, and chimera removal were performed by DADA2. Amplicon sequence variants (ASVs) clustering was performed with 100% sequence similarity [27]. Alpha and beta diversity indices were evaluated using the Kruskal–Wallis post-hoc test and the Bray–Curtis distance, respectively. The similarity of the bacterial community structure was evaluated by principal coordinates analysis (PCoA). In addition, 30 important ASVs were selected by the random forest algorithm. The entire 16 s rRNA gene sequence dataset in this paper has been submitted to the Sequence Read Archive (SRA)(https://www.ncbi.nlm.nih.gov/sra/)(Accession No. SUB10505899).

2.4. Statistical analysis

Data were represented by mean ± S·D, and homogeneity and normal distribution were obtained by Student's *t*-test in SPSS software (version 20.0, Chicago, Illinois). The correlations were determined by Spearman correlation analysis. Differences were considered statistically significant when $P < 0.05$.

3. Result

3.1. Prevalence of G. duodenalis in pet dogs

Simultaneous analysis of 448 fecal samples for amplification of the three loci (*bg, gdh, and tpi*) revealed that 32 were positive, with a positive rate of 7.1% (Table 1). It was also found that *G. duodenalis* existed in pet dogs of all age groups, among which the highest detection rate (9.6%, 10/104) was 6–12 months old dogs and the lowest detection rate (5.6%, 8/144) in dogs less than 6 months old (Table 1). In different genders, the infection rate of female dogs (7.2%, 13/180) was higher than that of male dogs (7.1%, 19/268). There was no significant difference between gender and age ($P > 0.05$) (Table 1).

3.2. Genotypes of G. duodenalis in pet dogs

32 samples were genotyped at one or more loci, and 21 *bg*, 16 *gdh* and 10 *tpi* loci sequences were successfully obtained (Table 1). Sequence analysis identified two known *G. duodenalis* assemblages, namely, C (40.6%, 13/32) and D (43.8%, 14/32), and mixed infections of assemblages C and D were found in five samples (15.6%, 5/32) (Table 1). These assemblages were detected in all age and gender groups (Table 1).

For the *bg* locus, the 21 sequences obtained were identified as one assemblage C sequence (ON168745) and two assemblage D sequences (ON168746 and ON168747). The assemblage C isolate ($n = 9$) shared 100% similarity with isolates collected from dogs in Croatia (JN416534), China (MK968845).The sequence ON168746 ($n = 10$) was identical to a *Canis lupus familiaris* isolate in Spain (MF285585), while the other sequence ON168747 ($n = 2$) was 100% similarity with isolates collected from dog in China (MN044604).

Table 1
Prevalence and genotype distribution of *G. duodenalis* among pet dogs of different ages and genders in Zhengzhou, central China.

Factor	Variable	No. positive/no. tested (%)	*G. duodenalis* Assemblages (n)							*P*-value	95% CI	OR (95% CI)
			tpi	*gdh*	*bg*	*tpi/gdh*	*tpi/bg*	*gdh/bg*	*tpi/gdh/bg*			
Age (month)	≤6	8/144 (5.6%)		D (3) C (1)	C (1)	C/D (1)		D/D (1)	C/C/C (1)	0.47	1.8–9.3	Reference
	6–12	10/104 (9.6%)		D (1)	C (2)		C/D (2)	D/D (1)	C/C/C (1)		3.9–15.4	1.8
					D (1)		C/C (1)	C/D (1)				(0.7–4.8)
	≥12	14/200 (7.0%)	C (2)	C (1)	D (5)		C/C (1)	C/C (1)	C/D/D (1)		3.4–10.6	1.3
				D (2)	C (1)							(0.5–3.1)
Gender	Male	19/268 (7.1%)	C (1)	C (1)	D (2)		C/D (2)	C/D (1)	C/D/D (1)	0.957	2.8–8.4	Reference
				D (4)	C (4)			D/D (1)	C/C/C (2)			
	Female	13/180 (7.2%)	C (1)	C (1)	D (4)	C/D (1)	C/C (2)	C/C (1)			5.1–13.8	1.0
				D (2)				D/D (1)				(0.5–2.1)
	Total	32/448 (7.1%)	C (2)	C (2)	C (4)	C/D (1)	C/C (2)	C/C (1)	C/C/C (2)		4.7–9.5	
				D (6)	D (6)		C/D (2)	C/D (1)	C/D/D (1)			
								D/D (2)				

For the *gdh* locus, of the 16 *G. duodenalis* isolates successfully sequenced, 6 were identified as assemblage C sequence (ON243609), while 10 were identified as two assemblage D sequences (ON168748 and ON168749). The assemblage C sequence was identical to the reference sequence from *C. lupus familiaris* isolate in Japan (LC437367). The two assemblage D sequences (ON168748 and ON168749) were identical to the reference sequences from a dog isolate in China (MK968852) ($n = 4$), and a dog isolate in Japan (KY608978) ($n = 6$), respectively.

For the *G. duodenalis tpi* loci, 2 assemblage C sequences were identified in 10 isolates, and the sequence (ON168743) ($n = 6$) shared 100% homology with isolates from *C. lupus familiaris* in Japan (LC437552). Another assemblage C isolate (ON168744) ($n = 4$) shared 100% similarity with isolates collected from dogs in the Germany (KY608998), and China (KY979493).

3.3. Effects of G. duodenalis on the consistency and shape of feces in pet dogs

According to the collected questionnaire from owners and the fecal score provided by the experimenters, most of the dogs in the *G. duodenalis*-positive group demonstrated diarrhea, depression, and loss of appetite. The scores of consistency and shape of feces in the *G. duodenalis*-positive group were higher than those in the *G. duodenalis*-negative group ($P < 0.05$) (Table 2).

3.4. Effects of G. duodenalis on the diversity and composition

1,138,613 paired-end raw reads were obtained by the Illumina platform, and 843,058 Non-Chimeric reads were generated. Finally, a total of 477,099 high-quality reads were screened (mean: 47,709.9; range: 54,554–123,303).

As shown in Fig. 1A, Chao1, Observed species, Shannon and Simpson indices with a higher ratio than those in the *G. duodenalis*-negative group all occurred in the *G. duodenalis*-positive group ($P = 0.076$, $P = 0.047$, $P = 0.117$ and $P = 0.251$, respectively), while the differences in Chao1, Shannon and Simpson indices were not statistically significant. PCoA showed a significant separation of gut microbiota between the two groups (PCo1: 20.7%, PCo2: 17.9%, $P = 0.044$) (Fig. 1B).

Table 2
Effects of *G. duodenalis* (assemblage D) on fecal consistency and shape scores in pet dogs.

Item	Score feces consistency	Score feces shape
Negative group	2.83 ± 0.12	2.47 ± 0.41
Positive group	3.70 ± 0.07	3.57 ± 0.19
P-value	0.000001	0.002

3.5. Changes in the gut microbiota caused by G. duodenalis infection

As depicted in Fig. 2A–G, the top five phyla in the two groups are Firmicutes, Bacteroides, Proteobacteria, Fusobacteria, and Actinobacteria, and the proportion of Bacteroidetes and Proteobacteria in the *G. duodenalis*-positive group (35.22% and 21.03%, respectively) was relatively higher than *G. duodenalis*-negative group (30.97% and 12.02%, respectively), whereas the proportion of Firmicutes, Fusobacteria and Actinobacteria in the *G. duodenalis*-positive group (36.55%, 6.04%, and 0.91%, respectively) was lower than the pet dogs that *G. duodenalis*-negative group (43.18%, 11.50%, and 1.49%, respectively). Furthermore, a slightly higher ratio of Firmicutes to Bacteroides (F/B) occurred in the *G. duodenalis*-positive group ($P > 0.05$).

Among the Top 30 genera, *Bacteroides* spp., *Megamonas* spp., and *Blautia* spp. were dominant in the *G. duodenalis*-negative group, whereas *Prevotella* spp., *Shigella* spp., and *Blautia* spp. were dominant in the *G. duodenalis*-positive group (Fig. 3A). Furthermore, the Top 30 important genera was screen out by random forest analysis, and the highest importance included *Prevotella* spp., *Lactobacillus* spp., *Bacteroides* spp., and so forth (Fig. 3B). 16 genera were screened when analyzed the important Top 30 and the abundant Top 30 by Venn diagram, namely, *Prevotella* spp., *Lactobacillus* spp., *Bacteroides* spp., *Sutterella* spp., *Fusobacterium* spp., *[Prevotella]* spp., *Phascolarctobacterium* spp., *Megamonas* spp., *Streptococcus* spp., *Collinsella* spp., *Allobaculum* spp., *Eubacterium* spp., *Anaerobiospirillum* spp., *Butyricicoccus* spp., *Helicobacter* spp., and *Bifidobacterium* spp. (Fig. 3C). Next, we noticed that the abundance of *Lactobacillus* spp. and *Bacteroides* spp. was lower in the *G. duodenalis*-positive group, while *Prevotella* spp. and *Phascolarctobacterium* spp. were higher than *G. duodenalis*-negative group ($P = 0.113$, $P = 0.048$, $P = 0.04$ and $P = 0.166$, respectively; Fig. 3D–G). By Spearman correlation analysis, the consistency was significantly negatively correlated with *Lactobacillus* spp. ($r = -0.710$, $P = 0.022$; Fig. 3h), while it was significantly positively correlated with *Prevotella* spp. ($r = 0.881$ and $P = 0.001$). For shaping, *Lactobacillus* spp. ($r = -0.892$ and $P = 0.001$) showed a significant negative correlation, while it had a significant positive correlation with *Prevotella* spp. ($r = 0.646$ and $P = 0.044$).

4. Discussion

In our study, the detection rate of *G. duodenalis* in pet dogs mainly living indoors from Zhengzhou, central China, was similar to that reported in Brazil (6.9%)[28], and Poland (6.0%)[29]. However, it is lower than the prevalence of rural dogs in Argentina (44.4%)[30], and Italy (15%)[31]. In this study, pet dogs living indoors rarely had the chance to contact other animals and contaminated environments [32]. Moreover, pet dogs living with their owners tended to live in more hygienic living conditions, which may also be an important factor leading to the relatively low prevalence rate of *G. duodenalis* [33].

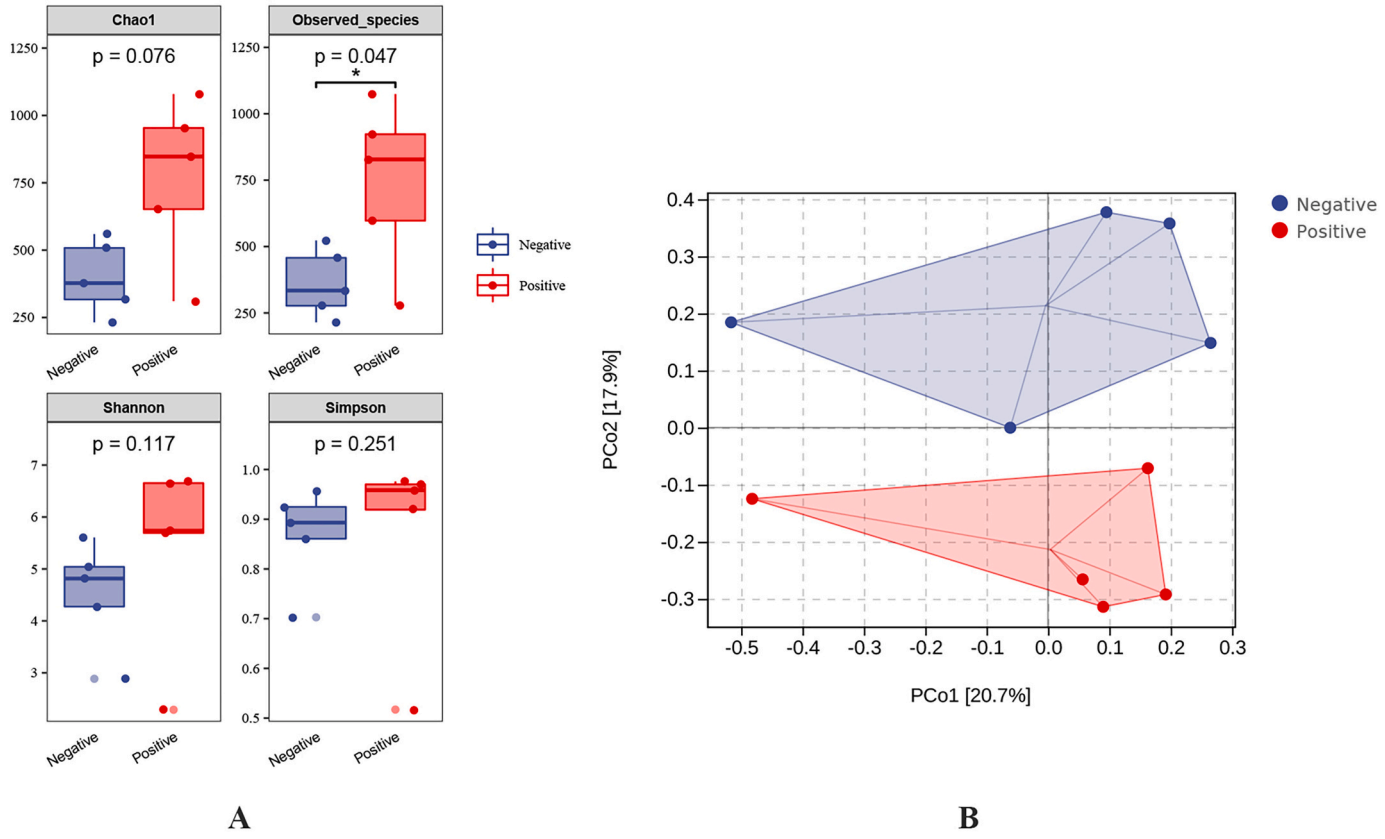

Fig. 1. Alpha diversity (A) and principal coordinate analysis (PCoA) by Bray-Curtis distance (B).

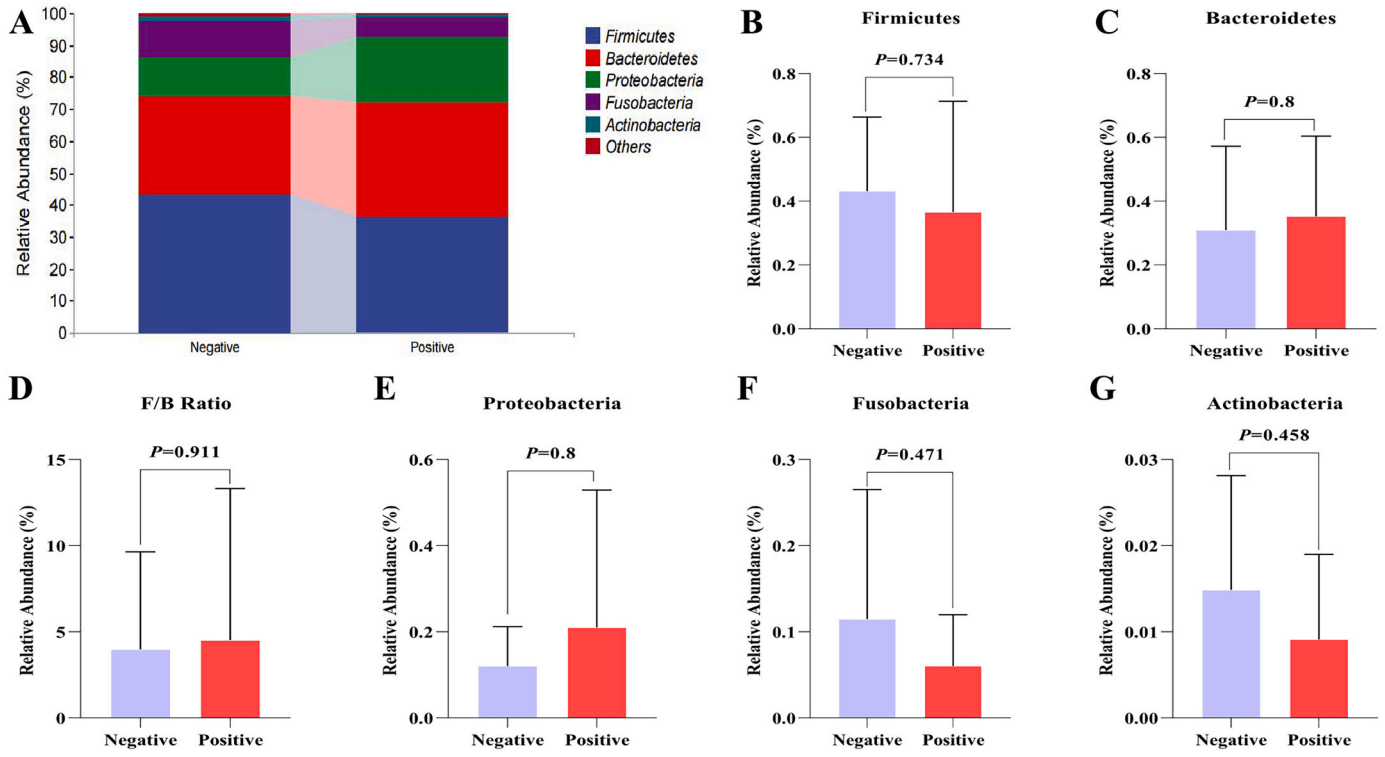

Fig. 2. Changes of gut microbiota at the phylum level during *G. duodenalis* infection. (A) The top 5 abundances of gut microbiota at the phylum level. (B–G) Differences in the abundance of Firmicutes, Bacteroidetes, Firmicutes/Bacteroidetes (F/B) ratio, Proteobacteria, Fusobacteria, and Actinobacteria, respectively.

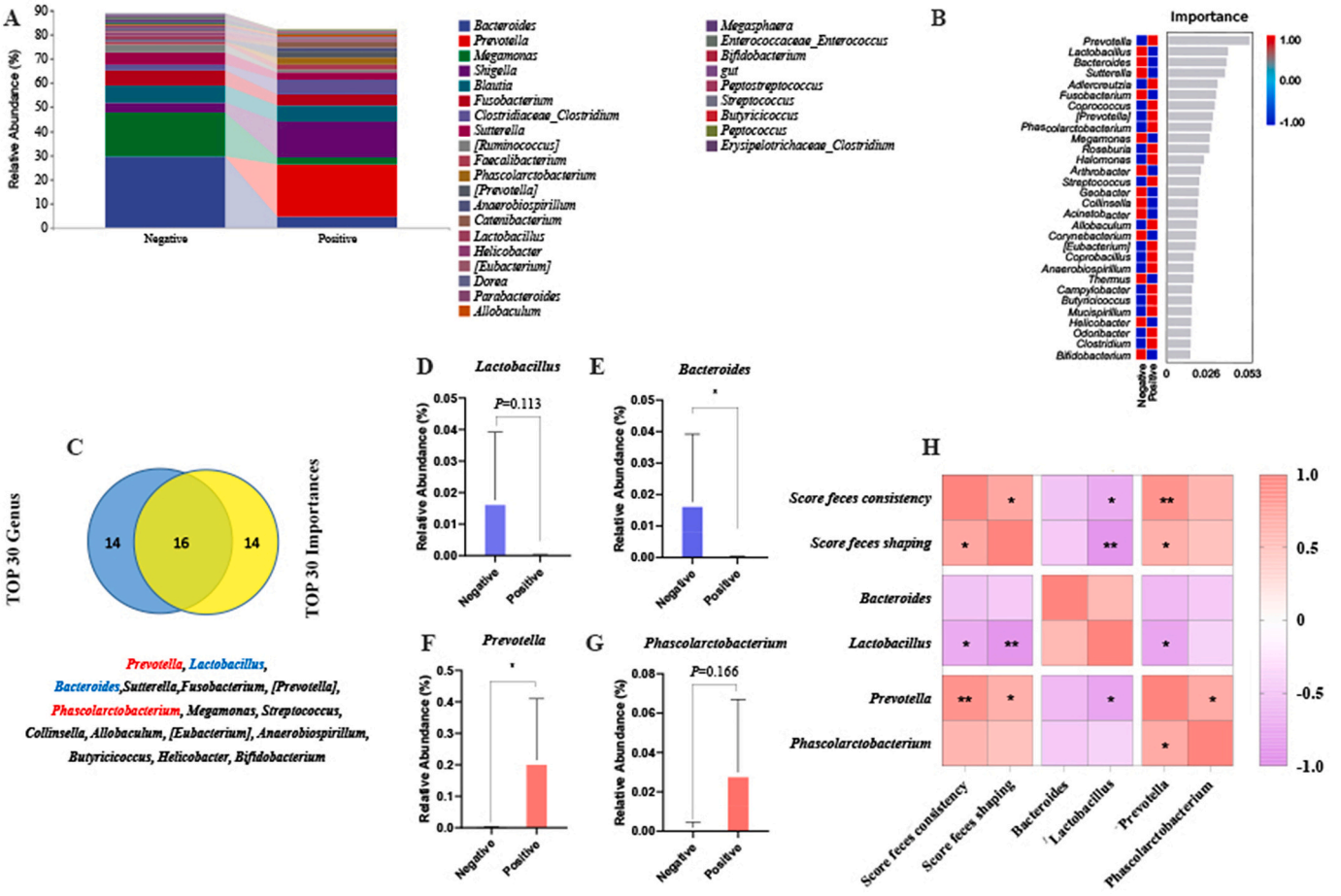

Fig. 3. Effects of *G. duodenalis* on the gut microbiota at the genus level in pet dogs. (A) The top 30 abundances of gut microbiota at the genera level. (B) Analysis of gut microbiota by the random forest algorithm. The value of the importance of genus is in the abscissa and the taxon name is in the ordinate. (C) Venn diagrams of the 30 genera with the highest abundance and the highest importance. (D–G) Differences of gut microbiota abundance at the genus level (*Lactobacillus* spp., *Bacteroides* spp., *Prevotella* spp., and *Phascolarctobacterium* spp., respectively). (H) Heat map of the correlation between gut microbiota and the consistency and shape of feces.

Sequence analysis identified only the host-specific assemblages C and D were detected in *G. duodenalis* isolates from dogs in the present study [19,34,35]. Although in Egypt [36], China [37], and Colombia [38], assemblages C and D were occasionally found in human, the potential for zoonotic transmission of *G. duodenalis* from dogs to humans is still largely an unresolved issue [36]. Compared with the zoonotic assemblages A and B that usually exist in humans, zoonotic transmission of giardiasis has rarely occurred between humans and dogs in Zhengzhou, central China in our study. Previous studies mostly reported that mixed infection of *G. duodenalis* in dogs is usually composed by C and D assemblages [32,35]. Similarly, mixed combination of C and D assemblages was found in dogs in this study, revealing the diversity of *G. duodenalis* in our investigation area.

Fecal characteristics represent important intestinal health indicators for dogs, especially fecal consistency and shape [39]. In this study, *G. duodenalis*-negative dogs had significantly lower fecal consistency and shape scores than *G. duodenalis*-positive dogs and were closer to the ideal optimal score (score 2) [40]. Thus, this study shows that *G. duodenalis* will have an adverse impact on the intestinal health of pet dogs.

In the intestines of humans and other mammals, *G. duodenalis* infection is often associated with host flora dysbiosis [41]. Using 16S sequencing analysis, we found higher diversity and species richness in the *G. duodenalis*-positive group. Similar to the gut protozoan *G. duodenalis*, higher gut microbial diversity was also reported in *E. bieneusi*-positive foxes and *Blastocystis*-positive children [42,43]. Beta-diversity showed significant changes between the two groups, and a similar phenomenon of significant dispersion during *G. duodenal* infection was also reported in domestic dogs in the United States [11].

In this study, the most abundant phyla in the two groups were Firmicutes and Bacteroidetes, together accounting for more than 70% of the total. The high abundance of Firmicutes and Bacteroidetes is consistent with previous studies on canines [44,45]. Both Firmicutes and Bacteroidetes participate in the metabolic processes of the host, and Firmicutes can produce volatile fatty acids and other by-products from the metabolic process [46]. Bacteroidetes can degrade proteins, carbohydrates, and compounds in plant cell walls [47]. The Firmicutes/Bacteroidetes ratio was slightly increased in the *G. duodenalis*-positive group, which may be related to the pathological changes of intestinal metabolic homeostasis and inflammatory markers [48]. There was no significant difference in the ratio of Firmicutes/Bacteroides between the two groups, which may be due to the small sample size of 16S amplicon sequencing.

Moreover, through genus level screening, we found four key genera, namely, *Prevotella* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Phascolarctobacterium* spp., among which, the relative abundance of *Prevotella* spp. and *Lactobacillus* spp. was significantly correlated with the consistency score and shape score of pet dog feces. *Prevotella* spp. plays a key role in host microbial interactions, especially in nutrition and metabolism [49]. Some studies have shown that *Prevotella* spp. may disrupt the intestinal microbiota and act as a proinflammatory mediator in the intestine by reducing the level of short-chain fatty acids and reducing

the protective mucus layer in experimental mouse models [50,51]. *Lactobacillus* spp. can effectively balance the intestinal microbial environment, improve the digestibility of nutrients, promote growth and immune status, and exert a beneficial impact on pet dogs [52].Our results showed that *G. duodenalis* may damage the fecal characteristics by increasing and decreasing the abundance of *Prevotella* spp. and *Lactobacillus* spp., respectively.

Furthermore, the direct cytotoxicity against *G. duodenalis* trophozoites and the same ecological niches highlight *Lactobacillus* spp. as a good probiotic candidate strain for the prevention and treatment of giardiasis [53–55]. These studies show that *Lactobacillus* spp. can be given priority in the future research of probiotic preparations, which may improve the intestinal health of pet dogs by treating giardiasis. However, the mechanism by which *Lactobacillus* spp. improves host fecal characteristics after *G. duodenalis* infection is not clear, which is worthy of further study.

5. Conclusion

The total prevalence of *G. duodenalis* in pet dogs in Zhengzhou, central China, was 7.1% based on *bg*, *gdh*, and *tpi* locus, where aggregates C and D were detected in all age and gender groups. Although no zoonotic genotypes were found in this study, the possibility of potential transmission of *G. duodenalis* should not be ignored to reduce the possibility of intraspecific transmission. Moreover, *G. duodenalis* may damage the fecal characteristics by increasing and decreasing the abundance of *Prevotella* spp. and *Lactobacillus* spp., respectively. Some measures that effectively minimize the threat posed to animals and public health by *G. duodenalis* should be taken in Zhengzhou, central China.

Ethics statement

The review and approval of this project was conducted by the Research Ethics Committee of Henan Agricultural University (approval No. IRB-HENAU-20180914-01) and was implemented in accordance with the Guide for the Care and Use of Laboratory Animals. Prior to collecting fecal samples, the owner's consent has been obtained.

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CRediT authorship contribution statement

Yuzhen Sui: Writing – original draft, Software, Formal analysis, Visualization. **Xiangqian Zhang:** Investigation, Formal analysis. **Haidong Wang:** Methodology. **Liping Zheng:** Methodology. **Yunan Guo:** Investigation. **Ying Lu:** Investigation. **Minghui Chen:** Methodology. **Bukang Wang:** Methodology. **Hongyu Dai:** Methodology. **Fang Liu:** Methodology. **Haiju Dong:** Resources, Supervision, Project administration. **Chao Tong:** Supervision, Resources. **Longxian Zhang:** Supervision, Resources.

Declaration of Competing Interest

None to report.

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