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Gut region induces gastrointestinal microbiota community shift in Ujimqin sheep (*Ovis aries*): from a multi-domain perspective

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Summary

Gastrointestinal (GI) microbiota is one of the most complicated microbial ecosystems and is vital in regulating biological processes associated with nutrient absorption and homeostatic maintenance. Although several efforts have been achieved in characterizing bacterial communities across gut regions, the variation of non-bacterial communities across GI tracts is still largely unexplored. To address this, we investigated microbial biogeography throughout the whole GI tracts of Ujimqin sheep (*Ovis aries*) by amplicon sequencing which targeted bacteria, fungi, and archaea. The results indicated that the community structures of all three domains were significantly distinguished according to GI tracts (stomach, small intestine, and large intestine), and a more strong and efficient species interaction was detected in small intestine based on cross-domain network analysis. Moreover, a between-domain difference in microbial assembly mechanism of among-GI regions was revealed here, wherein bacterial community is dominantly governed by variable selection (explaining ~62% of taxa turnover), while fungal and archaeal communities mainly governed by homogenizing dispersal (explaining ~49% and 60% of the turnover, respectively). Overall, these data highlight the GI section- and domain-dependence of GI microbial structure and assembly mechanism, suggesting that multi-domain should be explicitly considered when evaluating the influences of GI selection on gut microbial communities.

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Introduction

For colonized gut microbiota, the gut ecosystem presents some similarities with biogeographic islands, as the community inside occupies a habitat with a well-defined border and constantly undergoes immigration and extinction (MacArthur and Wilson, 1967; DeLong, 2014). Gut 'island' can filter the foreign microbes and establish a unique microbial community that can successfully colonize and competes in the gut habitats (Li *et al*., 2016; Yang *et al*., 2019). Due to their important roles in host nutrient uptake and immunity regulation, the gut microbiota is especially attractive for addressing questions regarding the function of gut microbes and host–microbes interaction (Flint *et al*., 2012). In previous studies, gut microbial communities were widely investigated by using faeces materials to represent the composition of multiple gut regions owing to their non-invasive and repeatable samplings. However, a growing body of evidence suggests that different regions of the gastrointestinal (GI) tract may harbour distinct microbes (Perea *et al*., 2017; Yeoman *et al*., 2018; Li *et al*., 2019; Chong *et al*., 2020) given that the physiochemical conditions (e.g., pH and oxygen availability) can vary widely throughout the gut (Kohl *et al*., 2013; Donaldson *et al*., 2016). As such, it is of great importance to illuminate the gut 'island' biogeography throughout the GI tracts, as the functions of gut microbiota and host–microbe relationships can also exhibit differential characteristics across gut regions (Mowat and Agace, 2014).

Microbial ecosystems along the GI tracts harbour a unique diversity of microbes including bacteria, fungi, and archaea (Wegener Parfrey *et al*., 2011). Although several reports have shed light on the diversity and structure of bacterial communities throughout the GI tracts, the characteristics of non-bacterial members have not been examined in as much detail. It has been well

acknowledged that gut fungi and archaea are widely involved in host metabolisms, such as immune homeostasis, medical treatment, and metabolism of nutrients (Dollive *et al*., 2013; Gaci *et al*., 2014; Leonardi *et al*., 2018). The interaction of inter-domain microbes is critical to reveal gut microbial community structure, as these microbes can form intricate networks through various types of relationships (Kerr, 1994; Hoffmann *et al*., 2013). For example, Pareek *et al*. (2019) found that fungal members *Candida* spp. can facilitate colonization of bacterial counterparts *Prevotella* spp. in human and mice intestine, and further evidence indicated a dietary metabolite mediated interaction between them. Archaeal members such as hydrogenotrophic methanogens can affect the niche and metabolic pathways of fermentative bacteria to optimize their fermentation and thus modify the structure of bacterial communities in the gut environment (Nakamura *et al*., 2010; Hoffmann *et al*., 2013). To obtain a comprehensive understanding of complex interspecies relationships, multi-domain-based network analysis would offer new insights into the structural traits of GI microbial communities.

Elucidating the assembly mechanism of the GI microbial community is an important step to better predict the responses of GI communities to various factors. Of the two major assembly mechanisms, the niche-based theory underlying the deterministic process (ecological selection in Vellend's theory) in which abiotic and biotic factors govern the microbial assembly (Chase and Leibold, 2003; Vellend, 2010). In contrast, the neutral theory emphasized the role of the stochastic process (dispersal and drift in Vellend's theory) in governing the microbial assembly. In fact, the deterministic and stochastic processes usually regulate the microbial assembly simultaneously though their contribution may vary in response to a range of driving factors. For example, Yan *et al*. (2016) investigated the assembly of gut bacteria over the fish development process and demonstrated that the gut bacteria were mainly shaped by environmental selection, but the deterministic effects that govern the bacterial assembly decreased as the fish developed. In addition, although increasing the knowledge of gut microbial assembly has been accumulated in bacterial communities (Zhu *et al*., 2016; Holt *et al*., 2020), we know virtually nothing about the relative contribution of differential ecological processes that govern GI fungal and archaeal communities. Therefore, illuminating the relative importance of deterministic and stochastic processes governing the GI microbiota among multiple microbial domains would fill the gap in assembly patterns of GI microbial community and also facilitate us to predict how multi-domain communities respond to the GI habitat selection.

Ujimqin sheep (*Ovis aries*), one of the terrestrial ruminant herbivores, are widely distributed in pastoral and agricultural areas of Inner Mongolia Autonomous Region, China. In this study, we analysed the microbial communities by using Illumina sequencing of amplicon libraries that targeted bacteria, fungi, and archaea, to thoroughly decipher the gut 'island' biogeography along the GI tracts of Ujimqin sheep. We hypothesized that (i) GI microbial community composition differs among sheep GI tracts and each GI tract can harbour distinct core microbes, and (ii) GI microorganisms may form intricate co-occurrence networks that may exhibit differential topological features across gut regions. To address these hypotheses, we also focused on unravelling the assembly mechanism of three microbial domains (i.e., bacteria, fungi, and archaea) in the sheep GI ecosystem. This study represents the first biogeographical map of the complete sheep digestive system and is expected to support further studies to investigate how gut microbiota across different GI tracts relates to host health.

Results

Microbial diversity and general turnover of the sheep GI microbiota

Through our current sequencing efforts, we detected a total of 4376, 1213, and 569 operational taxonomic units (OTUs) from all GI tracts, which respectively corresponded to bacteria, fungal and archaeal libraries. Firmicutes OTUs were mostly dominant, ranged from 12.16% to 27.99% of the bacterial community across different GI tracts of sheep. Dothideomycetes and Thermoplasmata OTUs were predominant in fungal and archaeal communities, which accounted for 31.46% and 31.08% on average of total OTUs in each community (except unclassified or norank taxon), respectively. The richness (Chao 1 estimator) of the prokaryotic community in the stomach was significantly greater than those in the small and large intestine (Duncan's test, $P < 0.05$), while the highest fungal species richness was revealed by the large intestine (Table 1). The trends of other alpha diversity indexes among GI tracts were generally consistent with the results of Chao 1 estimator, except for the Shannon index in the fungal communities (Supporting Information Tables S1–S3).

The beta-diversity analysis, which was based on the overall community composition of GI microbiota, showed clear changes between different GI tracts (Table 2; Supporting Information Fig. S1). A dissimilarity test based on Bray–Curtis distances suggested that the gut microbiota differed significantly between any compared GI tracts based on results of permutational multivariate analysis of variance (PERMANOVA, $P < 0.001$) and multiple-

Table 1. Comparison of alpha diversity indexes among different gastrointestinal tracts.

	Obs. OTUs	Chao 1	Shannon	PD	Good's coverage
Bacteria					
Stomach	1574 ± 120a	1894 ± 134a	5.82 ± 0.28a	130 ± 8a	0.9878 ± 0.0011b
Small intestine	802 ± 361c	1045 ± 416c	3.61 ± 1.62b	84 ± 27c	0.9934 ± 0.0050a
Large intestine	1231 ± 71b	1444 ± 75b	5.53 ± 0.42a	95 ± 6b	0.9916 ± 0.0006a
Fungi					
Stomach	155 ± 38b	199 ± 44b	2.30 ± 0.77b	34 ± 9ab	0.9991 ± 0.0004b
Small intestine	124 ± 44b	141 ± 51c	3.43 ± 0.36a	28 ± 9b	0.9997 ± 0.0001a
Large intestine	237 ± 64a	302 ± 70a	2.66 ± 0.46b	40 ± 13a	0.9984 ± 0.0004c
Archaea					
Stomach	79 ± 39a	98 ± 48a	2.16 ± 0.24a	18 ± 14a	0.9995 ± 0.0003b
Small intestine	57 ± 29ab	66 ± 32b	1.72 ± 0.23b	24 ± 11a	0.9998 ± 0.0002a
Large intestine	41 ± 15b	50 ± 18b	1.44 ± 0.28c	7 ± 5b	0.9998 ± 0.0001a

Obs. OTUs, the observed number of operational taxonomic units (OTUs); Chao 1, Chao 1 richness estimator; Shannon, Shannon diversity index; PD, phylogenetic diversity; Good's coverage, Good's coverage estimates. Values in this table indicated means ± S.D of each index. Different letters within the same column represent significant differences across gut regions (Duncan's test, $P < 0.05$).

Table 2. Dissimilarity test based on permutational multivariate analysis of variance (PERMANOVA) and multiple-response permutation procedure (MRPP) between different gastrointestinal tracts.

	PERMANOVA		MRPP	
	Pseudo *F*	*P*	*A*	*P*
Bacteria				
Stomach and small intestine and large intestine	22.6782	0.001	0.2987	0.001
Stomach and small intestine	10.4061	0.001	0.1283	0.001
Stomach and large intestine	42.5500	0.001	0.3244	0.001
Small intestine and large intestine	20.2253	0.001	0.2550	0.001
Fungi				
Stomach and small intestine and large intestine	7.8338	0.001	0.1522	0.001
Stomach and small intestine	5.4141	0.001	0.0775	0.001
Stomach and large intestine	10.8153	0.001	0.1527	0.001
Small intestine and large intestine	7.4409	0.001	0.1173	0.001
Archaea				
Stomach and small intestine and large intestine	52.8647	0.001	0.4731	0.001
Stomach and small intestine	10.1489	0.001	0.1474	0.001
Stomach and large intestine	69.2627	0.001	0.4465	0.001
Small intestine and large intestine	134.8806	0.001	0.5767	0.001

response permutation procedure (MRPP, $P < 0.001$). These different GI tract community patterns were also confirmed by the non-metric multidimensional scaling (NMDS) analysis based on Bray–Curtis distances (Fig. 1A–C), revealing that the gut microbiota of the same GI section was generally grouped together.

Microbial composition shift across the sheep GI tract

The composition of gut microbiota presented clear patterns across sheep GI tract at phylum or class level (Fig. 1D–F). The relative abundance of the detected bacteria suggested Firmicutes was significantly abundant in the large intestine (Duncan's test, $P < 0.05$; Supporting Information Fig. S2A). Instead, other bacteria such as Bacteroidetes were most enriched in the stomach, while Actinobacteria and Proteobacteria were the most abundant phyla in the small intestine. In fungal communities, the large intestine was largely colonized by Dothideomycetes and Leotiomycetes, while the stomach and small intestine were preferentially colonized by Neocallimastigomycetes and Sordariomycetes, respectively (Supporting Information Fig. S2B). The majority of the archaeal community consisted of methanogenic groups including Methanobacteria and Methanomicrobia (both accounted for 82.60% of total archaeal sequences), followed by Thermoplasmata (average of 16.56% in all GI tract samples). The distribution patterns of these archaea among different GI tracts also shifted notably. For example, Methanobacteria were significantly dominant in the small intestine (>90%), while Methanomicrobia and Thermoplasmata were significantly dominant in the large intestine (>70%) and stomach (>30%), respectively (Supporting Information Fig. S2C).

To illuminate the microbial variation at a more refined level, taxonomic assignments along the sheep GI tract

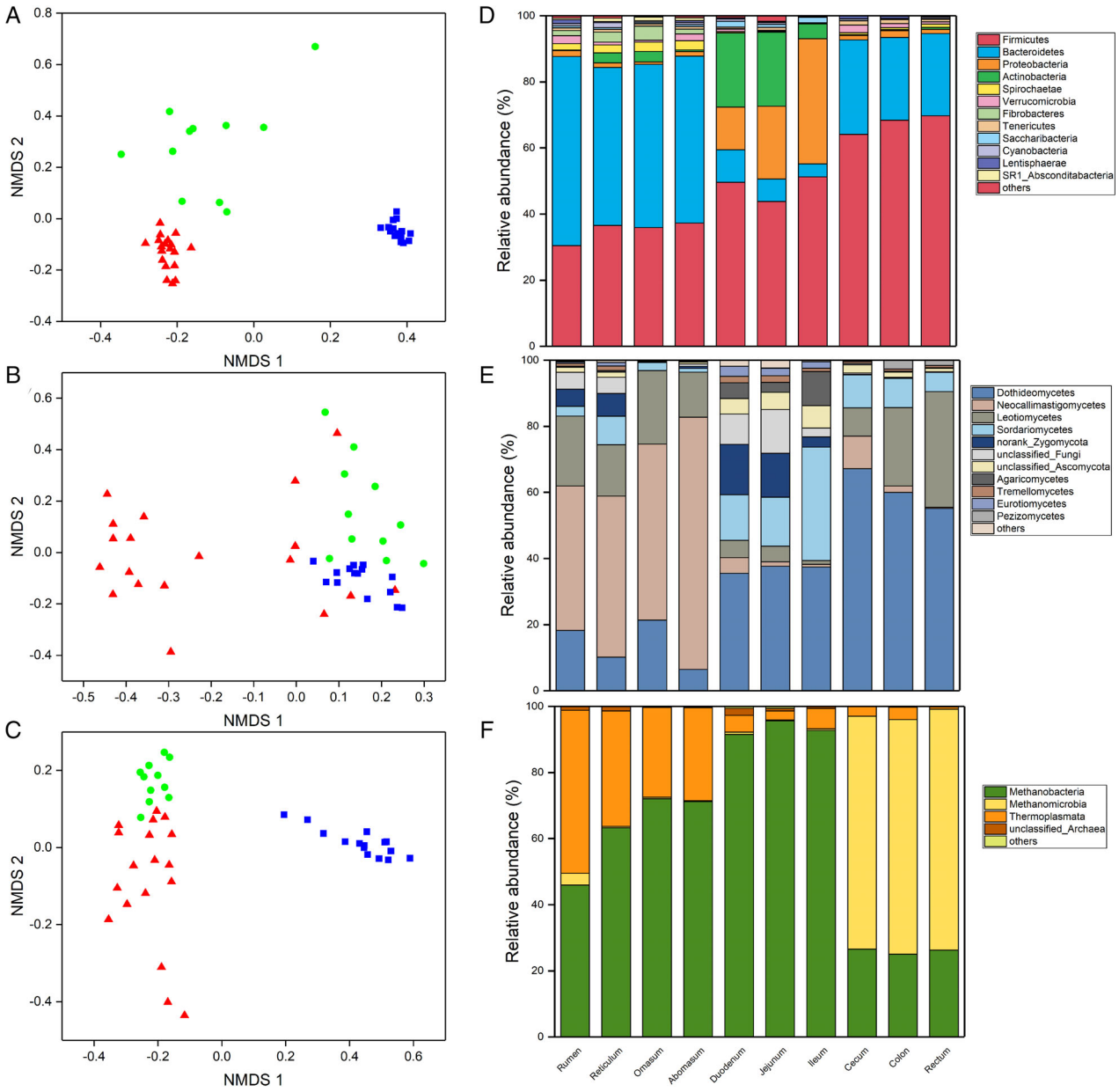

Fig 1. Non-metric multidimensional scaling analysis for bacteria (A), fungi (B), and archaea (C) and according to taxonomic information throughout the sheep gastrointestinal tract. Symbols with green circles indicate stomach samples, blue squares indicate the small intestine, and red triangles indicate the large intestine (A–C). Taxonomic information of bacterial communities was visualized at the phylum level, while archaeal and fungal communities were visualized at the class level. The phyla or classes with an average abundance of less than 1% were classified as 'others'.

were mapped onto the genus level. The taxa shared by most (>90%) of gut communities in each GI tract with relatively high abundance (>0.1%) were identified as core gut microbiota (Yan *et al*., 2016). These core genera, which in total accounted for more than 80% of the microbial abundance, also showed clear variations throughout the GI tracts of sheep. As shown in Fig. 2, similar dominant microbes inhabited in sheep GI were generally clustered within a particular GI tract region and thus generated similar colour pattern, but patterns across three major GI tract regions can shift significantly. For example, the co-varying taxa of *Succiniclasticum*, *Fibrobacter*, and *Anaeromyces* were found together in relatively large quantities in the stomach but were relatively absent in the small and large intestine. Moreover, 32 representative genera were also observed in three GI tracts based on linear discriminant analysis of effect size (LefSe) analysis, which revealed the microorganisms that

Fig 2. Heatmap showing the core genera of bacterial (A), fungal (B), and archaeal (C) communities across different gastrointestinal tracts. The abundance present here was $\log_2$-transformed.

most likely explained the observed differences among samples from the three major GI tract regions (Supporting Information Fig. S3). Core bacterial genera including *Prevotella* and *Treponema* as well as archaeal genera *Methanomicrobium* were significantly predominated in the stomach ($P < 0.05$). Members including bacterial genera *Ruminococcus* and *Acetitomaculum*, fungi genus *Cryptococcus*, and archaeal genera *Methanosphaera* and *Methanobrevibacter* (including *Methanobrevibacter ruminantium*, *Methanobrevibacter millerae*, and *Methanobrevibacter* sp. AbM4) were significantly enriched in the small intestine ($P < 0.05$). Microbial genera including bacteria genus *Bacteroides*, fungal genus belonging to Sporormiaceae, and archaeal genus

Methanocorpusculum were significantly dominated in the large intestine ($P < 0.05$).

Changes of network topological features across three major GI tract regions

Molecular ecological networks were constructed to understand the species interactions of different GI tracts. The datasets were split into three categories: stomach, small intestine, and large intestine and resulting in 632, 338, and 591 nodes of each network, respectively (Fig. 3). In all cases, the network connectivity distribution curves fitted well with the power-law model ($R^2 > 0.86$; Table 3), which suggested that the constructed networks present characteristics of scale-free and small world. In general, the majority of the links in the stomach network were associated with Bacteroidetes (40.71%), while most links in the small (63.27%) and large intestines (67.64%) network were associated with Firmicutes species. The results also showed distinct topological properties of co-occurrence networks across GI tracts. Especially the small intestine network, which presented a higher average degree and clustering coefficient as well as a shorter average path distance than those in the stomach and large intestine network (Table 3), implied a stronger and more efficient species interaction here than other GI communities. Furthermore, we found a lower proportion of positive co-occurrence in the stomach network compared with those in the small and large intestine, which indicates a higher degree of competitive activities in stomach community than other GI communities. Notably, only 28 nodes were shared by the aforementioned three networks and most of them were unique, i.e., only detected in one GI tract region (Supporting Information Fig. S4), indicating that most microorganisms had their special niches in different GI tracts.

Moreover, the different roles of the nodes in the networks were identified based on Zi-Pi plots (Supporting Information Fig. S5). Most of the microbes were classified as peripherals (97.31%), indicating that there were few links associated with them or most of the links were inside their modules. Meanwhile, there were 28, 13, and 1 node classified as module hubs, connectors, and network hubs, respectively. These nodes were considered as keystone microbes due to their important connecting role either within their own module (module hubs), or among the modules (connectors), or both (network hubs) (Olesen *et al*., 2007). More keystone microbes were found in the stomach network (12 module hubs, 12 connectors, and 1 network hub) than those in other networks. Within these 25 keystone species, the majority of them belonged to Firmicutes and Bacteroidetes and refined taxonomy results of those keystone species could be found in the Supporting Information Table S4. Interestingly, some of the nodes were served as keystone species in one network but were present as peripherals in the other network, indicating that the same OTUs exhibited different roles depending on the particular GI environment.

Ecological processes governing the assembly of the sheep GI microbiota

To determine which processes govern the assembly of microorganisms in GI tracts of sheep, we calculated

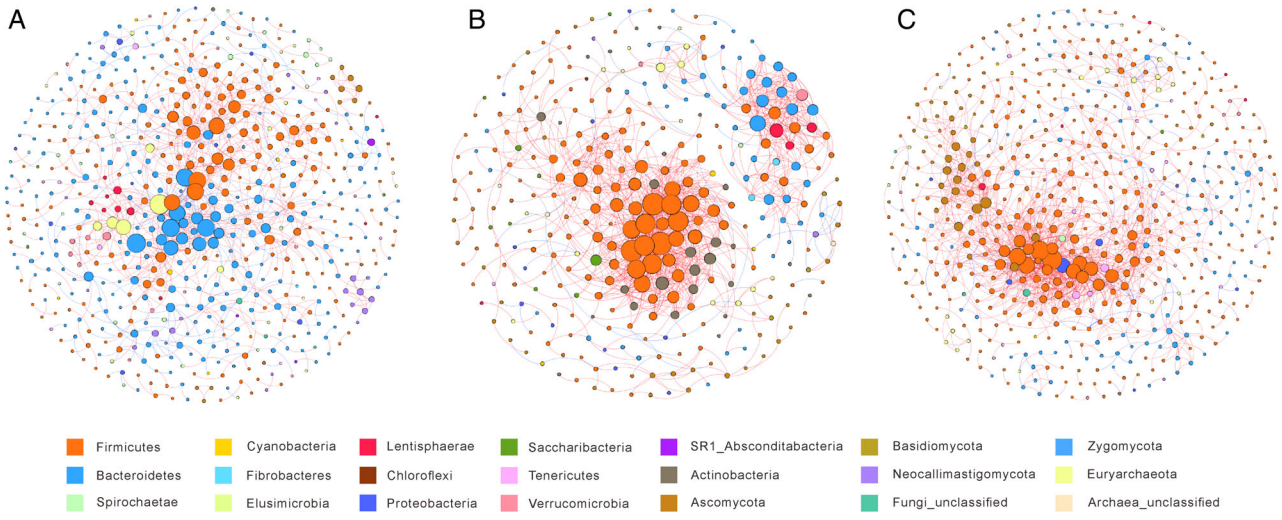

Fig 3. Co-occurrence patterns of gut microbiota in the stomach (A), small intestine (B), and large intestine communities (C). Nodes indicate taxonomic affiliations at the operational taxonomic unit (OTU) level. The size of each node is proportional to the number of degrees and the colours of nodes represent different taxonomic information (phylum level). Links between the nodes indicate a significant correlation between those OTUs ($P < 0.05$), and links with red and blue lines in the network represent positive and negative correlations, respectively.

Table 3. Topological features of microbial networks across gastrointestinal tracts and their associated random networks. The random networks were generated by rewiring the networks with 100 runs.

Topological properties	Stomach	Small intestine	Large intestine
Empirical networks			
Number of original OTUs	3189	2787	2072
Similarity threshold (st)	0.840	0.880	0.870
Total nodes	632	338	591
Total links	1012	1153	1463
R^2 of power law	0.909	0.863	0.927
Average path distance	6.307	3.806	5.930
Average clustering coefficient	0.150	0.313	0.222
Average degree	3.203	6.822	4.951
Density	0.005	0.020	0.008
Connectedness	0.416	0.285	0.506
Positive co-occurrence	0.775	0.961	0.939
Modularity	0.749	0.624	0.640
Random networks			
Average path distance	4.649 ± 0.047	3.166 ± 0.033	3.700 ± 0.036
Average connectedness	0.863 ± 0.020	0.960 ± 0.019	0.924 ± 0.020
Modularity	0.599 ± 0.005	0.316 ± 0.005	0.417 ± 0.004

β-nearest taxon index (βNTI) and Bray–Curtis-based Raup-Crick (RC_{Bray}) among three major GI tract regions (Fig. 4). As shown in Fig. 4A, the phylogenetic turnover of bacteria in most of the among-GI tract pairs was significantly different from the null expectation ($\beta NTI < -2$ in 1.82% cases, $\beta NTI > 2$ in 61.76% cases). In the remaining among-GI tract pairs, nearly half (~53%) presented a smaller taxonomic turnover than expected by chance ($RC_{Bray} < -0.95$). These results suggest that variable selection has dominated the bacteria assembly in most among-GI tract pairs, while homogenizing dispersal has played an important role in causing taxonomic turnover among others (Fig. 4G). In contrast, most (90.74%) among-GI tract pairs in fungal communities were not significantly different from the null expectation ($|\beta NTI| < 2$, Fig. 4B). Among these sample pairs cases, 54.35% exhibited smaller taxonomic turnover and 37.79% exhibited no significant difference from null expectation, suggesting homogenizing dispersal and drift tending to be the main drivers within the fungi assembly process (Fig. 4H). While for archaeal communities, we also found that the phylogenetic turnover among a large number of among-GI tract pairs (89.07%) was not significantly different from the null expectation (Fig. 4C), and nearly 67.12% of them exhibited smaller taxonomic turnover than null expectation (Fig. 4F). These results reveal that stochastic processes played the most important role in causing phylogenetic turnover of archaeal communities in most among-GI tract samples, and homogenizing dispersal was indicated as the predominant driver of the observed taxonomic turnover (Fig. 4I).

Moreover, the processes governing community turnover in within-GI tract pairs were generally regulated by homogenizing dispersal. To be specific, more than 75% of large intestine samples (including three microbial domains) were governed by homogenizing dispersal on average, followed by 65% in the stomach and 46% in small intestine samples. Drift also play important role in the assembly of GI microbiota within GI tracts, which especially dominated in small intestine sample pairs (30.30% on average). Notably, the proportion of structuring processes governing within-GI tract microbiota also varied among three microbial domains (Supporting Information Fig. S6), which further confirmed the requirements of independent analysis in shaping microbial assembly among different microbial domains.

Discussion

The importance of the mammalian GI microbial ecosystem for host health and performance has been well established (Nicholson *et al*., 2005); however, most studies have focussed on only bacteria and knowledge of the fungal and archaeal community in host GI tracts is comparably limited to date. The objective of our study was to explore the pattern of microbial diversity, composition, and interaction for all three microbial domains along GI tracts of Ujimqin sheep and further to illuminate the mechanisms that underlie GI community assembly. In this study, we showed differential diversity patterns along GI tracts of sheep in three microbial domains (Table 1). Consistent with other ruminants (de Oliveira *et al*., 2013; Mao *et al*., 2015), our results showed that sheep stomach harboured a more diverse bacterial community compared with the small and large intestine, and this could be associated with allochthonous microbes due to the continuous ingestion from the outer environment (Zhang *et al*., 2016). In contrast, the most diverse community in

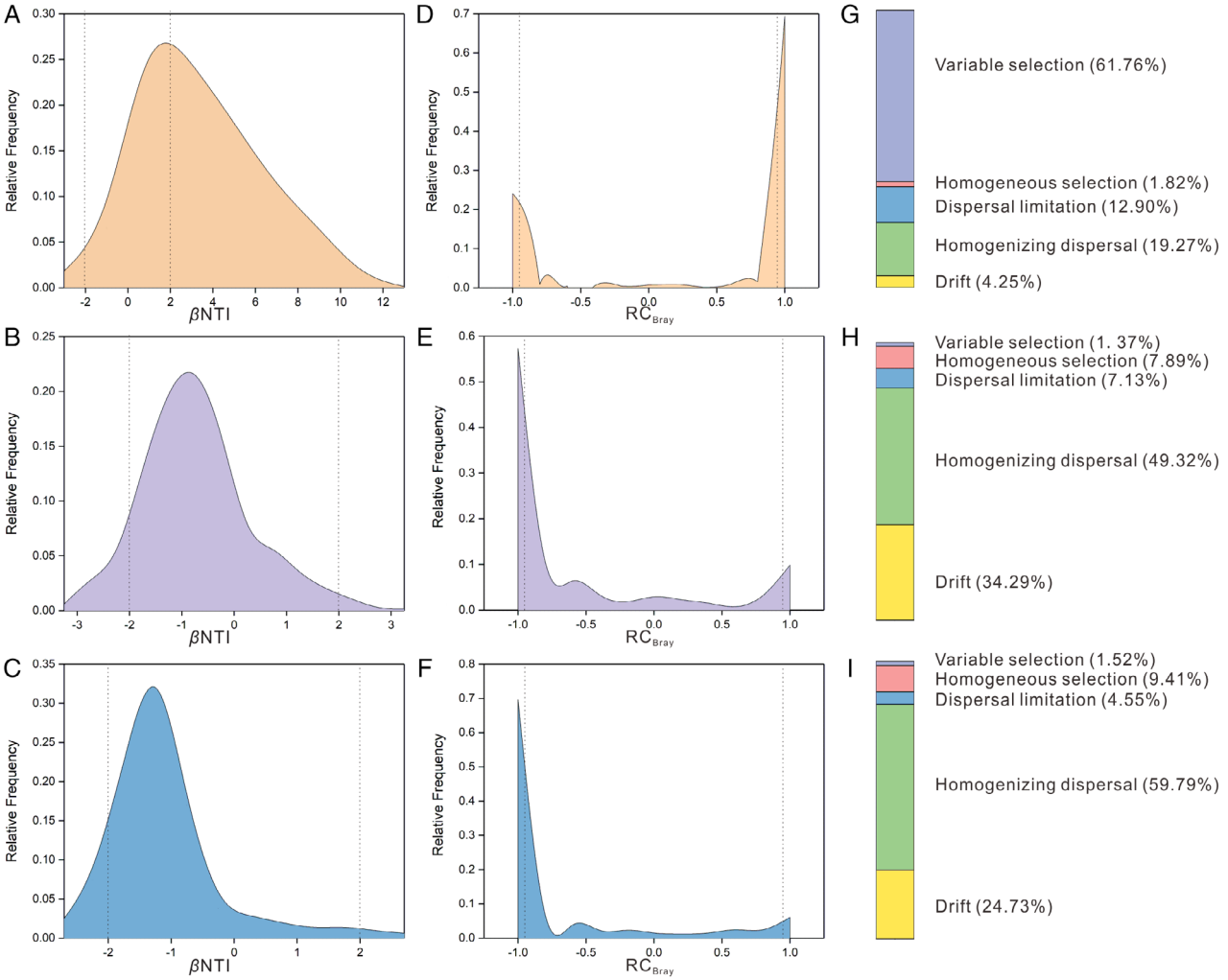

Fig 4. The distribution of standardized phylogenetic turnover (βNTI; panel A–C represented bacteria, fungi, and archaea, respectively) and taxonomic turnover (RC_{Bray}; panel D–F represented bacteria, fungi, and archaea, respectively), and the summary of the contribution of the ecological processes that govern community assembly of bacteria (G), fungi (H), and archaea (I) among gut regions. The dashed lines labelled the positions of −2 and 2 in βNTI panels, and −0.95 and 0.95 in RC_{Bray} panels.

fungal communities was found in the large intestine. Accumulating studies have disclosed the tight correlations of fungal and bacterial communities that can affect the community assembly (Hoffmann *et al*., 2013; Sam *et al*., 2017), and our results indicated that the relatively weak competition between microbial communities might explain the high fungal diversity in the large intestine. Based on the co-occurrence network, we found more negative links between bacterial and fungal OTUs (87.5%) in the stomach compared with the small intestine (50%) and large intestine (10.11%). The negative interaction between species may be induced by competition for limited resources or spaces and therefore repel each other (Coyte *et al*., 2015). Thus, it seems plausible that strong competitive relationships between fungi and bacteria in the stomach suppress certain fungal growth, whereas fungal diversity in the large intestine increased as this competitive relationship tends to be weak. Unlike bacterial and fungal communities, the diversity of archaeal communities was quite limited, only six archaeal species were discerned to an explicit species while the majority of them were defined as new and still-non-described species (Supporting Information Table S5). Notably, nearly 40% of these unclassified archaea were only detected in the stomach, which indicates that more cultivation efforts or functional metagenomics screening would be required to explore the diverse archaeal communities in sheep stomachs in the future.

The microbial community structures across different sheep GI tracts were observed to be differential, no matter of which microbial domains were compared (Table 2). Although individual variation may also affect the

assembly of GI microbes (De Filippo *et al*., 2010; Spor *et al*., 2011; Karl *et al*., 2018), our results showed that GI regions exert a stronger determinant of the composition of sheep GI microbiota than individual differences. The differential microbial structure across GI tracts can be driven by multiple factors, including pH, oxygen levels, nutrient supplies, and flow rate (Salonen and de Vos, 2014); therefore, similar conditions within the same GI tract can selectively assemble core microbes that shared by most communities (Fig. 2). The characteristics and functionality of several core microbes detected here have been reported in previous literature. For example, *Prevotella*, enriched in the stomach in our study, has been widely confirmed with a positive association with a high carbohydrate and fibre diet due to its potential to break down complex polysaccharides into smaller poly- and monosaccharide (De Filippo *et al*., 2010; Wu *et al*., 2011). *Bacteroides*, a representative core genus in the large intestine, have been reported that widely distributed in individuals tending to consume a low-fibre but high-protein diet (Wu *et al*., 2011; Nakayama *et al*., 2017). Notably, *Prevotella* and *Bacteroides* were enriched in different GI tracts in our study, which was consistent with previous works which demonstrated that subjects with high levels of *Prevotella* usually have lower levels of *Bacteroides* (Arumugam *et al*., 2011; Koren *et al*., 2013). These results imply that these two genera can be antagonistic, e.g., competition for the same niche in the local intestinal environment (Kovatcheva-Datchary *et al*., 2015; Ley, 2016). Furthermore, we also found some significant correlations among these core genera in GI tracts of sheep based on network analysis. For instance, genera *Ruminococcus* and *Methanobrevibacter*, both enriched in the small intestine, presented significant positive links with each other. Stams and Plugge (2009) have shown that *Ruminococcus* can provide methanogens with H_2, while methanogens can stimulate *Ruminococcus* to generate more ATP from the same amount of substrate, thus presenting a syntrophic relationship between these two genera. In contrast, beneficial bacteria including *Prevotella* (producing propionate) and *Saccharofermentans* (producing succinate) revealed a widely negative interaction with genus *Treponema*, many strains of which were reported as stealth pathogen in several mammals (Riviere *et al*., 1996; Radolf *et al*., 2016; Mamuad *et al*., 2020). The interaction between these beneficial microbes and pathogens may imply their potential in the maintenance of host homeostasis and health. Overall, sheep GI tracts may selectively filter particular microbial members to function as GI residents, which participate to form intricate interspecies interactions to serve the host.

Based on the topological properties of the co-occurrence network, it was found that the small intestine microbiome had a stronger biotic interaction than those in other GI tracts. It has been reported that the mean retention time of digesta in the small intestine of sheep is only 1.5–4.5 h (Coombe and Kay, 1965; De Vega *et al*., 1998), which develops a comparatively adverse condition for microbes to colonize and proliferate in the small intestine. The limited retention time can facilitate the colonization of certain microbes which could adapt to the adverse condition as well as those with shared niches, while microbes that cannot form tight links within the short periods would pass into the large intestine instead. Meanwhile, a short average path distance and a high average degree indicated that there is a more well-organized pathway for metabolites and information exchange among microbial taxa (Zhou *et al*., 2010; Faust and Raes, 2012). A closer biotic relationship can ensure the efficiency of metabolism in the small intestine and thus promote the digestion and absorption of nutrients at the local site (Jackson and McLaughlin, 2009). Moreover, we also found a higher proportion of negative/competitive co-occurrence in the stomach network compared with the small and large intestine. Strong competition in a diverse community is potentially favoured allelopathic species (Inglis *et al*., 2009), while an increasing allelopathic relationship possibly improves the resident microbial community barrier against exotic pathogen invasion (Zhang *et al*., 2009; Jousset *et al*., 2011). The wide distribution of negative interaction in the stomach network may highlight the importance of the stomach, as the first digestive part that contact a wide range of food materials, to effectively resist foreign pathogens and protects the health of the host (Coyte *et al*., 2015).

Furthermore, assembly mechanisms of the microbial community in GI tracts of sheep were also investigated based on a well-established ecological framework (Stegen *et al*., 2013). In fact, accumulating studies have disclosed the assembly mechanisms of the GI bacterial community (Berg *et al*., 2016; Adair *et al*., 2018), while few efforts were conducted to systematically monitor the underlying mechanism of fungal or archaeal communities in the GI ecosystem. In this study, we revealed the contrasting assembly mechanisms in three microbial domains based on across-GI tracts comparison. Most of the bacterial communities among GI tracts were governed by the deterministic process, in which variable selection was the most important process structuring bacterial communities. As shown in numerous previous studies, a wide range of factors, such as host species, development stage, diet, and gastrointestinal tracts, can have a large effect on GI bacterial assembly (De Filippo *et al*., 2010; McFrederick *et al*., 2014; Yun *et al*., 2014). One possible explanation here is that the gut habitat filtering or host-specific selection drives the GI bacterial community assembly, given that the sheep individuals in

our study were under the same development stage (adult) and diet mode (free grazing). In contrast, the stochastic process plays a predominant role in the assembly of fungal and archaeal communities among GI tracts. In other words, there is less effect of variable or selective environmental factors to the assembly of GI fungi and archaea than bacteria, which might attribute to the physiological/morphological differences of them compared with bacteria and therefore better adapt to the gut environment. For instance, the hyphal network of fungi (beneficial for water and nutrient uptake), and the long-time evolutionary history of archaea (a wide range of tolerance to extreme conditions), can facilitate their resistance against varied environments throughout GI tracts (Heath, 1988; Gaci *et al*., 2014; Adam *et al*., 2017). Besides, homogenizing dispersal contributed to the majority of archaeal and fungal assembly processes across gut regions, which indicated that between-GI tracts dispersal appears to be determined primarily by immigration (Stegen *et al*., 2013). As shown in the Venn diagram, a high abundance of fungal and archaeal OTUs were co-existed between these GI tracts (94.98% and 98.66% of total sequences for fungi and archaea, respectively, Supporting Information Fig. S7), which cross-verified the possibility of immigration of these microorganisms. Notably, the contrasting mechanism underlying the assembly of different microbial domains inhabiting the same community has been revealed in a range of systems including marine waters (Logares *et al*., 2018), biological soil crusts (Xu *et al*., 2020), glacier (Jiang *et al*., 2018), and lake island (Wang *et al*., 2020). Our study first confirmed that different domains of microbes inhabiting GI ecosystems can also be structured by different processes. Further studies will also be needed to determine how different microbial groups impose the selection/dispersal function in GI ecosystems.

In conclusion, our study characterized the gut microbiota along GI tracts of Ujimqin sheep from a multi-domain perspective. Our results illuminated that all microbial structures showed significant differences among GI tracts of sheep no matter for bacterial, fungal, or archaeal communities, which suggested a strong compositional differentiation and gut habitat filtering. Different sheep individuals can selectively shape core microbes within the same GI tracts and function as residents to participate in nutrient metabolism and host health. Co-occurrence network analysis based on multi-domain microbes underlined the different topological features among three major GI tract regions, with the strongest microbial interaction to be detected in the small intestine. Moreover, we surprisingly revealed the contrasting assembly mechanisms among three microbial domains in among-GI tracts samples here. We found within the same metacommunity, bacteria were mainly structured by the variable selection, while fungi and archaea were predominantly structured by homogenizing dispersal. These findings expand our knowledge of how GI tracts affect the microbial community structure and interaction, as well as to elucidate the ecological mechanisms that shape GI microbial assemblages in three microbial domains. Further experimental tests are still required to identify the determinants shaping the variation of microbial communities across the GI tracts.

Experimental procedures

Animals and sampling collection

Adult Ujimqin sheep ($n = 5$) were collected from Xilingol steppe, which is located in northern China and the steppe is dominated by the plant species of *Leymus chinensis*, *Agropyron cristatum*, and *Stipa grandis*. The animals used in the present study were 3-year-old, weighing 45.5–53 kg, healthy wether sheep and were free-fed while grazing. All animals were sacrificed following the national animal care guidelines and then aseptically dissected with a sterile scalpel to collect the intestinal content of each GI region. A total of 50 GI samples were collected and the whole GI of sheep was divided into three groups including stomach (rumen, reticulum, omasum, and abomasum), small intestine (duodenum, jejunum, and ileum), and large intestine (cecum, colon, and rectum). All GI samples were labelled and stored in a portable icebox until being transported to the laboratory and then kept at -80°C for further DNA extraction. The sampling took place in early August 2018.

DNA extraction, PCR, and sequencing

Genomic DNA from the 0.25 g GI samples was extracted using a FastDNA Spin Kit (MP Biomedicals, CA, USA) according to the manufacturer's instructions. The DNA concentration was determined using a Nanodrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the DNA quality was determined using gel electrophoresis analyses. For the Illumina MiSeq sequencing, bacterial, fungal, and archaeal libraries were created, respectively. The 16S rRNA genes of bacteria were amplified from genomic DNA using primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Mori *et al*., 2014). The ITS genes of fungi were amplified using primers ITS1F (5' CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCT GCGTTCTTCATCGATGC-3') (Bokulich and Mills, 2013). The 16S rRNA genes of archaea were amplified using primers 524F-10-extF (5'-TGYCAGC CGCCGCGGTAA-3') and arch958R (5'-YCCG GCGT TGAVTCCAATT-3') (Segata *et al*., 2011). PCR mixtures

(20 μl) were prepared which contained 10 ng of DNA template, 4 μl of 5 × FastPfu Buffer, 2 μl of 2.5 mM of dNTPs, 0.8 μl of 5 μM each primer, 0.4 μl of FastPfu Polymerase (TransGen, Beijing, China), and 0.2 μl of bovine serum albumin (BSA). To ensure the accuracy and reliability of the subsequent data analysis, random pre-tests were conducted to determine the number of cycles for each sample. The PCR reactions were performed with the following program: 95°C for 3 min; 37 cycles (29 cycles for bacteria) at 95°C for 30s, 55°C for 30s, 72°C for 45 s; and then 72°C for 10 min. A negative control PCR containing 1 μl sterile water was conducted for each batch of amplification. The PCR products were quantified and homogenized using a QuantiFluorTM-ST (Promega, Madison, WI, USA). The sequencing was completed using the Illumina MiSeq PE300 platform at the Genomic Research Centre at Majorbio Bio-pharm Technology (Shanghai, China).

Sequence data analysis

A total of 45 GI samples remained in the subsequent analyses. All sequence reads were quality checked using Mothur software (Schloss *et al*., 2009), and raw sequences with a low-quality score (<30) as well as ambiguous base ('N') were removed. The remaining sequences were further filtered before subsequent analysis to minimize the effects of random sequencing errors. Barcode, primer sequence, sequences shorter than 200 bp or longer than 460 bp, and those identified as chimeras were also removed (De Beeck *et al*., 2014; Gomes Lopes *et al*., 2019). The rest of the sequences were trimmed and compared against the Silva database (for bacterial and archaeal libraries) and UNITE database (for fungi libraries) using the ribosomal database project (RDP) classifier, which is based on the Bayesian algorithm on the QIIME platform (Wang *et al*., 2007; Caporaso *et al*., 2010). Similar sequences were clustered into OTUs with a 97% similarity using USEARCH (Edgar, 2010). To correct the differences in sequencing depth that may affect the estimation of the microbial diversity, all GI samples were rarefied to the same sequencing depth by randomly subsampling within each domain. In total, 30 216, 40 499, and 39 035 high-quality sequences per sample were yielded for the bacterial, fungal, and archaeal libraries, respectively.

Statistical analysis

Alpha diversity indices including Chao 1 estimator, Shannon index, Good's coverage, and phylogenetic diversity (PD) were calculated using Mothur. To explore the variability of microbial community structure between different GI tracts, NMDS based on Bray Curtis distance matrix was conducted using R (function metaMDS in vegan package). PERMANOVA and MRPP were used to test the significance of the microbial community structure among GI tracts (function adonis and mrpp in vegan package, respectively). To better convey the biological information in our study, the overview of the microbial composition was displayed at the phylum level for bacterial communities and class level for fungal and archaeal communities. One-way analysis of variance (ANOVA) followed by Duncan's test was conducted to determine whether the group differences of microbial taxa between GI tracts were significant or not. The LEfSe was performed with the online LEfSe tool in the Galaxy framework (Segata *et al*., 2011).

The network analysis was used to investigate the relationship of the microbial community within each GI tract, which was performed with Molecular Ecological Network Analysis Pipeline (Zhou *et al*., 2010; Zhou *et al*., 2011; Deng *et al*., 2012). Only representative OTUs (>five sequences within each GI tract) were kept and the following analysis was performed using the default settings in the pipeline. In brief, Pearson's correlation coefficient between pairwise OTUs was used to construct the similarity matrix, and then a similarity threshold (St, the cutoff value for the subsequent network construction) was chosen according to the *P*-value of this similarity matrix. After that, a condensed OTU table was generated for the following network calculation. After the calculations of 'global network properties', 'individual nodes' centrality', and 'module separation and modularity calculation', the networks were visualized with the interactive platform Gephi (Bastian *et al*., 2009).

The mechanisms of the microbial assembly were determined following the methods of Stegen *et al*. (2013). Pairwise phylogenetic turnover β-mean-nearest-taxon-distance (βMNTD) among GI samples were calculated by using R (function comdistnt in picante package) (Kembel *et al*., 2010). Randomization (1000 times) was then used to generate a null distribution of βMNTD after shuffling taxa labels and abundances according to the description by Stegen *et al*. (2013). The standard effect size measure of βMNTD, which is also known as the βNTI, was calculated as the magnitude of deviation between observed βMNTD and null βMNTD. We can interpret the primary mechanisms of microbial assembly by βNTI value: significant fraction of pairwise community comparisons with $|\beta NTI| > 2$, indicating a dominant role of the deterministic process; while non-significant fraction with $|\beta NTI| < 2$, indicating a greater effect of the stochastic process. Then, those non-significant βNTI values were further evaluated with RC_{Bray} by comparing observed Bray–Curtis dissimilarity (BC_{obs}) and its randomization model (BC_{null}). The RC_{Bray} were standardized between -1 and $+1$, and a value of $|RC_{bray}| > 0.95$ was considered

significant. The potential ecological process of the microbial community was further inferred as followed: βNTI > 2 indicate variable selection; βNTI < −2 indicate homogeneous selection; $|\beta NTI| < 2$, in combination with either $RC_{Bray} > 0.95$ or < −0.95, indicate dispersal limitation or homogenizing dispersal, respectively; $|\beta NTI| < 2$ and $|RC_{Bray}| < 0.95$ indicate drift (referred to as 'undominated' processes) (Stegen *et al*., 2015).

Data availability

Sequence data obtained in the course of the current study have been deposited in the NCBI short read archive under a bioproject accession number PRJNA605513, with biosample number SAMN14069766.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Supporting Information