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Distinct gut microbiotas between southern elephant seals and Weddell seals of Antarctica[§]

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The gut microbiome provides ecological information about host animals, but we still have limited knowledge of the gut microbiome, particularly for animals inhabiting remote locations, such as Antarctica. Here, we compared fecal microbiota between southern elephant seals (*Mirounga leonina*) and Weddell seals (*Leptonychotes weddelli*), that are top predatory marine mammals in the Antarctic ecosystem, using 16S rRNA amplicon sequencing and assessed the relationships of the gut microbial communities to functional profiles using gut metabolite analysis. The bacterial community did not differ significantly by host species or sex at the phylum level, but the distinction at the family level was obvious. The family Ruminococcaceae (Firmicutes) was more abundant in southern elephant seals than in Weddell seals, and the families Acidaminococcaceae (Firmicutes) and Pasteurellaceae (Gammaproteobacteria) were uniquely present in Weddell seals. The fecal bacterial community structure was distinctively clustered by host species, with only 6.7% of amplicon sequence variants (ASVs) shared between host species. This result implies that host phylogeny rather than other factors, such as diet or age, could be the major driver of fecal microbiotic diversification. Interestingly, there was no apparent sex effect on bacterial community structure in Weddell seals, but the effect of sex was pronounced in adult southern elephant seals mainly due to the prevalence of *Edwardsiella* sp., suggesting that extreme sexual dimorphism may modulate the gut microbiota of southern elephant seals. Unlike the clear distinction in the taxonomic composition of fecal bacterial communities, there were no discernible differences in the profiles of potential microbial functions and gut metabolites between host species or sexes, indicating that functional redundancy dominates the gut microbiota of seals surveyed in this study.

Keywords: gut microbiome, marine mammal, Phocidae, Antarctic seal, NMR

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Introduction

The gut microbiome provides ecological information about host animals. Recent studies have revealed that the gut microbiome plays a role in the immune system, metabolites, and health of host species (Kinross *et al*., 2011; Rooks and Garrett, 2016). Host animals may benefit from harvesting gut microorganisms by enhancing nutrient uptake and immune functions (reviewed in Leser and Mølbak, 2009). Despite the new findings on gut microbiomes, most studies have been conducted on humans, focusing on clinical applications (Bäckhed *et al*., 2012). Even in nonhuman studies, the majority of studies are conducted on small captive rodents, such as rats and mice, which are known as lab animals (Barko *et al*., 2018). However, we still have limited knowledge of the gut microbiome in wild animals.

In mammalian studies, the gut microbiome is closely associated the host through the sharing of bacterial communities within the same species (Ley *et al*., 2008; Youngblut *et al*., 2019). Mammalian guts are germfree at birth and colonized during development (Favier *et al*., 2003). Because mammalian species provide parental care to their offspring, it has been hypothesized that host and gut microbiome coevolved through vertical transmission (Ley *et al*., 2006a; Moran, 2006). This hypothesis has been tested in hominids by demonstrating species-specific gut microbiomes in five great ape species (Ochman *et al*., 2010). Thus, mammalian gut microbiomes are predicted to have adapted within the host taxa and coevolved with hosts. A recently proposed model suggests a cospeciation relationship between hosts and microbiomes through their congruent phylogenies (Groussin *et al*., 2020).

Other ecological factors may also drive the microbiome structure. Diet is a dominant candidate for determining the formation of microbes (Delsuc *et al*., 2014). Distant species may share gut microbiomes by harboring unique taxa that feature similar functions for digestion (for instance, bacterial similarities between whales and other terrestrial mammals that facilitate similar metabolic processes; Sanders *et al*., 2015). In a comparative analysis across 33 mammalian species, the relationship of fecal microbiomes to diet in different mammalian phylogenies was found to be mediated by microbial genes, such as those encoding carbohydrate-active enzymes and proteases, which can affect the evolution of the diet of the host animal (Muegge *et al*., 2011). Another key factor is the sex of host animals (Markle *et al*., 2013). Regulation by sex hormones often results in sex-specific differences in host immunity (Markle and Fish, 2014) and fat reserves (Min *et al*., 2019). In a sexually dimorphic species, sex differences are detected even at early developmental stages (Stoffel *et al*., 2020).

In the Antarctic ecosystem, marine mammals, such as seals

and whales, are top predators that feed on fish and invertebrates (Bowen, 1997; Staniland *et al*., 2018; Fearnbach *et al*., 2019). Because marine top predators are key species that respond to rapid environmental changes in Antarctica (Ducklow *et al*., 2007), it is important to study their gut microbiomes, which can interact with host status. Considering host adaptation to extreme conditions, it is important to understand the gut microbiome, which can interact with its host to affect its survival and reproduction. Due to the low accessibility of this region, however, limited studies have been conducted in Antarctic environments (Nelson *et al*., 2014, 2015).

In this study, we investigated the fecal microbiomes of southern elephant seals (*Mirounga leonina*, hereafter: SES) and Weddell seals (*Leptonychotes weddelli*, hereafter: WS) during austral summer. SES and WS are widely distributed in Antarctic coastal regions. Considering the genetic distances and the similarities in dietary habits between the two species, they are ideal species for estimating phylogenetic effects on gut microbiomes under a similar diet. If host phylogeny has affected the divergence of the gut microbiomes of SES and WS, we predict that the two seals may exhibit distinctive microbial community structures. Additionally, the two species have distinct mating systems. Southern elephant seals are sexually dimorphic, and one large alpha male often monopolizes a number of females (Modig, 1996), while WS has a low level of dimorphism and moderate polygyny (Stirling, 1969). Thus, we aimed to compare the sex differences in the gut microbiomes between the two species. Here, we conducted a bacterial taxonomic analysis using 16S rRNA amplification and compared the two species in terms of their bacterial community structure and diversity. Additionally, we performed a predictive functional analysis and described the metabolite profiles through nuclear magnetic resonance (NMR) analysis.

Materials and Methods

Study site and populations

A field study was conducted near the King Sejong Station on King George Island, Antarctica (62°13′22″S 58°47′18″W, Fig. 1). SES and WS are regularly observed in this area. SES lie on the sand along the coast, and WS often rests on snow. We collected fresh feces from nine SES individuals and five WS individuals in December 2019 and January 2020. For sample collection, we used DNA/RNA Shield fecal collection tubes (Zymo Research). We carefully collected the upper portion of fecal material to avoid soil contamination, using a sterile spoon attached to the collection tube screwcap. Approximately 10 ml of each fecal sample was transferred into a tube and mixed with buffer inside the tube. After sample collection, the tubes were stored at -20°C until DNA was extracted.

DNA extraction, PCR, and 16S rRNA gene amplicon sequencing

Fecal DNA was extracted from 180–220 mg of each sample using the QIAamp Fast Stool Mini Kit (QIAGEN) according to the manufacturer's instructions. DNA was amplified targeting the V3–V4 region of the bacterial 16S rRNA gene using the primers 341F (5′-CCTAGGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) (Fadrosh *et al*., 2014), and amplification was performed using the following protocol: one denaturation step at 94°C for 3 min; 5 cycles of denaturation at 94°C for 15 sec and extension at 65°C for 60 sec; 20 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 20 sec and extension at 72°C for 30 sec; followed by a final extension at 72°C for 5 min. Sequencing library construction and amplicon sequencing were performed at Macrogen using a 2 × 300 bp Illumina MiSeq sequencing system (Illumina).

Quantitative real-time PCR

Quantitative PCR (qPCR) was performed on a Rotor-Gene Q 5plex HRM (QIAGEN). Partial 16S rRNA genes were amplified using a bacteria-specific primer set, Eub338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and Eub518R (5′-ATTACCGCGGCTGCTGG-3′) (Fierer *et al*., 2005). Each PCR was carried out in a final volume of 10 μl, comprising of 5 μl of SYBR Premix Ex Taq (Bio-Rad), 3 μl of H_2O, 1 μl of 10 pmol of forward and reverse primer mix, and 1 μl of template DNA. The reaction was carried out using the following reaction cycle: initial denaturation at 95°C for 20 sec followed by 40

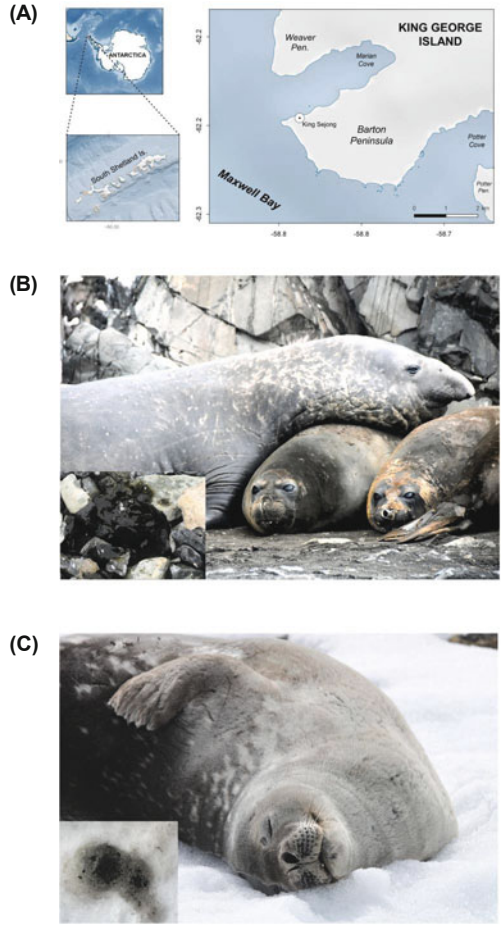

Fig. 1. Our study site and species. (A) Map of the sampling locations on King George Island, Antarctica. (B) A male and two female southern elephant seals and feces (lower left). (C) A female Weddell seal and feces (lower left).

cycles of denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec, extension at 72°C for 50 sec, and final extension at 72°C for 30 sec. Ten-fold dilutions ranging from 10^{-2} to 10^{-5} were run in duplicates for each sample together with negative controls containing no DNA template. Standard curves were generated using the 16S rRNA gene amplicons obtained from *Pseudomonas stutzeri*.

Bioinformatic analysis

The adapter and primer sequences were removed using Cutadapt v2.10 (Martin, 2011), and the resultant sequences were processed by DADA2 v1.16 (Callahan *et al*., 2016) to infer amplicon sequence variants (ASVs), which allowed a single-nucleotide resolution. When performing the quality trimming process, we applied a relaxed filtering option on the reverse reads as maxEE = c (2, 5), and the low-quality tails of each forward and reverse read were removed with truncLen = c (270, 210). Representative ASV sequences were then taxonomically assigned to genus-level phylotypes against the EzBiocloud database (May, 2020) (Yoon *et al*., 2017) by the combination of BLASTN and pairwise sequence alignment (Kim and Chun, 2014). Sequences matched to eukaryotes, mitochondria, or chloroplasts were omitted from the dataset. When collecting fecal samples of wild animals, the complete avoidance of neighboring materials (e.g., soil or rock) is almost impossible. To control for disturbed sequences that may come from nearby soils by chance, we computationally removed sequences belonging to genera that are commonly represented in the soil 16S dataset of the Barton Peninsula (Kim *et al*., 2019). Raw sequence data were submitted to the NCBI Sequence Read Archive (SRA) database with the accession number PRJNA665590.

Fecal metabolite analysis

The fecal samples were analyzed by NMR-based metabolomics to investigate the differences in fecal metabolites between host species and between sexes. All the fecal samples were vortexed and freeze-dried to remove the preservation solution and to homogenize samples. Then, 600 µl of deuterium oxide (D_2O) was added to each homogenized fecal sample (5 mg), and 550 µl of the supernatant was placed in a 5-mm high-resolution NMR tube (Optima). ^{1}H NMR spectra were acquired on Bruker Avance 600 MHz spectrometers at the NCIRF (National Center for Interuniversity Research Facilities at Seoul National University). All the NMR spectra were processed using the AlpsNMR R package following the default pipeline, including spectral interpolation/alignment, solvent region exclusion, outlier detection, and normalization (Madrid-Gambin *et al*., 2020). The resultant metabolite profiles normalized by probabilistic quotient normalization (PQN) were visualized by principal component analysis (PCA). The spectral ^{1}H NMR region from $\delta = 0.5$ to $\delta = 9.0$ was segmented into regions with widths of 0.04 ppm, giving 208 integrated regions in each NMR spectrum. The region in the range of 4.68–4.88 ppm was excluded to remove water resonance. ^{1}H NMR spectra were identified and quantified based on the chemical shifts and splitting patterns using the Chenomx NMR suite software (version 8.6, Chenomx, Inc.). All NMR spectra were subjected to the total intensity normalization.

Statistical analyses

ASV richness and diversity were compared between samples at equal sample coverage (coverage = 1 as all the samples reached asymptotes) using the iNEXT R package (Hsieh *et al*., 2016). The Bray-Curtis dissimilarities between samples were calculated using the Hellinger-transformed ASV abundance table and were visualized using principal coordinates analysis (PCoA). Permutational multivariate analysis of variance (PERMANOVA) was used to test the effects of host species and sex on fecal microbiota beta diversity, and p-values were obtained using 999 permutations (Anderson, 2001). The relative contribution of each ASV to the community dissimilarity between groups was calculated by similarity percentage analysis (SIMPER) in PRIMER v6 (Clarke and Gorley, 2006). Functional abundances were predicted by PICRUSt2 v2.3.0b (Douglas *et al*., 2020) with 16S ASV representative sequences as the input. The differential gene abundance between host species was identified based on the Wilcoxon test in the ALDEx2 R package (Fernandes *et al*., 2014). A Venn diagram was constructed with the nVenn R package for visualization, and all the plots were generated using basic functions in R v4.0.2 (www.r-project.org) unless otherwise specified (Pérez-Silva *et al*., 2018).

Results

General characteristics of the seal gut microbiota

We obtained a total of 927,355 high-quality 16S rRNA gene sequences with an average of 66,240 reads per sample, which resulted in 1,290 ASVs (197 ASVs per sample on average) (Supplementary data Table S1). The sample-size-based rarefaction and extrapolation curves revealed that bacterial richness reached asymptotes across all samples, suggesting that the current sequencing depth was sufficient to cover the full breadth of bacterial diversity (Fig. 2A). Bacterial abundances by qPCR did not differ between the two species (t-test, $t = 0.91$, $P = 0.12$; SES: 10.48 ± 0.83 [average, SD] ranged from 9.10 to 11.04 and WS: 11.14 ± 0.39 ranged from 10.80 to 11.81; in log [16S rRNA gene copies/ml]) (Supplementary data Table S1).

Effects of host species and sex on bacterial alpha diversity and taxon abundances

There were discernible differences in fecal bacterial richness and diversity between SES (n = 9) and WS (n = 5) (t-tests; $t = 5.45$, $P < 0.001$ and $t = 2.50$, $P < 0.05$, respectively) (Fig. 2B). The bacterial richness and diversity in WS were significantly higher than those in SES by factors of 1.76 and 1.62, respectively. To determine the relative contributions of bacterial taxa to the total richness, we partitioned total richness into family-level subsets. The numbers of ASVs belonging to Bacteroidaceae and Fusobacteriaceae were 3-fold greater in WS than in SES (27 ± 7 vs. 9 ± 3 ASVs for Bacteroidaceae and 34 ± 9 vs. 11 ± 3 ASVs for Fusobacteriaceae, respectively).

The bacterial 16S rRNA gene sequences mostly spanned five bacterial phyla, dominated by Firmicutes (35.1 ± 18.3%), Bacteroidetes (33.5 ± 17.0%), Fusobacteria (25.3 ± 6.3%) and, to a lesser extent, Proteobacteria (5.4 ± 7.7%) and Tenericutes

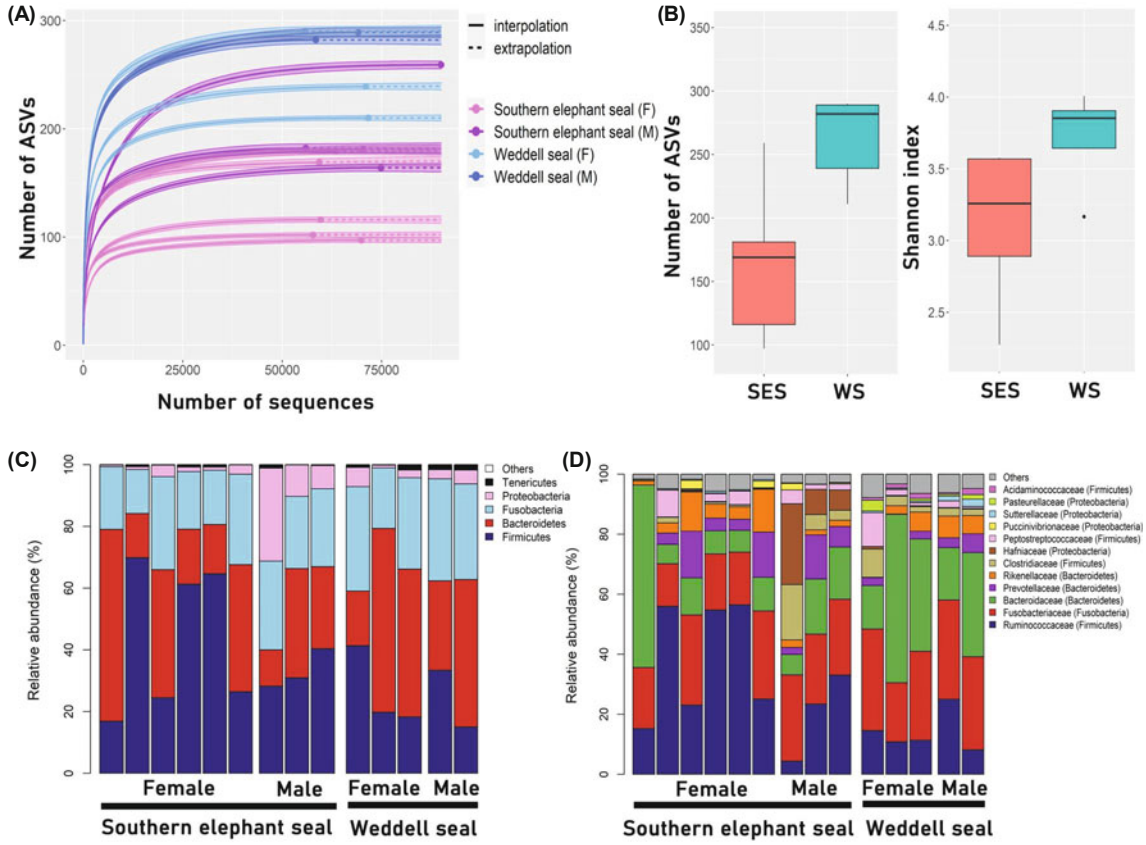

Fig. 2. Rarefaction curves, diversity indices, and relative abundance. (A) Sample size-based rarefaction and extrapolation curves. (B) Comparison of the number of ASVs and Shannon index between southern elephant seals and Weddell seals. Gut microbiota composition at the (C) phylum and (D) family levels. SES and WS denote southern elephant seals and Weddell seals, respectively.

(0.7 ± 0.6%) (Fig. 2C). There were no distinct differences in the relative abundances of the major bacterial phyla between seal species groups overall, but variations were observed among individual female SES, with stark differences in the ratio of Firmicutes to Bacteroidetes (Fig. 2C). Differences in the abundance of bacterial taxa between seal species and sexes were apparent at the family level. The relative abundance of the family Bacteroidaceae (Bacteroidetes) was 1.95 times greater in WS (32.1 ± 16.9%) than in SES (16.5 ± 17.2%), but the difference was not statistically significant (Wilcoxon rank-sum test; $P = 0.06$). A higher relative abundance of the family Ruminococcaceae (Firmicutes) was observed in SES (32.4 ± 19.2%) than in WS (14.0 ± 6.6%) ($P < 0.05$). The families Acidaminococcaceae (Firmicutes) and Pasteurellaceae (Gammaproteobacteria) were uniquely present in WS and almost absent in SES, but their contribution to the total abundance was low (1.30 ± 1.37% and 1.31 ± 0.61%, respectively). Interestingly, the family Hafniaceae (mostly *Edwardsiella*) was significantly more abundant in male SES (14.1 ± 11.2%) than in female equivalents (0.19 ± 0.17) ($P < 0.05$).

Effects of host species and sex on gut microbiota beta diversity

Fecal bacterial communities were distinctively clustered by host species (PERMANOVA; pseudo-F = 12.1, $P < 0.001$) (Fig. 3A). The effect of sex on bacterial beta diversity differed by seal species. PCoA analysis revealed that the bacterial communities of male SES formed a distinct cluster apart from those of female individuals (PERMANOVA; pseudo-F = 2.7, $P < 0.05$), although a juvenile male was placed alongside the female cluster. There was no apparent distinction in bacterial community structure between male and female WS ($P > 0.05$). We further determined the extent to which taxa contributed to the three distinct clusters (WS, male SES, and female SES) by plotting the species scores coupled with the SIMPER results on the ordination space (Fig. 3B). Representative ASVs of *Sporobacter*, *Faecalibacterium*, *Alistipes*, and *Alloprevotella* contributed primarily to the cluster of female SES, whereas ASVs belonging to *Edwardsiella* led to the separation of male SES from others. Interestingly, different ASVs of *Bacteroides* and *Fusobacterium* contributed differently to the clustering of WS and female SES, suggesting that species- or subspecies- level distinction was achieved based on these two bacterial genera.

We also attempted to identify bacterial ASVs shared between different groups. Only 6.7% (86/1,290) of the total ASVs were shared between SES and WS (Fig. 3C). When compared among the four groups categorized by host species and sex, a very small proportion (2.6%, 33/1,290) commonly occurred across the four groups (Fig. 3D). Female WS harbored the largest proportion of unique ASVs (22.3%), followed by male SES (21.7%), and male WS had the lowest proportion (13.4%).

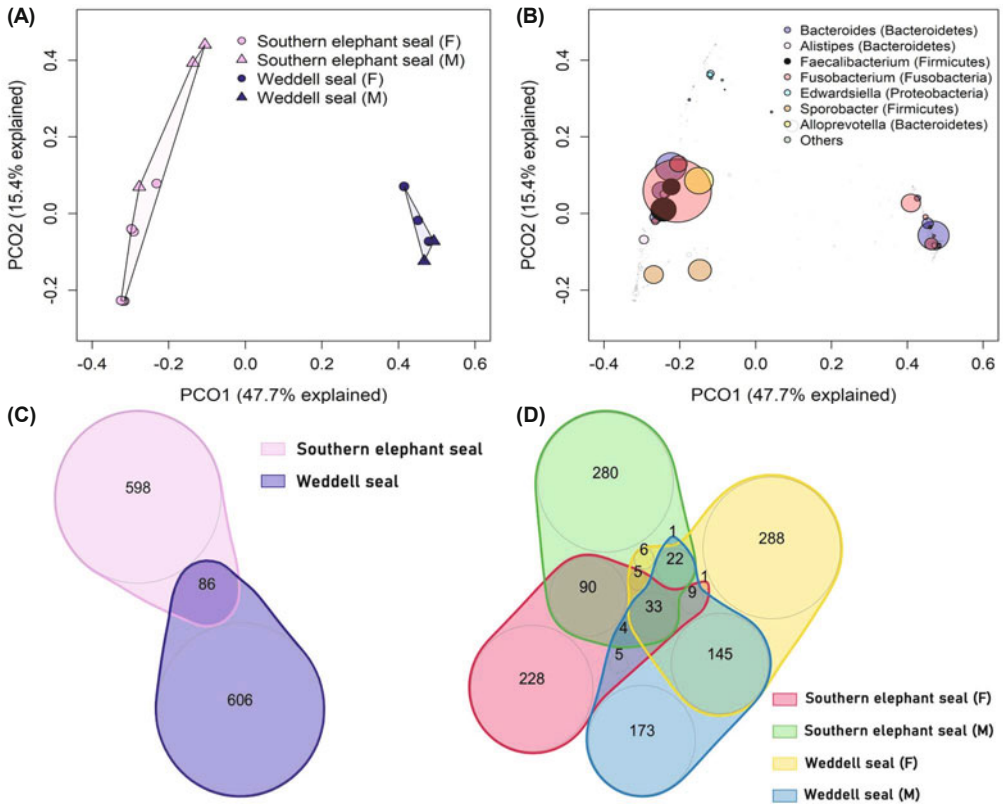

Fig. 3. Fecal bacterial communities clustered by species and sex. (A) PCoA plot and (B) species score plot of the ASV-level bacterial community composition. Venn diagram representing the numbers of ASVs within and between (C) southern elephant seals and Weddell seals and (D) among the four different groups (combinations of seal species and sex). In (B), the species scores are represented as gray circles and the top 10 ranks by SIMPER as different colors at the genus level, with the circle size proportional to the mean relative abundance of each ASV across all samples.

When examining the presence of the core gut microbiota, over 70% of seal individuals shared 20 ASVs, including *Sporobacter* (5 ASVs), *Edwardsiella* (4 ASVs), *Fusobacterium* (2 ASVs), and *Butyricicoccus* (2 ASVs) (Table 1).

Table 1. BLAST matches of the core gut bacterial ASVs present in at least 70% of seal individuals

ASVs	Closest BLAST matches[a]	Accession number	Sequence similarity (%)
ASV1	*Fusobacterium perfoetens* ATCC 29250	JHXW01000023	99.75
ASV4	*Alloprevotella* sp. WSp144	GQ867379	97.63
ASV5	*Faecalibacterium* sp. WSp133	GQ867370	98.51
ASV6	*Sporobacter* sp. CFT19D9	DQ455860	98.27
ASV9	*Faecalibacterium* sp. WSp133	GQ867370	98.26
ASV10	*Sporobacter* sp. WSp118	GQ867359	100.0
ASV11	*Fusobacterium mortiferum* DSM 19809	HG324078	100.0
ASV12	*Clostridium perfringens* ATCC 13124	CP000246	100.0
ASV17	*Edwardsiella tarda* NBRC 105688	BANW01000030	99.77
ASV18	*Alistipes* sp. S7_29	GQ867418	99.77
ASV20	*Edwardsiella tarda* NBRC 105688	BANW01000030	100.0
ASV25	*Sporobacter* sp. WSp118	GQ867359	98.03
ASV33	*Edwardsiella tarda* NBRC 105688	BANW01000030	99.77
ASV39	*Butyricicoccus* sp. bdog1_aai78g08	EU772680	99.26
ASV40	*Edwardsiella hoshinae* NBRC 105699	BAUC01000062	100.0
ASV49	*Butyricicoccus* sp. bdog1_aai78g08	EU772680	98.77
ASV65	*Colidextribacter* sp. PBF_b35	FJ375805	100.0
ASV74	*Sporobacter* sp. CE3_aai06d03	EU773770	99.76
ASV80	Uncultured *Oscillospiraceae* sp. dgB-70	AB218327	95.76
ASV146	*Sporobacter* sp. CFT19D9	DQ455860	98.77

[a] Results were obtained by running BLAST searches against EzBioCloud DB (May, 2020)

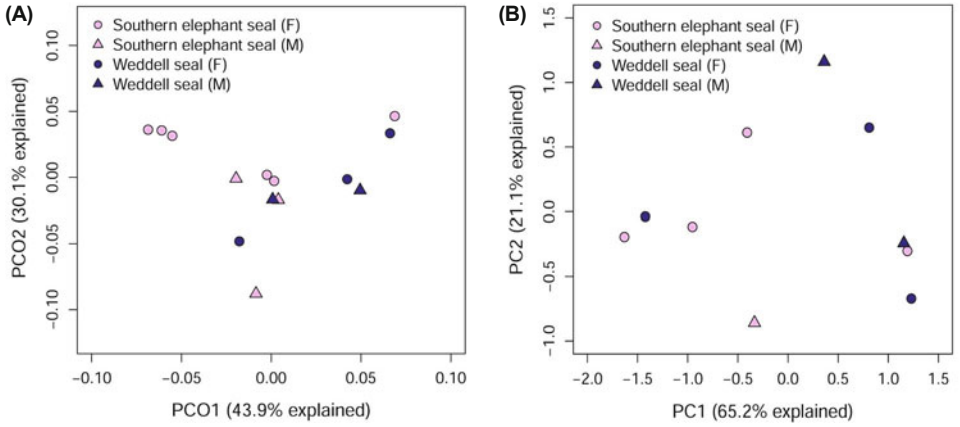

Fig. 4. Predicted microbial functions and metabolite profiles. (A) Community composition of PICRUSt2-predicted potential microbial functions and (B) NMR-based fecal metabolite profiles.

Profiles of potential microbial functions and gut metabolites

The metabolic potential of the seal gut microbiota was predicted from representative 16S rRNA gene sequences using PICRUSt2. Only 9 out of 1,290 ASVs scored greater than the nearest-sequenced taxon index (NSTI) value of 2, which is the maximum confidence cutoff by default in PICRUSt2. The weighted NSTI scores ranged from 0.034 to 0.085, far below the average values for mammals (Douglas *et al.*, 2020). Given the striking differences in bacterial taxonomic composition, we hypothesized that the microbial functional profiles may also exhibit a similar degree of distinction between host species or sex. Unlike the expectation, however, there were no significant differences in the functional profiles between host species or different sex groups (all $P > 0.05$), implying that the gut microbiota of seal individuals are likely to be functionally redundant (Fig. 4A). Among various microbial metabolic pathways, we particularly examined genes which have previously been identified to differentiate between carnivorous and herbivorous mammals (Muegge *et al.*, 2011; David *et al.*, 2014). For example, in central pyruvate metabolism seal gut microbiomes were enriched in genes encoding oxaloacetate decarboxylase (EC:4.1.1.3), pyruvate kinase (EC:2.7.1.40), phosphoenolpyruvate phosphotransferase (EC:2.7.3.9), and pyruvate-phosphate dikinase (EC:2.7.9.1) (Supplementary data Fig. S1). In glutamate metabolism, genes encoding glutamate synthase (EC:1.4.1.13) and glutaminase (EC:3.5.1.2) were more enriched in seal gut microbiomes (Supplementary data Fig. S1). We also compared the abundances of genes involved in the microbial production and utilization of short-chain fatty acids (SCFAs), the typical end products of the fermentation of dietary carbohydrates by the gut microbiota, but there were no significant differences between different host species or sex groups (all $P > 0.05$). Butyrate-producing communities were mostly represented by acetyl-CoA pathway (PWY-5676 in MeTaCyc), whereas glutarate (P162-PWY), 4-aminobutyrate (PWY-5022), and lysine (PWY-5677) pathways were relatively less represented (Supplementary data Fig. S2A). The members of *Fusobacterium*, *Sporobacter*, and *Bacteroides* contributed mostly to the acetyl-CoA pathway in butyrate production (Supplementary data Fig. S2B). We further analyzed gut metabolites using ^{1}H NMR to identify possible links with taxonomic or functional traits of the gut microbiota. The gut metabolite profiles did not differ significantly by host species or sex (all $P > 0.05$), as was the case for the predicted functional profiles (Fig. 4B). A large proportion of gut metabolites comprised of trehalose (38.1% on average), followed by trimethylamine (0.88%), acetate (0.48%), ethanol (0.45%), and propionate (0.43%) (Supplementary data Table S2). Alanine, sarcosine, isovalerate, suberate, and salicylate were also present but at relatively lower abundance. Butyrate was not detected in NMR analysis despite the high representation of potential functions in the PICRUSt2 result.

Discussion

Our results suggest that there are significant differences in fecal bacterial diversity between SES and WS, with both richness and diversity being consistently higher in WS than in SES. A recent study has demonstrated that body mass is one of the major drivers determining gut bacterial diversity in vertebrates, showing an increase in bacterial diversity with increasing animal body mass (Godon *et al.*, 2016). If body mass has an influence, then SES must have higher bacterial diversity in their gut environments than WS, considering their greater body mass than WS (up to 3700 kg in SES, see Tarnawski *et al.*, 2013; 181–502 kg in WS, Goetz *et al.*, 2017). Factors other than body mass could be potential determinants of bacterial diversity. The differences in the degree of bacterial diversity between seal species can be explained by the high flexibility of the WS foraging behavior (Green and Burton, 1987; Lake *et al.*, 2003). Although WS mainly forages for fish and cephalopods (Casaux *et al.*, 1997), they exhibit spatial, seasonal and individual variations in food items. In particular, WS is capable of using benthic and pelagic water across the sea ice over the continental shelf (Lake *et al.*, 2003). Thus, such flexible diets and habitats may be responsible for the diverse food sources causing higher levels of bacterial diversity in WS than in SES.

We also found that the bacterial communities were distinctively clustered by host species. This result corresponded to our prediction that the two seal species may have distinctive microbial community structures if host phylogeny affects the divergence of the gut microbiomes of the two species. The two species descended from a common ancestor over 10 million years ago (Arnason *et al.*, 2006). Additionally, both species both forage on fish and cephalopods, although the diets

vary among populations (Bradshaw *et al.*, 2003; Goetz *et al.*, 2017). Thus, we reason that host phylogeny can be one of the major drivers of the gut microbial composition. The effect of sex was clear in adult SES but not in WS. SES is one of the most dimorphic animals (Tarnawski *et al.*, 2013). Related to the size differences between sexes, SES exhibits sex-specific foraging, and resources are partitioned according to sex (Lewis *et al.*, 2006). Therefore, due to the sexual dimorphism and sex-specific strategies of SES, we expected to find bacterial differences between the sexes. Such sexual differences in the gut microbiota have also been reported in a closely related species, the northern elephant seal (Stoffel *et al.*, 2020). Although the exact mechanisms of sex-specific inheritance of gut microbiomes were not revealed, sexual differences appeared in the early stages of development (Stoffel *et al.*, 2020). Differences in bacterial community traits between males and females should be considered carefully as the sample size within each category (e.g. n = 3 for male SES, n = 2 for male WS) is not large enough to do robust statistical tests.

The phylum-level composition of the gut microbiota observed in the current study was somewhat dissimilar to the results of previous studies. Nelson *et al.* (2013) reported that members of Bacteroidetes are consistently more abundant in SES than in leopard seals (*Hydrurga leptonyx*). Actinobacteria was one of the major bacterial phyla found in fecal samples of WS in McMurdo Sound and White Island, Antarctica (Banks *et al.*, 2014). Contrary to previous findings, we could not find any host species-specific patterns in the microbiota composition at the phylum level. The proportion of Bacteroidetes varied greatly among SES individuals (11.8–62.2% in relative abundance), and Actinobacteria was a very minor component of the gut microbiota in WS (less than 0.5%). The abundances of bacterial phyla varied rather markedly between seal individuals, with differences in the Firmicutes to Bacteroidetes ratio (F:B ratio) being more pronounced, within the range of 0.27–4.87. The F:B ratio is one of the most extensively used indices in human gut microbiome studies, and its close association with obesity, an imbalance in health conditions, has been frequently reported (Ley *et al.*, 2006b; Castaner *et al.*, 2018). High individual-level variability is also observed in other pinniped species, such as sea lions and Pacific harbor seals (Bik *et al.*, 2016; Pacheco-Sandoval *et al.*, 2019). Evidence is currently not conclusive, but additional individual-level data together with clinical information will help elucidate the causal relationship between the phylum-level distribution and the health condition of seals.

The presence of a large proportion of Fusobacteria across all seal individuals is consistent with the result of a recent study comparing the gut microbiotas of terrestrial and marine mammals (Nelson *et al.*, 2014). Nelson *et al.* (2014) suggested the possibility that their codominance in the guts of both Canidae (e.g., dogs) and Phocidae (e.g., seals) may be ascribed to the evolutionary proximity between the two host families. The members of the family Fusobacteriaceae generally inhabit the oral and intestinal mucosae of animals and are capable of producing various SCFAs by fermenting carbohydrates or amino acids (Olsen, 2014). The genus *Fusobacterium* is among the most commonly represented genera among seal individuals, indicating that these bacteria may play important roles in seal gut metabolism.

Edwardsiella is one of the core bacterial taxa present in almost all individuals in this study. Representative 16S rRNA gene sequences of *Edwardsiella* shared the highest sequence similarity (99.8–100% identity) with that of *E. tarda*. *Edwardsiella tarda* is an opportunistic bacterial pathogen with a broad host range and commonly infects aquatic mammals, reptiles, and fish (Park *et al.*, 2012 references therein). *Edwardsiella tarda* is widespread across various Antarctic mammals and birds, with *E. tarda* isolates obtained from 281 (15.1%) of the 1,855 Antarctic wildlife samples (Leotta *et al.*, 2009). Edwardsiellosis in fish generally occurs under disturbed environmental conditions when the natural balance of an ecosystem is disrupted, and infected fish show abnormal behavior or external lesions (Park *et al.*, 2012). However, there were no apparent clinical signs of this disease in surveyed Antarctic animals (Leotta *et al.*, 2009). The ability to infer the reason why *E. tarda* is prevalent only in male SES is at this stage limited due to the lack of accompanying physiological or clinical data from individual seals. Given that *E. tarda* is prevalent in both juvenile and adult males, the physiological traits related to the pronounced sexual dimorphism of SES could constitute a reason.

Seal gut microbiomes were different from those of terrestrial carnivores in the abundance of genes involved in central pyruvate and glutamate metabolisms. Seal gut microbiomes were enriched in genes encoding enzymes catalyzing the replenishment of tricarboxylic acid cycle intermediates by the anaplerotic supply of oxaloacetate from phosphoenolpyruvate and pyruvate, which is in line with that of baleen whale microbiomes (Sanders *et al.*, 2015). Gene abundance profiles in glutamate metabolism was also more similar to those of baleen whales than to those of terrestrial carnivores, likely reflecting the similarities in diets of marine mammals. Seal gut microbiomes were also enriched in genes encoding enzymes catalyzing the production and utilization of SCFAs, and the presence of acetate and propionate in the gastrointestinal tract of seals were evidenced by the NMR-based metabolite result. Interestingly, butyrate was not detected in NMR-based metabolite profiles across all fecal samples despite the high representation of butyrate-forming pathways. Given that the high protein and low carbohydrate diets lead to a disproportionate reduction in the human fecal butyrate content (Duncan *et al.*, 2007), protein-rich diets such as fish, crustaceans, etc. may result in the decreased concentration of butyrate in the seal fecal metabolites. The profiles of the reconstructed microbial functions and gut metabolites are not significantly differentiated by host species or sex, despite the marked degree of distinction in taxonomic composition. Remarkably similar functional profiles of gut microbiota, while varying considerably in taxonomic groups, have been commonly reported in human gut microbiome studies (Lozupone *et al.*, 2012; The Human Microbiome Project Consortium, 2012). Likewise, functional redundancy (various taxonomic groups sharing similar functions) also seems applicable to the gut microbiota of Antarctic seals. There is a growing body of evidence indicating that interindividual functional variation in the human microbiome occurs at the species or strain level (The Human Microbiome Project Consortium, 2012). Although there were distinct ASV-level differences in bacterial genera (i.e., *Bacteroidetes* and *Fusobacterium*) between the two seal

species, strain-specific functional differentiation was not achieved in PICRUSt-based prediction results due to the scarcity of neighboring genomes. Multiomics approaches, including genome-resolved metagenomics and metabolomics, will enable a better understanding of individual-level functional variations and host–microbiome interactions in seals.

Conclusion

This study illustrates that the gut microbiotas of SES and WS exhibit distinct taxonomic community compositions, with only a minor fraction of shared taxa. The marked degree of community differentiation between the two seal species, despite the similarities in their dietary habits, suggests that the host evolutionary history may have profoundly affected the gut microbiota of Antarctic seals. To a lesser extent, the gut microbiotas of SES differed significantly by sex, presumably due to traits associated with the pronounced male-biased sexual dimorphism. Unlike the taxonomic composition results, the microbial functional community composition and gut metabolite profiles were not significantly differentiated by host species or sex. This lack of functional differentiation may result either from the prevalence of functional redundancy of the gut microbiota across seal individuals or the presence of strain-level functional variation, which was not sufficiently resolved with the 16S rRNA gene-based functional inference method. Given the limited knowledge on the gut microbiomes of wild animal populations, these results will advance our understanding of how the health of Antarctic wildlife is responding to recent climate change as well as the interactions and coevolution between host species and the gut microbiota.

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Conflict of Interest

The authors declare no competing interests.

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