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Seasonal characteristics of gut microbiota in varied tits and its relationship with immunoglobulin A

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Abstract. Gut microbiota play a central role in immune defense and adapting to environmental fluctuations. Varied tits (*Sittiparus varius*) are socially monogamous birds with a narrow geographic distribution, whose habitat and diet undergo significant seasonal changes. We hypothesized that the diversity and composition of gut microbes in varied tits would exhibit seasonal differences, and that the relative abundance of gut microbes would be correlated with host's immunity. To test these hypotheses, we characterized the fecal bacterial community composition of varied tits by sequencing the V3–V4 region of the 16S ribosomal RNA gene, comparing the differences in gut microbiota composition across seasons, and exploring the relationship between bacterial abundance and Immunoglobulin A (IgA) concentrations. A total of 4847 operational taxonomic units (OTUs), 40 phyla, 108 classes, 269 orders, 477 families, and 1109 genera were obtained from 16S metabarcoding. The intestinal microbiota of varied tits was dominated by the phyla Proteobacteria (35.29%), Firmicutes (26.66%), Cyanobacteria (13.99%), Actinobacteriota (9.62%) and Bacteroidota (7.32%). Significant seasonal variations in gut microbiota composition were observed, while the abundance-based coverage estimator (ACE) index of alpha diversity in spring was significantly higher than in summer, and overall community structure (beta diversity) differed markedly between winter and spring. Linear discriminant analysis effect size (LEfSe) revealed the enrichment of specific bacterial taxa in each season (LDA>4, $P<0.05$), such as winter-associated Proteobacteria and *Pantoea*, spring-associated Bacteroidota and *Bacteroides*, and summer-associated Firmicutes and *Erwinia*. However, no significant correlation was found between the abundance of these gut microbes and IgA concentrations. Our findings provide empirical insights into the seasonal dynamics of gut microbiota in wild birds, contributing to a better understanding of their ecological adaptive strategies, which are essential for developing adequate conservation strategies.

Keywords. Intestinal microbiota, seasonal variation, immunoglobulin A, varied tits.

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Introduction

Gut microbiota, as the largest and most sophisticated micro-ecosystem in organisms, play critical roles in host environmental adaptation (Waite & Taylor 2014; Goossens *et al*. 2021), nutrient metabolism (Rowland *et al*. 2018), and immune regulation (Naeem & Bourassa 2025). Systematic analyses

on composition, diversity and seasonal dynamics of avian gut microbiota provide valuable insights into microbial ecology, and deepen our understanding of the hosts' ecological adaptative strategies. Furthermore, a deeper understanding of the microbiota-immunity nexus provides an additional foundation for developing effective conservation strategies.

Wild birds vary widely in their life history components (such as reproductive and migrative behavior, for example), and to cope with changes in their environment, birds not only need to make physiological and behavioral adaptations, but also their gut microbial communities play a crucial role. For example, research of Hooded crane (*Grus monacha*) shows that the diversity and abundance of gut microbiome differ significantly among seasons (Zhang *et al*. 2020). The intestinal microbiota of Sichuan partridge (*Arborophila rufipectus*) shows the lowest alpha diversity and higher proportions of Firmicutes and Actinobacteriota during winter (Tang *et al*. 2023). The study of four migratory *Catharus* thrushes finds that the alpha diversity of microbial community in the breeding season was higher than during the migratory period (Skeen *et al*. 2023). These studies highlight the plasticity of avian gut microbiota in response to environmental fluctuations, underscoring their potential role in facilitating host adaptation.

Immune defense serves as a primary mechanism for resisting pathogenic microorganisms and coping with environmental stressors (Buehler *et al*. 2008). Gut microbiota contribute to host adaptation by modulating immune responses, particularly through interactions with immunoglobulins (Beller *et al*. 2020). Among immunoglobulins, secretory IgA was selected for analysis in the current study because it is the predominant antibody in mucosal secretions, and exerts a key role in maintaining gut microbial homeostasis (Tizard 2002; Schofield *et al*. 2018). When bound to or encapsulating resident symbiotic bacteria in the gut, IgA helps to prevent infections from pathogenic microorganisms and to maintain a stable intestinal microbial composition (Sutherland *et al*. 2016). While the gut microbiota-IgA relationship has been extensively studied in mammals, its dynamics in wild birds remain poorly understood.

Varied tit (*Sittiparus varius*) is a socially monogamous bird with a narrow geographic distribution across Korea, Japan, and parts of China. This species exhibits marked seasonal shifts in its ecology. Its diet changes from being predominantly animal-based during the breeding season (March–July) to plant-derived resources, such as nuts and seeds of *Cornus controversa* and *Sorbus alnifolia*, in winter (Cai 2014). Concomitantly, the ambient temperatures of varied tits' habitat fluctuate dramatically, from below -10°C in winter to over 30°C in summer, imposing strong selective pressures on physiological and microbial adaptations. Given these extreme seasonal variations, the varied tit serves as an ideal model for investigating host-microbiota-environment interactions. We hypothesized that the diversity and composition of gut microbes in varied tits may have significant seasonal differences, and that there will be a significant correlation between the relative abundance of gut microbes and IgA concentrations. By bridging the gap in the knowledge of fecal microbes of varied tits, our study will advance understanding of adaptive strategies in birds under fluctuating environmental conditions, and contribute to research on ecological and conservation biology of this and other bird species.

Methods

Sample collection

The study was conducted in the Xianrendong National Nature Reserve, Liaoning Province, China (122°53′24″~123°03′ 30″ E, 39°54′00″~40°03′00″ N) during 2018–2020. We captured adult varied tits with misting nets. About 100 μL of blood samples were taken from the brachial vein within 3 min after capturing. The individuals were then placed in clean paper bags for approx. 10–20 min, after which fecal samples were collected from the paper surface. The collection of fecal and blood samples was completed at the same time. A total of 31 fecal samples were collected, including 15 samples in

spring, 8 samples in summer and 8 samples in winter. Twenty-four blood samples (8 per season) were collected for IgA analyse. All of the fecal and blood samples were flash frozen in liquid nitrogen and stored at -80°C until DNA extraction. The details of sampling information are provided in the Appendix.

DNA extraction, PCR amplification, and high-throughput sequencing

DNA was extracted from the fecal samples using the E.Z.N.A. ® Soil DNA Kit (OMEGA, USA) in accordance with the manufacturer's protocols. Extracted DNA was quantified using Nanodrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, USA) and 1% agar-gel electrophoresis, respectively.

The 16S rRNA is an important component of ribosomal RNA in prokaryotes. Its V3–V4 region is highly variable providing sufficient genetic information for microbial analysis at the phylum/genus levels, and is widely used in studies analyzing microbial diversity (Yoon *et al*. 2017; Pérez-Bustamante *et al*. 2024). During the current study, the V3–V4 region of 16S rRNA gene of the 31 samples was amplified with the universal primer set of Dai *et al*. (2022): 338F (5'-barcode-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). These primers have been extensively validated and widely used in other microbial ecology studies (Dai *et al*. 2022). They provide a robust balance between read length (required for accurate taxonomic classification) and amplification specificity, effectively covering a broad range of bacterial taxa while minimizing amplification of host (eukaryotic) DNA. Polymerase chain reactions (PCR) were performed in a mixture containing 4 μL FastPfu Buffer (5×), 2 μL dNTPs (2.5 mM), 0.8 μL forward and reverse primer (5 μM), 0.4 μL FastPfu Polymerase, 10 ng template DNA and ddH_2O to a final volume of 20 μl. PCR amplifications were conducted under the following conditions: an initial denaturation step of 3 min at 95°C; followed by 28 cycles of 30 sec at 95°C, 30 sec at 53°C, 45 sec at 72°C; and a final extension of 10 min at 72°C. High-throughput sequencing analysis was performed on Illumina MiSeq platforms at Majorbio Bio-pharm Technology Co., Ltd (Meiji, Shanghai, China).

Detection of IgA content by immunoglobulin A analysis

Plasma samples were collected after centrifugation of blood at 3000 rmp for 10 min. Levels of IgA were quantified with enzyme-linked immunosorbent assays (ELISAs) (MM-0913O1, Kete Biological Company) in accordance with the manufacturer's protocols. Briefly, the plasma and detection antibody labeled with horseradish peroxidase (HRP) were added into microtiter plate wells embedded with purified chicken IgA antibody. After incubation and washing, the HRP substrate solutions were added into the microtiter plate wells. The reaction was terminated by adding a termination solution and the absorbance (OD) at 450 nm was measured. The IgA levels were calculated based on the OD and a standard curve. Each sample was measured three times, and the mean value was calculated as the final IgA level.

Data analyses

Quality control and sequence processing

Raw sequence data quality was assessed using FastQC (ver. 0.20.0; https://github.com/OpenGene/fastp). Initial processing of the raw dataset included quality filtering to remove short and low-quality readings. Paired-end reads were assembled using FLASH (ver. 1.2.7; https://sourceforge.net/projects/flashpage) (Magoč *et al.* 2011). The raw sequences were processed as follows: bases with a quality score below 20 were trimmed, and reads shorter than 50 bp or containing more than three ambiguous bases (N) were discarded. Paired-end reads were merged requiring a minimum overlap of 10 bp and allowing a maximum mismatch rate of 0.2 within the overlap region; reads failing to merge under these criteria were filtered out. Demultiplexing was performed based on barcode and primer sequences, which were trimmed prior to further processing. No mismatches were allowed in the barcodes, while a maximum of 2 mismatches was permitted in the primers. Following demultiplexing, the barcode and primer sequences were trimmed, and high-quality reads without primer sequences were retained.

OTU picking and taxonomic classification

Open-reference operational taxonomic unit (OTU) identification was performed at 97% sequence similarity using USEARCH (ver. 7.1; http://drive5.com/usearch/) and converted into an OTU table. The clustering procedure was as follows: Singletons (single sequences without duplication) sequences were first removed from the merged reads to eliminate potential sequencing errors. The remaining sequences were then clustered using the cluster_otus command in USEARCH. Subsequently, chimeric sequences were filtered out from clustered OTUs. All high-quality sequences were mapped to a reference database, and sequences with more than 97% similarity to representative OTUs were selected to generate the OTU table. Rarefaction curves, OTU cumulative curves, and curves of core OTUs of all samples were used to analyze sequencing depths.

Microbial diversity analysis

The number of identified OTUs was used to analyze alpha diversity indices (Shannon index, Simpson index, ACE index and Chao1 index) with >97% identity using mothur (ver. 1.30.2; Oakley *et al*. 2009) by R (ver. 3.3.1; R Core Team 2023). Because of unequal sample sizes, a Kruskal-Wallis H test was used to analyze the differences in alpha diversity among the three seasons by function "kruskal.test" in R. Non-metric multidimensional scaling (NMDS) was used to assess beta-diversity with the vegan package (ver. 2.6-4; Oksanen *et al*. 2022) in R (ver. 3.3.1; R Core Team 2023). The fecal microbiota community of varied tits were compared across seasons using the similarity analysis test (ANOSIM, analysis of similarities) with 999 permutations with the vegan package in R.

Identification of dominant taxa and statistical associations

Excluding unidentified sequences (3.15%), the dominant fecal microbiome was defined as taxa present in > 50% of samples with a relative abundance > 1% in the samples where it is found (Grond *et al*. 2018; Liu *et al*. 2023). Linear discriminant analysis effect sizes (LEfSe) were used to identify differentially taxa ranging from the phylum to the genus level across seasons using a parametric Kruskal-Walli's rank-sum test (Alpha-value: 0.05; effect size threshold: 4).

Taking bacteria abundance and season as independent variables and sampling year as covariate, multiple linear regressions tested for a possible correlation between IgA concentrations and the abundance of intestinal microbial communities in varied tits by lm () function in R with the stats package (Bach *et al*. 2018). Bonferroni corrections of *P*-values for multiple comparisons were also conducted in R with the stats package.

Results

After quality filtering, we obtained 1 589 427 high-quality readings with individual sequence lengths ranging from 101 to 537bp (Appendix). A total of 4847 OTUs were identified based on 97% sequence similarity (Figure 1a). The rarefaction curves of the sequencing reads per sample reached plateaus, indicating that the sequencing depth was adequate to capture the microbial diversity present (Figure 1b). The cumulative curve of the number of OTUs also tended to flatten, further supporting the adequacy of the sequencing depth (Figure 1c). The rarefaction curve for the core microbiota, defined at the OTU level (97% similarity), showed a gradual decrease with increasing sample size, and the number of core OTUs stabilized at 5 when the sample size reached 20 or more (Figure 1d).

Seasonal variation of alpha and beta diversity

When analysing alpha diversity, the microbial richness (ACE index) in spring was significantly higher than that in summer ($P=0.042$, Figure 2), while the Chao index ($P=0.230$, Figure 2), the Simpson ($P=0.260$, Figure 2) and the Shannon diversity index ($P=0.260$, Figure 2) showed no significant differences across the three studied seasons.

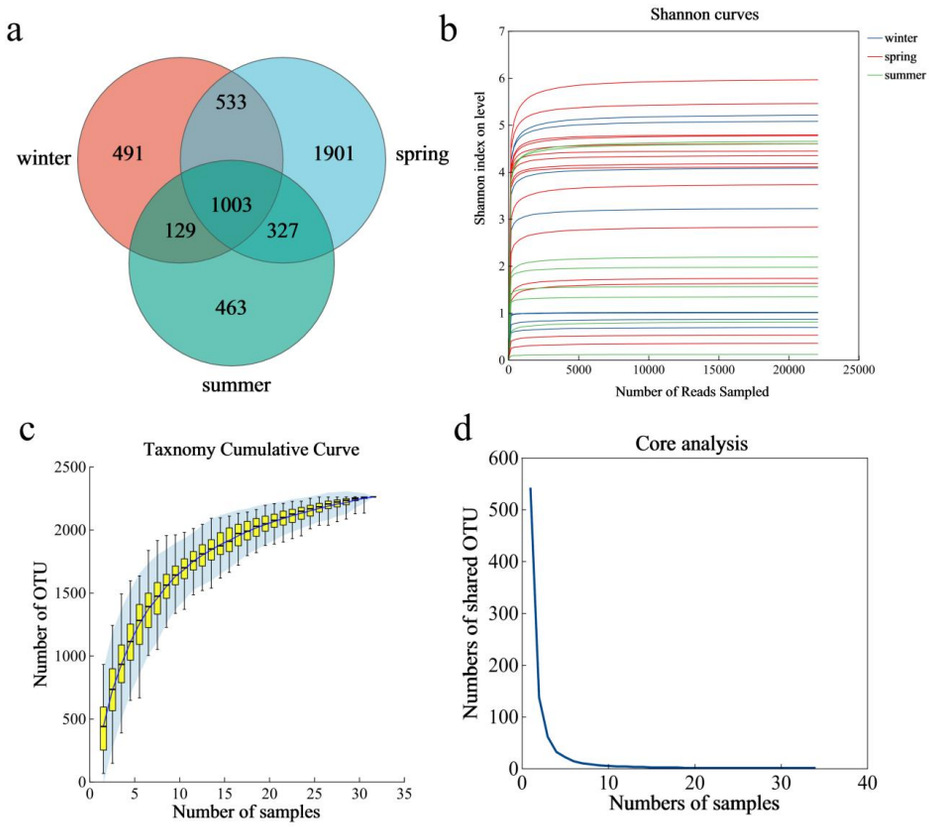


Figure 1 – Venn diagram showing the number of shared OTUs of all groups of varied tits (a), rarefaction curves of 31 samples (b), species cumulative curve on OTU level (c), core OTUs curve (d).

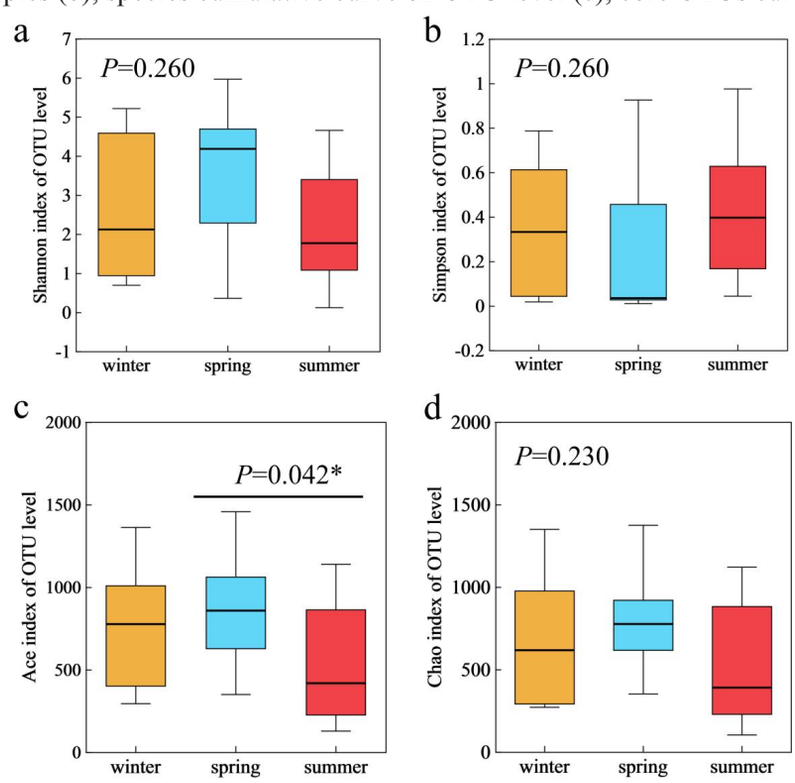
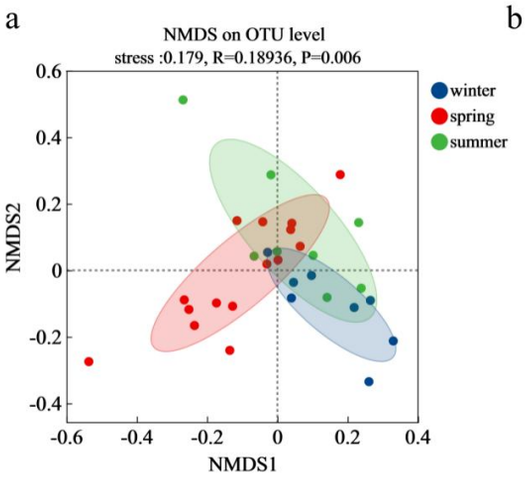


Figure 2 – Intestinal microbial alpha diversity index Shannon index (a), Simpson index (b), Ace index (c), Chao index (d) of varied tits across the three seasons. *$P<0.05$.

a



b

The analysis of similarities (ANOSIM) test was used to compare differences in the intestinal bacteria of varied tits across three seasons.

Treatment	ANOSIM	
	R	P-value
Summer vs Winter	0.204	0.104
Summer vs Spring	0.264	0.062
Spring vs Winter	0.278	0.006**

**P<0.01

Figure 3 – NMDS analysis between different individuals based on OTUs (a). Each point in the graph represents a sample, the distance between points indicates the degree of difference, samples in the same group are represented in the same color, when the stress <0.2, NMDS can accurately reflect the differences between groups and within groups of samples. Analysis of similarity (ANOSIM) analysis between different groups (b). **P< 0.01.

The NMDS analysis showed that the fecal bacteria of varied tits from the same season had a tendency to aggregate (Figure 3a). Based on the ANOSIM analysis, the fecal microbiota community of varied tits varies significantly between winter and spring (P=0.006, Figure 3b).

Seasonal variation in gut microbiota composition

A total of 40 phyla, 108 classes, 269 orders, 477 families, 1109 genera and 2203 species were identified for varied tit microbiota. Proteobacteria (average of 35.29% ±6.16%), Firmicutes (average of 26.66% ±5.34%), Cyanobacteria (average of 13.99% ±4.41%), Actinobacteriota (average of 9.62% ±1.89%) and Bacteroidota (average of 7.32% ±2.13%) were the dominant phyla across all 31 samples regardless of the season.

In winter, varied tits had the most Proteobacteria (72.90% ±11.85%), which then markedly dropped in spring (15.94% ±3.87%) and summer (33.95% ±13.11%) (Figure 4a–c, P=0.003). In spring, the dominant phyla were Firmicutes (31.08% ±6.99%), Cyanobacteria (18.16% ±5.63%), Proteobacteria (15.94% ±3.87%), Actinobacteriota (11.24% ±2.88%) and Bacteroidota (12.47% ±3.41%) (Figure 4b). The most abundant phyla changed again in summer to Firmicutes (36.29% ±15.33%), Proteobacteria (33.95% ±13.11%) and Cyanobacteria (16.91% ±8.98%) (Figure 4c).

At the genus level, taxa surpassing 1% abundance in varied tits' microbiota composition were *Pantoea*, *Lactobacillus*, *Pseudomonas*, *Serratia* and *Ralstonia* in winter (Figure 4d), *Enterococcus* in spring (Figure 4e), and *Pantoea*, *Lactobacillus*, *Enterococcus*, *Weissella*, *Klebsiella*, *Ralstonia* and *Carnobacterium* in summer (Figure 4f).

Specific fecal bacterial taxa in varied tits were further identified by LEfSe and significant differences were found across the three studied seasons. As shown in Table 1, one phylum (Proteobacteria (P=0.003)), one class (Gammaproteobacteria (P=0.006)), one order (Enterobacterales (P=0.006)), two families (Pseudomonadaceae (P=0.001), Erwiniaceae (P=0.002)) and three genera (*Pantoea* (P=0.002), *Pseudomonas* (P=0.001), *Ralstonia* (P=0.008)) were significantly more abundant in winter than in spring and summer. One phylum (Bacteroidota) (P=0.037), three classes (Bacteroidia

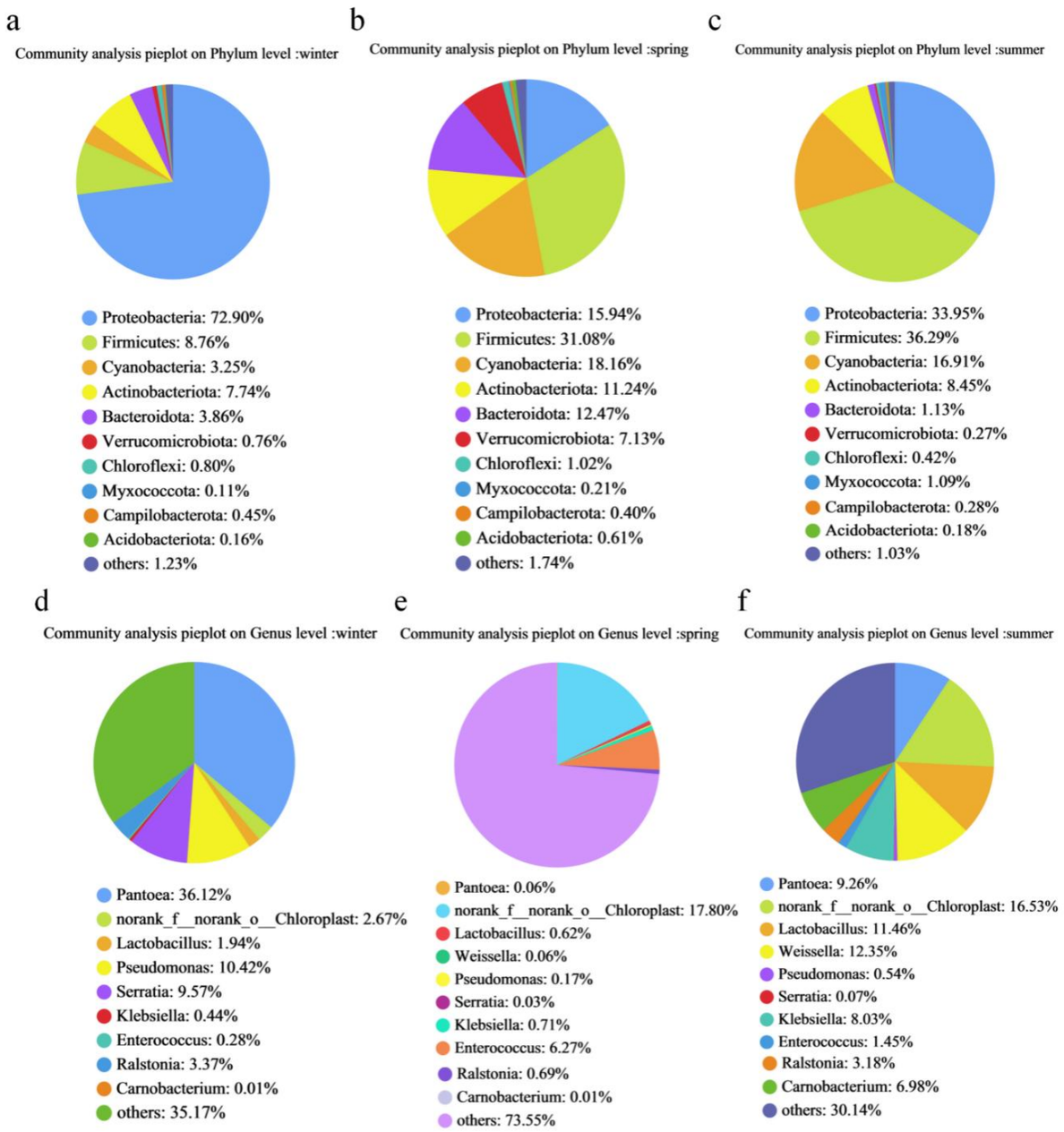


Figure 4 – The composition of gut microbiota communities at the phyla level in winter (a), spring (b), and summer (c), and at the genera level in winter (d), spring (e), and summer (f).

($P=0.037$), Chlamydiac ($P=0.003$), Clostridia ($P=0.006$)), four orders (Bacteroidales ($P=0.017$), Lachnospirales ($P=0.004$), Oscillospirales ($P=0.001$), Chlamydiales ($P=0.003$)), three families (Lachnospiraceae ($P=0.004$), Bacteroidaceae ($P=0.018$), Ruminococcaceae ($P=0.002$)) and three genera (*Bacteroides* ($P=0.018$), *Blautia* ($P=0.001$), and *Faecalibacterium* ($P=0.003$)) were more abundant in spring. And one phylum (Firmicutes ($P=0.036$)), and one genus (*Erwinia* ($P=0.040$)) were more so in summer.

TABLE 1

Bacterial taxa significantly enriched in each season identified by LEfSe analysis (LDA score > 4, P < 0.05).

Season	Taxonomic level	Taxon name	LDA Score	P_value
Winter	Phylum	Proteobacteria	5.44289	0.00333
	Class	Gammaproteobacteria	5.4319	0.00602
	Order	Enterobacterales	5.35784	0.00656
	Family	Pseudomonadaceae	4.71797	0.0013
	Family	Erwiniaceae	5.22347	0.00193
	Genus	*Ralstonia*	4.2517	0.00779
	Genus	*Pseudomonas*	4.71797	0.0013
	Genus	*Pantoea*	5.22324	0.00182
Spring	Phylum	Bacteroidota	4.74789	0.03673
	Class	Clostridia	4.94318	0.00649
	Class	Bacteroidia	4.74795	0.03673
	Class	Chlamydiae	4.58085	0.00322
	Order	Oscillospirales	4.51525	0.00158
	Order	Chlamydiales	4.58085	0.00322
	Order	Bacteroidales	4.7445	0.0173
	Order	Lachnospirales	4.71888	0.00411
	Family	Ruminococcaceae	4.36288	0.00162
	Family	Lachnospiraceae	4.7187	0.00411
	Family	Bacteroidaceae	4.40608	0.01837
	Genus	*Blautia*	4.00779	0.00141
	Genus	*Bacteroides*	4.40608	0.01837
	Genus	*Faecalibacterium*	4.07183	0.00268
Summer	Phylum	Firmicutes	5.08728	0.03635
	Genus	*Erwinia*	4.37441	0.03969

Relationship between intestinal microbiome and individual immunoglobulin concentration

Differential bacterial taxa were first defined as those taxa showing statistically significant differences in abundance across the three seasons combining the results of the LEfSe analyzes with a parametric Kruskal-Walli's rank-sum test (Alpha-value: 0.05; effect size threshold: 4). Multiple linear regression analysis was then applied to investigate a possible correlation of IgA concentrations and the relative abundance of dominant bacteria phyla (defined as those being present in > 50% of samples and exceeding 1% of average relative abundance) and differential bacterial taxa among the three seasons (Table 2).

No significant correlations were observed between plasma IgA concentrations and the relative abundance of the dominant bacteria phyla at the phylum level (Proteobacteria: $P = 0.07$, Firmicutes: $P = 0.112$, Cyanobacteria: $P = 0.903$, Actinobacteriota: $P = 0.836$, Bacteroidota: $P = 0.935$) or any of the seasonally differential bacterial taxa at various taxonomic levels (all $P > 0.05$, for detailed statistics, see Table 2).

TABLE 2

Taking bacteria abundance as independent variables, the year of sampling as a covariate, multiple linear regression analysis was used to analyze the effects of bacteria abundance, season, and sampling year on IgA concentration in varied tits.

Dependent variable	Independent variable	Estimate	Std. Error	t	P_value	P_{adj}
IgA concentration	Intercept	0.556	0.135	3.063	0.030	0.159
	Proteobacteria abundance	0.657	0.343	1.915	0.070	0.353
	Season	-0.782	0.579	-1.350	0.192	0.964
	Season × Proteobacteria abundance	-0.556	0.414	-1.340	0.196	0.979
	Year	-0.473	0.541	-0.875	0.392	1.000
IgA concentration	Intercept	0.078	0.197	0.399	0.694	1.000
	Firmicutes abundance	-0.387	0.232	-1.668	0.112	0.558
	Season	0.01	0.391	0.026	0.979	1.000
	Season × Firmicutes abundance	0.388	0.303	1.278	0.217	1.000
	Year	-0.008	0.431	-0.020	0.985	1.000
IgA concentration	Intercept	-0.141	0.275	-0.515	0.612	1.000
	Cyanobacteria abundance	0.048	0.395	0.124	0.903	1.000
	Season	0.109	0.454	0.241	0.812	1.000
	Season × Cyanobacteria abundance	-0.326	0.403	0.808	0.429	1.000
	Year	0.077	0.499	0.155	0.879	1.000
IgA concentration	Intercept	-0.03	0.231	-0.130	0.898	1.000
	Actinobacteriota abundance	-0.05	0.240	-0.210	0.836	1.000
	Season	-0.071	0.479	-0.149	0.883	1.000
	Season × Actinobacteriota abundance	-0.111	0.230	-0.481	0.636	1.000
	Year	-0.196	0.466	-0.211	0.835	1.000
IgA concentration	Intercept	0.015	0.230	0.068	0.947	1.000
	Bacteroidota abundance	0.029	0.353	0.083	0.935	1.000
	Season	-0.108	0.486	-0.223	0.826	1.000
	Season × Bacteroidota abundance	0.082	0.295	0.280	0.783	1.000
	Year	-0.135	0.479	-0.283	0.781	1.000
IgA concentration	Intercept	0.857	0.252	4.013	0.013	0.065
	Gammaproteobacteria abundance	0.657	0.403	1.630	0.119	0.597
	Season	-0.813	0.643	-1.264	0.221	1.000
	Season × Gammaproteobacteria abundance	-0.579	0.460	-1.260	0.223	1.000
	Year	-0.516	0.558	-0.926	0.366	1.000
IgA concentration	Intercept	0.025	0.234	0.108	0.915	1.000
	Clostridia abundance	0.045	0.309	0.146	0.885	1.000
	Season	-0.107	0.502	-0.214	0.833	1.000
	Season × Clostridia abundance	0.093	0.260	0.359	0.724	1.000
	Year	-0.136	0.484	-0.282	0.781	1.000
IgA concentration	Intercept	0.015	0.230	0.068	0.947	1.000
	Bacteroidia abundance	0.029	0.353	0.084	0.934	1.000
	Season	-0.108	0.486	-0.223	0.826	1.000
	Season × Bacteroidia abundance	0.082	0.295	0.281	0.782	1.000
	Year	-0.135	0.479	-0.282	0.781	1.000
IgA concentration	Intercept	0.393	1.291	0.305	0.764	1.000
	Chlamydiae abundance	1.738	5.916	0.294	0.772	1.000
	Season	-0.376	1.016	-0.370	0.715	1.000
	Season × Chlamydiae abundance	-1.527	4.934	-0.309	0.760	1.000
	Year	-0.118	0.479	-0.248	0.807	1.000
IgA concentration	Intercept	0.714	0.183	5.860	<0.001	<0.001
	Enterobacterales abundance	0.873	0.356	2.453	0.024	0.120
	Season	-0.984	0.551	-1.784	0.090	0.451
	Season × Enterobacterales abundance	-0.8	0.398	-2.010	0.058	0.294
	Year	-0.643	0.483	-1.332	0.198	0.992

Dependent variable	Independent variable	Estimate	Std. Error	t	P_value	P_{adj}
	Intercept	0.013	0.233	0.056	0.956	1.000
	Oscillospirales abundance	-0.008	0.315	-0.028	0.978	1.000
IgA concentration	Season	-0.129	0.506	-0.255	0.801	1.000
	Season × Oscillospirales abundance	0.05	0.263	0.193	0.849	1.000
	Year	-0.153	0.489	-0.315	0.756	1.000
	Intercept	0.684	0.116	4.443	0.007	0.035
	Chlamydiales abundance	0.477	0.752	0.635	0.533	1.000
IgA concentration	Season	0.017	0.503	0.036	0.972	1.000
	Season × Chlamydiales abundance	0.35	0.670	0.523	0.607	1.000
	Year	-0.265	0.558	-0.475	0.640	1.000
	Intercept	0.013	0.230	0.060	0.953	1.000
	Bacteroidales abundance	0.022	0.351	0.065	0.949	1.000
IgA concentration	Season	-0.109	0.486	-0.224	0.825	1.000
	Season × Bacteroidales abundance	0.073	0.294	0.251	0.804	1.000
	Year	-0.136	0.479	-0.284	0.779	1.000
	Intercept	0.023	0.230	0.100	0.921	1.000
	Lachnospirales abundance	0.063	0.308	0.207	0.838	1.000
IgA concentration	Season	-0.092	0.501	-0.185	0.855	1.000
	Season × Lachnospirales abundance	0.1	0.258	0.391	0.700	1.000
	Year	-0.118	0.488	-0.243	0.811	1.000
	Intercept	-0.116	0.998	-0.117	0.908	1.000
	Pseudomonadaceae abundance	-0.398	3.320	-0.120	0.906	1.000
IgA concentration	Season	0.021	1.058	0.020	0.984	1.000
	Season × Pseudomonadaceae abundance	0.333	2.777	0.120	0.906	1.000
	Year	-0.113	0.485	-0.233	0.818	1.000
	Intercept	0.668	0.274	3.943	0.015	0.075
	Erwiniaceae abundance	0.852	0.409	2.080	0.051	0.256
IgA concentration	Season	-0.795	0.529	-1.503	0.149	0.747
	Season × Erwiniaceae abundance	-0.821	0.416	-1.974	0.063	0.315
	Year	-0.529	0.466	-1.134	0.271	1.000
	Intercept	0.031	0.226	0.141	0.889	1.000
	Ruminococcaceae abundance	-0.027	0.286	-0.096	0.924	1.000
IgA concentration	Season	-0.185	0.485	-0.383	0.706	1.000
	Season × Ruminococcaceae abundance	0.153	0.247	0.621	0.542	1.000
	Year	-0.191	0.474	-0.404	0.690	1.000
	Intercept	0.023	0.230	0.100	0.921	1.000
	Lachnospiraceae abundance	0.063	0.308	0.207	0.838	1.000
IgA concentration	Season	-0.092	0.501	-0.185	0.855	1.000
	Season × Lachnospiraceae abundance	0.1	0.258	0.391	0.700	1.000
	Year	-0.118	0.488	-0.243	0.811	1.000
	Intercept	-0.002	0.220	-0.012	0.991	1.000
	Bacteroidaceae abundance	-0.022	0.237	-0.093	0.927	1.000
IgA concentration	Season	-0.135	0.468	-0.288	0.776	1.000
	Season × Bacteroidaceae abundance	0.159	0.198	0.805	0.431	1.000
	Year	-0.138	0.472	-0.293	0.773	1.000
	Intercept	-0.044	0.205	-0.218	0.829	1.000
	Ralstonia abundance	-0.42	0.208	-2.019	0.057	0.289
IgA concentration	Season	0.089	0.420	0.212	0.834	1.000
	Season × *Ralstonia* abundance	0.32	0.324	0.990	0.334	1.000
	Year	-0.006	0.416	-0.016	0.987	1.000
	Intercept	-0.116	0.998	-0.117	0.908	1.000
	Pseudomonas abundance	-0.398	3.320	-0.120	0.906	1.000
IgA concentration	Season	0.021	1.058	0.020	0.984	1.000
	Season × *Pseudomonas* abundance	0.333	2.777	0.120	0.906	1.000
	Year	-0.113	0.485	-0.233	0.818	1.000

Dependent variable	Independent variable	Estimate	Std. Error	t	P_value	P_{adj}
IgA concentration	Intercept	0.963	0.219	4.762	0.002	0.010
	Pantoea abundance	1.279	0.567	2.254	0.036	0.181
	Season	-1.011	0.568	-1.778	0.091	0.457
	Season × *Pantoea* abundance	-1.17	0.521	-2.245	0.036	0.184
	Year	-0.579	0.463	-1.250	0.226	1.000
IgA concentration	Intercept	0.024	0.251	0.098	0.923	1.000
	Blautia abundance	-0.015	0.399	-0.040	0.969	1.000
	Season	-0.155	0.524	-0.297	0.769	1.000
	Season × *Blautia* abundance	0.071	0.334	0.212	0.834	1.000
	Year	-0.175	0.491	-0.356	0.726	1.000
IgA concentration	Intercept	-0.002	0.220	-0.012	0.991	1.000
	Bacteroides abundance	-0.022	0.237	-0.093	0.927	1.000
	Season	-0.135	0.468	-0.288	0.776	1.000
	Season × *Bacteroides* abundance	0.159	0.198	0.805	0.431	1.000
	Year	-0.138	0.472	-0.293	0.773	1.000
IgA concentration	Intercept	0.304	0.128	2.457	0.043	0.215
	Faecalibacterium abundance	-0.136	0.245	-0.555	0.585	1.000
	Season	-0.3	0.448	-0.672	0.510	1.000
	Season × *Faecalibacterium* abundance	0.378	0.323	1.170	0.256	1.000
	Year	-0.3	0.445	0.674	0.508	1.000
IgA concentration	Intercept	-0.041	0.219	-0.189	0.852	1.000
	Erwinia abundance	0.374	0.221	1.689	0.108	0.537
	Season	7.377	13.300	0.554	0.586	1.000
	Season × *Erwinia* abundance	37.226	64.600	0.576	0.571	1.000
	Year	-0.29	0.455	-0.637	0.532	1.000

Discussion

The current study characterized the dynamics of gut microbial composition in varied tits using DNA sequence data from the 16S rRNA gene. At phylum level, the gut microbiota of varied tits are predominantly composed of Proteobacteria, Firmicutes, Cyanobacteria, Actinobacteriota and Bacteroidota, similar to the results of other studies on avian gut microbiota (Wang *et al*. 2017). These phyla may be associated with the food uptake or metabolic balance. For instance, Firmicutes are linked to the breakdown of carbohydrates, polysaccharides, sugars and fatty acids which are utilized by the host as energy sources (Hart *et al*. 2018). Bacteroidota possess large numbers of genes encoding carbohydrate active enzymes, allowing them to switch readily between different energy sources in the gut depending on availability (Thomas *et al*. 2011). Proteobacteria are rich in cellulase, playing an important role in the digestion of plant-derived fiber-enriched diets and the accumulation of energy (Morrison *et al*. 2009; Colston *et al*. 2016). Actinobacteriota can generate some antibiotics that belong to secondary metabolites to resist pathogenic bacteria (Ventura *et al*. 2007).

Significant inter-seasonal variations in gut microbial diversity were found across the three seasons in the current study. More specifically, the microbial richness (ACE index of alpha diversity) was significantly higher in spring than in summer (Figure 2c), and also overall community structure (beta diversity) showed marked differences between spring and winter (Figure 3). There were also significant differences in the abundance of gut microbiota across seasons (Figure 4, Table 1) at different taxonomic levels. Such differences could be in response to altering external environments and shifts in life history periods that lead to changes in temperatures, food resources and reproduction status (Mueller *et al*. 2006; Drovetski *et al*. 2019). For example, compared with the non-breeding period, the relative abundance of Bacteroidota, *Prevotella* and *Alistipes* increased significantly in breeding common muskrat (*Ondatra zibethicus*), while the relative abundance of Firmicutes and Actinobacteriota decreased (Song *et al*.

2023). In the current study, there was no significant difference in alpha diversity estimated with the Chao, Simpson and Shannon indices between the three seasons, suggesting that environmental stress may primarily affect rare microbiota species rather than dominant groups (Berlow *et al.* 2021).

Pressures associated with seasonal lifestyle changes would disrupt the stability of the gut microbiota, leading to an increase in susceptibility to pathogens (Owen & Moore 2006; Risely *et al*. 2018; Anwar *et al*. 2021). During the current study, we observed a significant increase in the relative abundance of bacterial genera commonly associated with environmental sources or opportunistic pathogens (e.g., *Pantoea*, *Pseudomonas*) during winter (Figure 4, Table 1), coinciding with the harsher winter conditions (lower temperatures, scarcer food resources) experienced by varied tits, which are known to increase physiological stress and potentially susceptibility to pathogens in wild birds (Benskin *et al*. 2009; Brodin *et al*. 2017).While the direct health consequences of these compositional changes were not assessed in this study, the increase in relative abundance of such taxa during a period of heightened environmental challenge warrants further study of their potential role in winter survival. We also found a higher prevalence of the plant pathogen *Erwinia* in summer in varied tits, which may be associated with increased availability of infected plants and insect vectors (Pilgrim 2024). As varied tits primarily consume insects and invest heavily in reproduction during this season, their exposure to *Erwinia* was significantly elevated in summer (Cai 2014; Jing *et al*. 2019).

Growing evidence suggests that IgA plays an important role in regulating the colonization of commensal microbiota, and is recognized as a key factor in maintaining microbial homeostasis (Takeuchi & Ohno 2022). IgA deficiency leads to abnormal amplification of symbiotic anaerobic bacteria represented by *Clostridium* in the small intestine, seriously disrupting the balance of intestinal flora in mice (Suzuki *et al*. 2004). However, in the current study, we did not find any significant correlation between the abundance of gut microbiota and plasma IgA levels in varied tits (Table 2). This might be related to the interaction of different immunoglobulins on gut microbiota. For example, IgG assists IgM in coating bacteria, playing an important role in regulating intestinal microbiota (Eriksen *et al*. 2023). Besides, environmental factors might also have great effects on the interaction of gut microbes and IgA, which may result in a non-linear correlation between them (Huus *et al*. 2021). The complex interactions between gut microbes and host immunity are critical to understand avian evolution and adaptation, and further studies are needed to elucidate their interactions and biological significance.

The current study has several limitations that should be considered when interpreting the results. The primary limitation of this study is the uneven distribution of sample sizes across seasons, which may reduce statistical power. Another limitation is that the samples were collected across different years. Future studies should employ a more balanced sampling design with larger, evenly distributed sample sizes across seasons.

In conclusion, the study showed that the fecal microbes of varied tits were dominated by Proteobacteria, Firmicutes, Cyanobacteria, Actinobacteriota and Bacteroidota at phylum level. The gut bacterial community composition and diversity of varied tits changed with seasons, which might be a key factor to thriving in changing environments. Increased pathogenic bacterial infections were found in winter but there was no significant correlation between gut microbes and IgA concentration.

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Statements and declarations

Competing interests

The authors declare that there is no conflict of interest.

Ethics approval and consent to participate

The experiments comply with the current laws of China. Fieldwork was carried out under the permission of Liaoning Xianrendong National Nature Reserve. The animal study protocol was approved by the Animal Ethics Committee of Liaoning University (Liaoning, China).

Data availability statement

Data supporting the findings of this study are available from the corresponding authors upon request.

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Appendix

Sampling time and sequencing results of the 31 samples.

Sample	Seq_num	Base_num	Blood samples	Fecal samples	Season	Sampling time
A1	50510	21379620	Yes	Yes	Winter	2018.11
A2	57125	24489082	Yes	Yes	Winter	
A3	55820	23379317	Yes	Yes	Winter	
A4	39460	16883369	Yes	Yes	Winter	
A5	59099	25338863	Yes	Yes	Winter	
A6	44540	19057707	Yes	Yes	Winter	
A7	51840	21359032	Yes	Yes	Winter	
A8	47717	19845588	Yes	Yes	Winter	
B1	47958	19977421	Yes	Yes	Spring	2019.04
B2	58298	23745339	Yes	Yes	Spring	
B3	53371	21754201	Yes	Yes	Spring	
B4	40250	16600595	Yes	Yes	Spring	
B5	58391	23969137	Yes	Yes	Spring	
B6	69945	29944887	Yes	Yes	Spring	
B7	50646	20904361	Yes	Yes	Spring	
B8	67324	27644789	Yes	Yes	Spring	
C1	40750	17410747	Yes	Yes	Summer	2019.06
C2	45790	19478558	Yes	Yes	Summer	
C3	54228	23172975	Yes	Yes	Summer	
C4	55575	22859601	Yes	Yes	Summer	
C5	40162	17125325	Yes	Yes	Summer	
C6	45235	19396224	Yes	Yes	Summer	
C7	52136	21428046	Yes	Yes	Summer	
C8	45329	18571098	Yes	Yes	Summer	
D2	49014	20007057	No	Yes	Spring	2020.04
D1	37296	15189631	No	Yes	Spring	
D3	94930	23995130	No	Yes	Spring	
D8	56586	14296450	No	Yes	Spring	
D5	41781	10570623	No	Yes	Spring	
D4	28754	7277825	No	Yes	Spring	
D6	42433	10749468	No	Yes	Spring	