Dynamics of Bacterial Communities on Eggshells and on Nest Materials During Incubation in the Oriental Tit (*Parus minor*)

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Received: 1 June 2021 / Accepted: 10 November 2021 / Published online: 30 January 2022 © The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2021

Abstract

Eggshell bacterial communities may affect hatching success and nestling’s condition. Nest materials are in direct contact with the eggshells, but the relationships with the eggshell microbiome during incubation have not been fully elucidated. Here, we characterize eggshell and nest material bacterial communities and their changes during incubation in the Oriental Tit (*Parus minor*). Bacterial communities on the nest material were relatively stable and remained distinct from the eggshell communities and had higher diversity and greater phylogenetic clustering than the eggshell communities from the same nest, resulting in lower phylogenetic turnover rate of nest material microbiome during incubation than expected by chance. While the species diversity of both communities did not change during incubation, we found significantly greater changes in the structure of bacterial communities on the eggshell than on the nest material. However, eggshell microbiome remained distinct from nest material microbiome, suggesting independent dynamics of the two microbiomes during incubation. We detected an increase in the relative abundance of several bacterial taxa on the eggshell that likely come from the bird’s skin, feathers, cloaca/intestine, or uropygial secretion which suggests some exchange of bacteria between the incubating bird and the eggshell. Furthermore, incubation appeared to promote the abundance of antibiotic producing taxa on the eggshell, which may hypothetically inhibit growth of many bacteria including pathogenic ones. Our results suggest that the future studies should focus on simultaneous monitoring of absolute abundance as well as relative abundance in communities on eggshells, nest materials, and the incubating bird’s body.

Keywords *Parus minor* · Eggshell · Nest material · Bacterial community · Incubation · Dynamics
Introduction

Since bacterial communities on eggshells may affect hatching success and nestling’s physiological condition [1–3], the function of incubation behavior has been recently expanded from the classical function of controlling the thermal conditions of embryogenesis to manipulating the microbiome communities on eggshells [4–8]. Cook et al. (2005) [9] used a culture-based approach to study the eggshell bacterial communities of a tropical wild bird species, *Margarops fuscatus*, and found a decrease in the percentage of eggs infected with pathogenic bacteria after incubation. Brandl et al. (2014) [6] studied bacterial communities of reed warbler’s eggs and nestlings and found complete removal of hemolytic bacteria after incubation. Using high throughput sequencing, Lee et al. (2014) [8] studied bacterial community structure of eggshells of the Oriental Magpie *Pica serica* and found a decrease in the relative abundance of pathogenic bacterial genera (*Bacillus*) and increase in harmless bacterial genera (*Bacillus*) after incubation.

On the other side, there were efforts made to characterize potential source of eggshell microbiome [9, 10] since eggs are known to be internally sterile at the time when they are laid (shown in hens [11]). Using culture-based methods, Ruiz-De-Castañeda et al. (2011) [12] found a positive correlation between bacterial loads of maternal cloaca and eggshells in the broods of the pied flycatcher (*Ficedula hypoleuca*). Using ARISA fingerprinting, Martínez-García et al. (2016) [13] demonstrated an association between nest materials’ and eggshells’ bacterial communities of hoopoes (*Upupa epops*). Using high throughput sequencing method, Silvia Díaz-Lora et al. (2019) [14] also studied hoopoes (*Upupa epops*) and found difference in the eggs’ bacterial communities of nest boxes with and without old-soft materials. Van Veelen et al. (2018) [10] studied Woodlarks (*Lullula arborea*) and Skylarks (*Alauda arvensis*) and found brood patch skin, feather, and nest material being the most important source of the microbiome of freshly laid eggs rather than maternal cloaca.

Nest materials, especially from the nest cup (in which the eggs are located), have often been assumed to be a potential source of eggshell microbiome because they are in direct physical contact with the eggshells throughout incubation period. However, the dynamics and phylogenetic structure of the bacterial communities on nest materials and eggshells during the incubation period have not been analyzed. Since eggshell microbiome starts to develop when the eggs are laid, it is important to monitor the changes in eggshell microbiome alongside with nest material microbiomes as both may continuously interact. Here, we aimed to understand the dynamics of bacterial communities on eggshells and nest materials as well as interactions between these communities during incubation of the Oriental tit *Parus minor* using a high throughput sequencing technique. Under the general hypothesis that eggshell microbiome and nest material microbiome interact with each other and go through interactive dynamics during incubation by female birds, we aimed to verify the following four predictions that stem from four specific predictions from the following hypotheses.

**Prediction 1: Before the Onset of Incubation, the Bacterial Communities Formed on the Nest Material will Be More Diverse and will Show Stronger Phylogenetic Clustering than the Bacterial Communities on the Eggshells**

We hypothesize (Hypothesis 1) that nest materials of the Oriental tit, which include an underlying layer of green moss with overlaying grasses, rootlets, and animal hair forming the nest cup, contain bacterial communities that have already been established on those materials before they have been brought into the nests. Those communities may be ecologically and taxonomically specific for each type of nest material and each nest’s local habitat. We hypothesize that this would lead to the higher diversity and possibly also stronger phylogenetic clustering (where a group of closely related species shares a trait, or suite of traits, that allow them to persist on a given type of nest materials/in a given local habitat). On the other hand, we expect that a typical eggshell microbiome before incubation has low abundance and low diversity of bacteria that are mainly coming from one source: the egg laying female [9, 10, 12].

**Prediction 2: The Initial Communities of Microbes on the Eggshells and on Nest Materials will Become More Similar to Each Other During the Incubation Period**

As the nest materials and the eggshells maintain direct contact with each other during incubation and because female birds rotate their eggs from time to time during incubation, as well as move inside the nest touching eggs and nest materials, we hypothesize (Hypothesis 2) that the bacterial communities of eggshells and nest materials will become more similar to each other near the end of incubation period.
Prediction 3: There will Be a Higher Proportion of Beneficial Bacteria and Lower Proportion of Pathogenic Bacteria After Incubation in Both of the Communities, on the Eggshells and on the Nest Materials

The consistent presence of an incubating bird promotes growth of beneficial bacteria on the eggshells [6, 8, 9]. We hypothesize (Hypothesis 3) that due to the direct contact between female’s body and the eggshells and the nest cup materials throughout incubation, and due to transition of microbes between eggs and nest materials, beneficial bacteria will be present in higher proportion on both the eggshells and on the nest cup materials after incubation.

Prediction 4: Eggshell Bacterial Communities will Experience Greater Phylogenetic Turnover Compared to Nest Material Bacterial Communities During Incubation

We hypothesize (Hypothesis 4) that a nest material community within a nest remains more stable during incubation and experience less phylogenetic turnover because it is already established on the surface of nest materials before they are transported to the nest and because the main source of new taxa is the relatively less diverse eggshell community on eggs that rest on the nest materials (Prediction 1). On the other hand, eggshell bacterial communities are expected to experience more environmental changes during incubation as eggshell is in contact with the female bird that provides warmth, and by frequently leaving the nest for foraging, the female may bring more microbes from the environment. Additionally, as the typical eggshell microbiome is expected to start with a low abundance and low diversity community, a rapid succession of communities may be expected (mirroring the classical ecological succession) during incubation period. All these processes may result in higher phylogenetic turnover in eggshell communities.

Methods

Study Species and Field Sampling

We used nest boxes distributed on the slopes of Mt. Gwanak (37°27.63091′, 126°56.71676′) and in the campus of Seoul National University (37°27.60460′, 126°57.18539′), South Korea. Mt. Gwanak harbors temperate forests with an annual average temperature of 9°C and an annual average relative humidity of 74% at the summit (https://data.kma.go.kr/cmmn/main.do, Korea Meteorological Administration, 2018). The breeding season of Oriental tits starts from late March until late July. All nest boxes are cleaned before the breeding season starts. The nest is built by male and female who use freshly collected material (i.e., they do not reuse old nest) to make a nest consisting of several layers. First, the nest box bottom is covered with a layer of green mosses that may have a thickness of up to several centimeters. Then,

| Nest ID | GPS coordinates | First swabbed date | Second swabbed date | Number of days between 1st and 2nd sample | Duration of incubation covered by sampling | Mean temperature in nest box during incubation (°C) | Mean humidity in nest box during incubation (%) | Clutch size |
|---------|-----------------|--------------------|--------------------|------------------------------------------|------------------------------------------|-----------------------------------------------|-----------------------------------------------|------------|
| N28     | 37.4465 N 126.952 E | 7–05–2018         | 21–05–2018        | 14                                       | 10 days                                  | 16.78                                         | 89.32                                         | 11         |
| N108    | 37.4481 N 126.953 E | 14–05–2018        | 26–05–2018        | 12                                       | 11 days                                  | 18.41                                         | 75.35                                         | 8          |
| N26     | 37.4464 N 126.951 E | 28–05–2018        | 9–06–2018         | 12                                       | 9 days                                   | 22.37                                         | 64.86                                         | 7          |
| CampJ5  | 37.4584 N 126.953 E | 29–05–2018        | 8–06–2018         | 10                                       | 9 days                                   | 23.59                                         | 55.26                                         | 7          |
| CampL4  | 37.4569 N 126.953 E | 2–06–2018         | 15–06–2018        | 13                                       | 9 days                                   | 21.09                                         | 74.95                                         | 9          |
| CampK1  | 37.4575 N 126.949 E | 3–06–2018         | 15–06–2018        | 12                                       | 9 days                                   | 21.03                                         | 73.65                                         | 8          |
| CampK7  | 37.4576 N 126.956 E | 8–06–2018         | 21–06–2018        | 13                                       | 9 days                                   | 20.6                                          | 78.01                                         | 8          |
| CampA6  | 37.4669 N 126.955 E | 21–06–2018        | 29–06–2018        | 8                                        | 8 days                                   | 22.92                                         | 77.06                                         | 6          |
| CampI4  | 37.4595 N 126.953 E | 21–06–2018        | 4–07–2018         | 14                                       | 9 days                                   | 23.08                                         | 89.4                                          | 7          |
the nest cup is lined with dry grasses and soft materials like animal hair, bird feathers, and artificial soft materials such as yarins. Oriental tits lay 5–12 eggs and start incubation with the production of the last egg or a day or two before the last egg is laid. Incubation lasts for 12–15 days. Only the female incubates, and the male feeds the female during incubation period.

In 2018, we collected samples of eggshell microbes and nest materials from nine breeding nests (Table 1). In the study year, snake predation on nests was so severe that it was not possible to collect more than 9 nest samples. We wore disposable gloves cleaned with 90% ethanol before handling the eggs to prevent contamination. We used sterilized cotton-tipped swabs dipped in sodium phosphate buffer solution (pH 7.2, 0.2 M) to collect eggshell bacterial samples. To obtain sufficient biomass of microbes, we swabbed the surface of randomly selected three whole eggs before incubation started (“before incubation” samples) and a different set of three eggs near the end of incubation period (“after incubation” samples; 1–5 days before expected hatching date). Nest materials were collected from the nest cup right beneath the eggs on the day of the eggshell microbe sampling. Samples were transported to the lab in Seoul National University within 4 h after collection and stored at -20°C, awaiting DNA extraction for less than 3 months.

DNA Extraction, PCR, and Sequencing

DNA was extracted from the eggshell and the nest material samples using the PowerSoil Kit (Qiagen, Carlsbad, CA) following manufacturer’s instruction. Extracted DNAs were shipped to CGEB-IMR (Canada) for polymerase chain reaction (PCR) and high throughput sequencing. PCR targeting bacterial V4–V5 region of 16S rRNA gene was performed using modified 515f (5’-GTGCGACGCMGGCGGTAA-3’) and 806r (5’-GGACTACNVGGGTWTCTAAT-3’) primers described in Walters et al. (2016) [15]. Amplified DNAs were paired-end sequenced (2 x 300 bp) using the Illumina MiSeq platform.

Sequence Processing

Raw fastq sequence files were uploaded to NCBI SRA (sequence read archive) under project ID of PRJNA629431. Paired-end sequence reads were joined together using PAN-DAsq v.2.8 [16]. Sequences were further processed following the MiSeq SOP (https://www.mothur.org/wiki/MiSeq_SOP) using Mothur v.1.42.3 [17]. For sequence alignment and classification, Silva database v.132 [18] was used. Operational taxonomic units (OTUs) were assigned based on 97% similarity using the OptiClust algorithm [19]. Archaeal, chloroplast, and eukaryotic sequences were removed. Sequences were subsampled into 3,123 reads for statistical analysis.

Phylogenetic Analysis on the Community Structure

Maximum-likelihood phylogenetic tree was generated using FastTree v. 2.1.3 [20]. To evaluate phylogenetic clustering in the bacterial community of each sample, we calculated the standardized effect size of the mean nearest taxon distance (SES.MNTD) [21, 22] using the “ses.mntd” function in R package “picante” [23] with 999 randomized null models. The MNTD represents the mean distance separating each species in the community from its closest relative and thus allows to evaluate phylogenetic aspects of the community structure. Standardized effect size of the mean nearest taxon distance (SES.MNTD) describes the difference between phylogenetic distances in the observed community compared to a null community generated by a randomization method and divided by the standard deviation of phylogenetic distances in the null data [22], and negative values of SES. MNTD indicate closely related taxa that co-occurred more often than predicted in the null model.

To assess the community changes (i.e., phylogenetic turnover) during incubation, we determined pairwise evolutionary distances between communities before and after incubation using β-nearest taxon index (βNTI) [22] in the Phylocom software [24]. The values of βNTI within the range from -2 to +2 are interpreted as indicating phylogenetic turnover not significantly different from a null expectation [22], while values below this range indicate significantly lower phylogenetic turnover during incubation than expected by chance.

Statistical Analysis

To compare beta diversity and SES.MNTD of the samples before and after incubation, paired Wilcoxon signed rank test was used. As each variable represents a different specific hypothesis and we were interested in specific sets of comparisons (thus “planned” comparisons, e.g., before vs after incubation, eggshell vs nest materials), we did not use correction of the significance level for multiple comparisons (e.g., Bonferroni correction, Holm-Bonferroni method [25]). To compare OTU richness and Shannon diversity between the four different types of samples (eggshell before incubation, eggshell after incubation, nest material before incubation, nest material after incubation), we used linear mixed effect models and Tukey’s post hoc test with nest ID included as a random variable. Bray–Curtis dissimilarity between samples were calculated based on square root transformed OTU table and visualized through non-metric multidimensional scaling (nMDS) plots using the Primer 6 software [26]. Analysis of similarity (ANOSIM) test was performed using the Primer
6 software [26] with 999 permutations. To test if there is any difference in community composition at phylum level and genus level after incubation, differential abundance analysis [27, 28] were performed using the “DESeq2” package in R [29]. Pseudocount of 1 was applied for each of the samples at both phylum level and genus level to calculate log2-fold change during incubation. p Values were adjusted for differential abundance analysis using the method described by Benjamini and Hochberg (1995) [30]. The overall bacterial abundance data were not collected in this study due to lack or resources, and only the relative abundance of bacterial taxa were compared.

Results

Changes in Bacterial Composition During Incubation

Eggshell bacterial communities were distinct from nest material bacterial communities both before and after incubation (Fig. 1, Table S1). There was a significant difference in the composition of eggshell bacterial communities before and after incubation (Table S1). In contrast, no significant difference was found in the nest material bacterial communities before and after incubation (Table S1). Bray–Curtis dissimilarities of the bacterial communities before and after incubation were higher in eggshells compared to nest materials (Fig. 2a). The eggshell bacterial communities were similarly distinct from the nest material bacterial communities before and after incubation as indicated by no significant difference in their dissimilarities (Fig. 2b).

The most abundant phylum found on eggshells was Proteobacteria, followed by Firmicutes, Actinobacteria, Bacteroidetes, and Planctomycetes (Fig. 3). The major phylum composition was similar in nest material bacterial community, where Proteobacteria was the most abundant phylum, followed by Bacteroidetes, Firmicutes, Actinobacteria, and Acidobacteria. Differential abundance analysis (Table S2) shows that Proteobacteria became significantly less abundant after incubation on the eggshells. On the other hand, Firmicutes and Actinobacteria became significantly more abundant after incubation on the eggshells. There was no significant change in the abundance of bacterial phyla in nest materials.

The most abundant genus found on eggshells was *Psychrobacter*, followed by *Streptomyces, Rhodococcus, Sphingobacterium*, and *Pedobacter* (Fig. 4). On the other hand, the most abundant genus found in nest materials was *Massilia*, followed by *Pedobacter, Flavobacterium, Chryseobacterium, Acinetobacter*, and *Sphingobacterium*. Differential abundance analysis (Table S3) shows a significant decrease in the abundance of *Psychrobacter* during incubation in the eggshell microbiome. In contrast, *Paenibacillus* became more abundant after incubation on the eggshell.

![Fig. 1 nMDS plot showing Bray–Curtis dissimilarities between samples](image-url)
Change in Bacterial Diversity During Incubation

OTU richness and Shannon diversity were lower in the eggshell bacterial communities compared to nest material bacterial communities both before and after incubation (Fig. 5, Table S4–S5). There was no significant change in OTU richness and Shannon diversity during incubation both on the eggshells and in nest materials (Fig. 5, Table S4–S5).

Change in the Phylogenetic Community Structure During Incubation

In all of the samples, closely related taxa co-occurred more often than predicted in the null model (SES.MNTD < 0) (Table S6). The SES.MNTD value was significantly lower in the nest material bacterial communities than in the eggshell bacterial communities before incubation compared with those of eggshell and nest material microbial communities after incubation. Median, thick horizontal line; upper and lower quartiles, upper and lower box edges; minimum and maximum, the end of the lower and upper whiskers; outliers, circles.
signed rank test, W = 0, p = 0.004) incubation (Fig. 6). This indicates that eggshell communities comprised taxa that were phylogenetically more distant from each other than were taxa in the nest material communities.

βNTI values for the nest bacterial communities were overall lower than -2 indicating that their phylogenetic structure remained significantly more stable during incubation than expected by chance (Fig. 7). However, βNTI of the eggshell bacterial communities ranged from -2 to +2, indicating no significant differences in phylogenetic turnover compared to what is expected by chance and suggesting that processes other than phylogenetic relationship among taxa were responsible for shaping the eggshell communities during incubation.

**Discussion**

**Composition of Bacterial Communities**

In this study, we have examined the bacterial communities on eggshells and nest materials. The composition of bacterial community on the eggshell was significantly different that on the nest material at OTU level, although the major phylum composition was rather similar.

The most abundant phylum found in this study was Proteobacteria, which is one of the dominant phyla found in soil [31], phyllosphere [32], potential nest material sources such as moss crusts [33], and also on avian eggshells [7, 435].
Firmicutes, which was significantly more abundant in eggshell samples collected after incubation compared to the ones collected before incubation, is often found in animal’s gut microbiota including that of wild bird species [36]. Considering the lower abundance of Firmicutes in nest materials compared to eggshells, it is possible that the species belonging to Firmicutes, which might have originated from parent bird’s gastrointestinal tract, are competitively dominant over the other taxa on eggshells probably because the temperature on the eggshell is closer to that in gastrointestinal tract (due to incubation). Lee et al. (2014) [8] also found overall higher averaged relative abundance of Firmicutes in incubated eggs and found Bacillus (belonging to Firmicutes), which includes species producing antibiotics [37], being significantly more abundant after incubation. It is however also possible that there is a consistent load of Firmicutes from external sources other than nest materials during the incubation.

The most abundant genus in the eggshell microbiome was Psychrobacter, and they were more abundant on the eggshell before incubation, and their relative abundance decreased significantly with incubation. As its name suggests, they are mostly found in cold environments such as Arctic glacier or Antarctic [38–40]. However, they have also been found in other eggshell studies. Wang et al. (2011) studied Western Bluebird, Tree Swallow, and Violet-green Swallow’s eggs in California and isolated Psychrobacter species from their samples [3]. Grizard et al. (2014) studied homing pigeons in Netherlands and found an OTU that has 99% sequence similarity with Psychrobacter glacinola [7]. Although it has not been discussed in detail in these studies, it seems that Psychrobacter sp. can also be found in temperate environments. Further studies on the sources of Psychrobacter in the eggshells and their survival strategies in an ambient temperature would be interesting.

Massilia, which was found to be the most abundant genus in the nest material microbiome, has been isolated from a variety of environments, encompassing soil, atmosphere, freshwater, phyllosphere, rhizosphere, and in blood samples [6, 41]. They have also been found in hen’s flea (Ceratophyllus gallinae) samples in great tit’s nests [42]. The presence of plants and rootlets in the nest may be related to the abundance of Massilla.

Goodenough and Stallwood (2010) [43] accessed bacterial and fungal composition of blue tit and great tit nest material using culture-based method and found species belonging to Bacillus, Pseudomonas, Enterobacter, and Staphylococcus dominating. Although some of those genera were also detected in our study, their relative abundance was much lower than it was found in Goodenough and Stallwood (2010) [43]. This could be due to the different methods used in the two studies. Animal pathogens have been studied very well and have been cultured for more than decades. In contrast, most of the bacterial taxa found in environmental samples are uncultured or recently culture lacking detailed information. The result from our study suggests that there are much more diverse bacterial groups present in bird’s nest and it is not necessarily dominated by pathogenic taxa.

Another finding in our study is that there is a lack of incubation effect on bacterial diversity even though there is an effect on bacterial composition. Grizard et al. (2014) [7]
found reduction in bacterial diversity after incubation on the eggshells of homing pigeon *Columba livia*. Lee et al. (2014) [8] found the same pattern on the eggshells of Oriental magpie, *Pica serica*. The different pattern found in our study could be due to the ecological difference between studied species, for example, breeding season and the use of different types of nest materials. Below, we discuss the remaining results in the framework of testing of the four predictions (see Introduction).

**Testing the Predictions**

**Prediction 1: Before the Onset of Incubation, the Bacterial Communities Formed on the Nest Material will Be More Diverse and will Show Stronger Phylogenetic Clustering than the Bacterial Communities on the Eggshells**

As expected, nest material bacterial communities had higher diversity compared to eggshell bacterial communities. Also, the co-occurrence of closely related taxa (which have high possibility to share genes with similar functions) was stronger in the nest material microbiome (lower SES. MNTD). These results support the idea that the nest materials contain already established microbiome, whereas the eggshell microbiome did not have sufficient time to be established. However, greater diversity in nest material bacterial communities than that on eggshells remained similar even after incubation. This suggests that, even with direct contact, eggshell microbiome experiences independent change from nest materials (see below).

**Prediction 2: The Initial Communities of Microbes on the Eggshells and on Nest Materials will Become More Similar to Each Other During the Incubation Period**

We found that the eggshell bacterial community does not necessarily become more similar to nest material bacterial community after incubation. In fact, the changes in nest material bacterial communities during incubation were minimal compared to those of the eggshell bacterial communities. The nest materials might have had less chance to be affected by incubation, because they are placed under the eggs and do not interact directly with the female’s brood patch. Another possibility is that the nest materials, as they are not in direct contact with the female’s body, they are maintained at lower temperature than eggshells throughout the incubation period, which may differentially limit the proliferation of certain bacteria. The huge changes in the eggshell microbiome during incubation may imply rapid successional processes occurring during incubation. It could be useful to collect another set of samples in the middle of incubation to have a glance at the spectrum of the changes.

**Prediction 3: There will Be Higher Relative Abundance of Beneficial Bacteria and Lower Relative Abundance of Pathogenic Bacteria After Incubation Both in the Eggshell and Nest Materials**

Overall, we found high variability between the samples in their taxonomic composition. However, we were still able to find *Psychrobacter* and *Paenibacillus* showing significant differences in their relative abundance after incubation. *Psychrobacter* are rare opportunistic human pathogens [44], and their pathogenicity against birds has not been reported. Their relationship with birds has been described only in a limited number of studies [45, 46], so it is difficult to conclude if they are beneficial or harmful to birds in general.

The relative abundance of *Paenibacillus* was higher in the eggshell samples collected after incubation than the ones collected before incubation. Many of *Paenibacillus* species are known to produce antibiotics [47, 48]. Their ability to produce antibiotics might be related to prevention of pathogenic bacterial growth, and it may contribute to overall change in the composition of bacterial communities of eggshells and nest materials. Some of the species belonging to *Paenibacillus* are known to have keratinolytic activity, potentially contributing to bird feather degradation [49]. The increase in *Paenibacillus* after incubation might be originated from the feather of female birds through continuous contact with their eggs and could possibly affect chick’s feather development.

In conclusion, there is no evidence that potentially pathogenic taxa decreased their relative abundance. However, as we did not measure the absolute abundance, we cannot exclude the possibility that indeed the abundance of potentially pathogenic bacteria decreased assuming that general abundance of bacteria might have decreased like in some previous studies [1, 4, 9]. We have evidence suggesting that the proportion of antibiotic-producing bacteria, hence potentially beneficial for birds, increased in the eggshell communities during incubation.

**Prediction 4: Eggshell Bacterial Community will Experience Greater Phylogenetic Turnover Compared to Nest Material Bacterial Community During Incubation**

As expected, the phylogenetic turnover in nest material bacterial communities was minimal. Similarly, with what we mentioned in Prediction 1, this result might be due to their relative “age,” as they have been already developed and established long before the nest materials are transported to the nest during nest building stage. On the other
hand, eggshell bacterial community showed similar level of phylogenetic turnover from what can be expected by chance, implying relatively that new bacterial communities were developing during incubation. This implies that the phylogenetic turnover occurring in eggshell bacterial community is not related with (or influenced by) the dynamics in nest material bacterial community. More likely is that the microbes originating from parents shape the dynamics in eggshell bacterial community during incubation \[10, 12, 50, 51\]. For instance, we found noticeable increase in the proportion of Firmicutes on the eggshell and Firmicutes include species of microbes present in uropygial gland \[52\] gastrointestinal tract \[43, 53, 54\], or feathers \[55\]. It is plausible that the microbes originating from parents, either through contact with cloaca (when laying) or with uropygial gland and feathers (during incubation), are transferred once or continuously to the eggshell and shape the dynamics and phylogenetic turnover in eggshell bacterial community.

**Conclusions**

In summary, we found significant changes in the structure of bacterial communities on eggshells during incubation but not in communities on nest materials. The result from the phylogenetic analysis supports the idea that the minimal changes in nest materials could be due to their relatively long successional history. In contrast to the findings from other studies, we found no evidence of a decrease in the relative abundance of taxa containing pathogenic species, which could be due to the difference in the life history traits of the studied bird species \[56\]. However, the increase in relative abundance of taxa producing antibiotics may be viewed as hypothetically adaptive direction of change of bacterial communities on eggshells because antibiotics may inhibit growth of pathogens. This study demonstrates for the first time the changes in bacterial community structure in the nest materials microbiome during incubation. As there has been no study which characterized bacterial composition of the Oriental tit eggs and nest materials using high throughput sequencing, the results from this study add knowledge for the future comparative analyses across different avian taxa.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00248-021-01927-0.

**Acknowledgements** The authors would like to thank Ms. Yeojoo Yoon for her assistance in field sampling.

**Author Contribution** Hokyung Song: Conceptualization; Investigation; Formal analysis; Writing, original draft; Visualization; Funding acquisition. Keesan Lee, Injae Hwang, Eunjung Yang, Jungmoon Ha, and Woojoo Kim: Investigation (nest monitoring and sample collection). Sungjin Park: Investigation (nest monitoring); Writing, review and editing. Hyunjoon Cho: Formal analysis. Sang-im Lee, Jae Chun Choe, and Piotr Jablonski: Conceptualization; Investigation; Writing, review and editing; Funding acquisition.

**Funding** This work was supported by National Research Foundation of Korea (NRF) via grants 3344–20180017 and 2–2019–1273–001–3, by DGIST Start-up Fund Program of the Ministry of Science and ICT via grant 2021010026, and by the BK Korea 21 (Grant number: 5253–20180100/21A20131212006).

**Data Availability** Raw fastq sequence files were uploaded to NCBI SRA (sequence read archive) under project ID of PRJNA629431.

**Code Availability** Not applicable.

**Ethics Approval** This research was conducted following national and international guidelines. The methods used in this study were reviewed and approved by Institutional Animal Care and Use Committee of Seoul National University (No. SNU-180727-1).

**Conflict of Interest** The authors declare no competing interests.

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