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Intraspecific variation and interspecific differences in the bacterial and fungal assemblages of Blue Tit (*Cyanistes caeruleus*) and Great Tit (*Parus major*) nests

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Abstract

Although interest in the relationship between birds and microorganisms is increasing, few studies have compared nest microbial assemblages in wild passerines to determine variation within and between species. Culturing microorganisms from blue tit (*Cyanistes caeruleus*) and great tit (*Parus major*) nests from the same study site demonstrated diverse microbial communities, with 32 bacterial and 13 fungal species being isolated. Dominant bacteria were *Pseudomonas fluorescens*, *P. putida* and *Staphylococcus hyicus*. Also common in the nests were the keratinolytic bacteria *P. stutzeri* and *Bacillus subtillis*. Dominant fungi were *Cladosporium herbarum* and *Epicoccum purpurascens*. *Aspergillus flavous*, *Microsporum gallinae* and *Candida albicans* (causative agents of, in order, avian aspergillous, favus and candidiasis) were present in 30%, 25% and 10% of nests, respectively. Although there were no differences in nest mass or materials, bacterial (but not fungal) loads were significantly higher in blue tit nests. Microbial species also differed interspecifically. As regards potential pathogens, the prevalence of *Enterobacter cloacae* was higher in blue tit nests, while *Pseudomonas aeruginosa* – present in 30% of blue tit nests – was absent from great tit nests. The allergenic fungus *Cladosporium cladosporioides* was both more prevalent and abundant in great tit nests. Using Discriminant Function Analysis (DFA), nests were classified to avian species with 100% accuracy using the complete microbial community. Partial DFA models were created using a reduced number of variables and compared using Akaike's Information Criterion on the basis of model fit and parsimony. The best models classified unknown nests with 72.5-95% accuracy using a small subset of microbes (n = 1-8), which always included *Pseudomonas agarici*. This suggests that despite substantial intraspecific variation in nest microflora, there are significant interspecific differences – both in terms of individual microbes and the overall microbial community – even when host species are closely related, ecologically similar, sympatric, and construct very similar nests.

Introduction

The complex relationships between birds and microorganisms are increasingly becoming the subject of ecological research [34]. Recent studies have shown that birds have distinctive feather bacteria that influence plumage condition and colouring [10, 11, 52], complex conjunctiva and nasal bacterial assemblages [53] and diet-determined differences in gut bacteria [6, 23]. Microbial loads and assemblages often differ between bird species (e.g. in plumage bacteria: [5]). However, few studies have been conducted on the microbial species associated with the nesting environment of free-living (non-aviary) passerines [4, 38] and consequently understanding of variation in nest bacterial and fungal assemblages within and between species in this environment is limited [26, 55]. Indeed, associations between wild birds and fungi in general are not well researched as the few microbial studies have been undertaken have typically focused on bacteria [12].

Microbial species can interact with birds in many different ways. Some are commensals, living *in vivo* as part of the normal feather or gut flora without apparently affecting their host. Some are avian pathogens, either obligatorily (e.g. *Chlamydia psittaci*) or opportunistically (e.g. *Pseudomonas aeruginosa*). Other microbes, particularly fungi such as *Cladosporium* and *Epicoccum*, have the potential to be allergens [26]. Both pathogenic and allergenic species can act to reduce fitness, making individuals more susceptible to competition and predation, while severe infections/reactions are significant causes of mortality [41]. Conversely, the presence of microbes can be beneficial; for example, *Enterococcus faecium* has been found to increase fitness of pied flycatcher (*Ficedula hypoleuca*) nestlings [39], while *Eupenicillium javanicum* contains the cyclic depsipeptide, Eujavanicin A, the antifungal properties of which are effective against *Aspergillus fumigates,* a cause of avian aspergillois [17, 40]. Keratinolytic microbes (i.e. those that decompose keratin through keritinase production) can also be important in the Aves due to their likely role in feather degradation. Microbe-induced feather degradation can potentially hinder flight (when wing feathers are involved) and mate attraction (when plumage condition or colour is important for mate selection), as well as thermoregulation [10,11, 52]; although the extent and magnitude of such effects is currently unknown [24]. Keratinolytic microbes previously associated with birds include the bacterium *Bacillus licheniformis* [47] and the fungi *Chrysosporium tropicum* and *Microsporum gallinae* [27].

From an evolutionary ecology perspective, it is not surprising that the microbes associated with individual birds often vary substantially between species from different avian lineages [23] or that inhabit very different environments [6]. Likewise, differences in nest microbial communities between species that differ substantially in taxonomy, breeding biology or breeding environment are both expected and explicable [26, 27]. What is currently unclear is the extent to which nest microbial communities differ between

species that are closely-related, sympatric, and that share very similar life history and breeding strategies. Studies of nest ectoparasites have shown there to be comparatively little difference in prevalence or abundance of specific species, such as hen fleas (*Ceratophyllus gallinae*), between closely-related species with similar nesting environments [62], suggesting that nest parasite communities are not always strongly host or situation-specific. A similar comparison for nest microbiota would advance understanding of the ecological co-evolutionary relationships that birds have with other taxa [48], especially when related to avian reproductive success.

In this study, bacterial and fungal species from the nests of blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*) are isolated to establish variations in nest microbial abundance and assemblage within and between these closely-related and ecologically-similar species. This is apparently the first time that a full profile (identification and quantification) of culturable nest microorganisms has been undertaken for these species. Interspecific nest-level differences are then determined using a series of univariate and multivariate statistical analyses and compared to avian reproductive success where possible. Profiling the microbial characteristics of avian nesting environments in this way complements the molecular studies of cloæae bacterial assemblages in parid and brood-parasite nestlings [33, 48] (which assess important genetic and environmental influences on microbial community structure but without identification of the majority of microbial species involved), as well as providing baseline information on avian-microbial ecology to act as a springboard for future research.

Methods

Study site

This study was undertaken at Nagshead Nature Reserve (Gloucestershire, UK, 2°34′0″W, 51°47′0″N), a 200-year oak plantation of English oak (*Quercus robur*). This site is home to the longest-running nestbox scheme in the UK [13], which is managed by the Royal Society for the Protection of Birds (RSPB). In 2006, the nestboxes were occupied most frequently by blue tits (*n* = 143) and great tits (*n* = 49).

Study design

In order to undertake meaningful and powerful research, blue tit and great tit nests were paired to ensure that known or potential environmental variables did not confound analyses of nest microbes. This ensured that any differences in microbiology could be ascribed reliably to interspecific differences. Pairing took account of: (1) type of nestbox (15mm ply-wood, internal measurements, 110 mm wide, 170 mm deep, 210 mm high); (2) orientation of nestbox (a known influence on microbial load: Goodenough and Stallwood, *in prep.*); (3) brood size; (4) time in the season (hatching date ± one day if necessary); and (5) woodland grazing regime (sheep-grazed or ungrazed). It was possible to pair a total of 40 nests in this way, giving a sample size of 20 blue tit and 20 great tit nests. When there was a choice of possible blue tit nests with which to pair a particular great tit nest (or *vice versa*), the nearest nest geographically was selected. This paired sampling design, together with the plantation origins of the study site, which has created two types of very homogeneous habitat (grazed and ungrazed mature woodland), accounted for a large number of potentially confounding variables. Data on two extra environmental factors, which were not built into the study design but could influence findings – height of each nestbox above the ground and the decay status of the tree to which each nestbox was attached (based on a ranking scale of 1-5, with 1 being no decay to 5 being dead standing) – were collected during fieldwork and empirical testing was undertaken to ensure that they did not differ between the blue tit and great tit datasets.

Microbial cultivation

Under licence from English Nature (number: 20060590), each nest was removed from its nestbox within 24 hours of the young fledging and placed in a sterile polyethylene sample bag with an air-tight seal (Fisher Scientific, Loughborough, U.K.) using sterile single-use plastic forceps (Williams Medical Supplies, Rhymney, U.K.). Gloves were worn throughout the nest-collection process.

Once in the laboratory, nesting material was swabbed thoroughly with a sterile rayon-tipped swab moistened with phosphate buffer (Steriswab™, Medical Wire and Equipment, U.K) for 30 seconds. This process was undertaken using a full aseptic technique within an ethanol-sterilised class 100 Laminar Flow Hood (Labcaire VLF6, Clevedon, U.K.), which provided a BS5726-accredited class A sterile environment. Immediately after swabbing, swabs were washed in 10ml of sterile 1.3% (w/v) nutrient broth

(Oxoid, Cambridge, U.K.). This process was felt to be more rigorous than taking a small amount of nesting material and washing this in nutrient broth directly [e.g. 55], since that approach assumes that microbes are uniformly distributed in the nest. However, it should be noted that the swab method of sampling could bias analyses toward microbes that are easily removed from the nesting material. The possibility of underestimating fungi using this sampling method (because of the potential for stronger attachment to the nest material by hyphae) is unlikely for species that were sporulating, since spores are usually easily detachable from hyphae, but cannot be discounted for species that explicit culturable viability following mycelial fragmentation [21]. Two air swabs (swabs exposed to the air for the same time as the swabbing procedure) were taken as experimental controls [32]. These were processed in the same way as the study swabs to check for contamination – these checks were negative at all stages of the culturing procedure.

Serial dilutions of the broth were undertaken down to 10^{-9} and a 10μl drop of each dilution was plated onto 2.8% (w/v) nutrient agar at pH 7.4 and incubated at 28°C for 24 hours. For each nest sample, 100μl of the two most suitable dilution factors (that with around 30 colonies per 10μl drop and the dilution immediately below this: typically 10^{-8} and 10^{-9} for nest swabs and 10^{0} for control swabs) was cultured. The choice of comparatively high dilution factors was deliberate to reduce any bias towards fast-growing species by reducing inter-isolate competition on the plate. Culturing was undertaken on nutrient agar to encourage bacterial growth and 3.9% (w/v) potato dextrose agar (PDA) at pH 5.6 to encourage fungal growth. Plates were incubated for 7 days at 28°C [51] before colony forming units (CFUs) were counted to ensure that any bias towards fast-growing species was limited. Plates were re-examined after 21 days and any additional CFUs were documented – this occurred in two cases only.

Microbial identification

Identification of fungi (including yeasts) was undertaken taxonomically on the basis of macroscopic and microscopic characteristics according to standard keys [16, 31, 56]. Identification of two cryptic isolates was verified by a specialist mycologist at CABI Bioscience (Nomica, Egham, Surrey, U.K.). In both cases the specialist identifications, which were undertaken on a blind basis, matched the initial identification made by the authors.

Bacteria were identified using automated fatty acid methyl ester (FAME) analysis using gas-liquid chromatography. This determined the types and concentrations of fatty acids in a bacterial colony and compared the resulting profile to over 200,000 identified isolates [30]. This process was undertaken through the Sherlock® Microbial Identification System (MIDI Inc., Newark, Delaware) using the Sherlock Rapid Methods® technique and the RTSB50 (environmental isolates) reference library. FAME and Sherlock® systems are validated identification methods have been widely used to successfully identify environmental bacteria [29, 44, 58], including isolates from bird feathers when the method produced results

that were comparable to DNA identifications [51]. Before analysis, isolates were sub-cultured on 4% (w/v) Trypticase Soy Broth agar and incubated at 28°C for 24 hours to ensure a typical FAME profile. Similarity indices of ≥ 0.500 (excellent species-level match between sample and reference isolates: [63]) were used as the demarcation for positive identification. Where two or more possible matches were given, the closest match was accepted provided that the similarity index separation between this and the second match was ≥ 0.200 (double the minimum recommended separation: [30]). Because atypical strains of fluorescent *Pseudomonas* spp. in ribosomal Ribonucleic Acid (rRNA) similarity group 1 [43] can occasionally be confused using FAME analysis [42], identifications of these isolates were verified by growth at high (42°) and low (3°C) temperatures and by their ability to hydrolyse gelatin [25].

When a FAME profile indicated marginal species match (similarity index < 0.500; n = 5 isolates), identification was confirmed by sequencing the 16S rRNA gene. DNA was extracted using a commercial kit (DNeasy® DNA extraction kit: Qiagen, Sussex, U.K.) following thermic shock. The required gene was amplified using Polymerase Chain Reaction (PCR) in a 50μl reaction containing 25μl Taq Master Mix (Qiagen), 21μl deionised water, and 2μl each of two oligonucleotide primers 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 530r (5'-ATTACCGCGGCTGCTGGC-3') (Operon, Cologne, Germany). The PCR conditions comprised an initial step of 15 minutes at 95°C, followed 30 cycles of 1 minute at 95°C, 1 minute at 56°C, and 1.5 minutes at 72°C [60]. This was followed by a final extension step of 10 minutes at 72°C. The amplified 16S rRNA gene was separated from the remaining DNA using submerged horizontal gel electrophoresis at 100 volts for 45 minutes using 1X TBE running buffer (Eppendorf, Cambridge, U.K.). The PCR bands were cut from the gel and DNA was extracted using a commercial gel extraction kit (QIAquick® Gel Extraction Kit: Qiagen) and 100% isopropanol. Sequencing was undertaken by MWG Biotech (Ebersberg, Germany). In all cases, the DNA sequencing analysis matched the original FAME identification, suggesting that the latter was a suitable and reliable identification method for the isolates in this study, as previously found for bird feather microbiota [51].

Nest analysis

After microbial analysis, the composition of each nest was established. Nests were thoroughly teased apart using forceps and each material present was identified (different species of moss, animal hair, etc.). The abundance of each material was then quantified using the DAFOR ranking scale (Dominant, Abundant, Frequent, Occasional or Rare). Nest weights were also recorded.

Avian reproductive success

Data on chick survival was collected by RSPB volunteers by means of regular nestbox visits. These data were made available so that microbial data could be related to avian reproductive success.

Statistical analysis

To quantify any interspecific differences in nest bacterial and fungal loads, paired *t*-tests were used after count data had been log (*ln*+1) transformed to achieve normalisation. Paired *t*-tests were also used to establish whether obvious differences in the abundance of individual microbial species were significant (the test was not applied without *a priori* reason to avoid pseudo-significance: [20]. To determine any significant associations between the prevalence of specific microbes and avian species, Fisher's exact test was used.

To establish whether the microbial community could be used to classify nests according to bird species, Discriminant Function Analysis (DFA) was used. This was undertaken on the basis that a high level of classification accuracy was good evidence of substantial interspecific differences, rather than simple intraspecific variation. This community-level approach thus complemented analyses of the prevalence and abundance of individual microbes by assessing interspecific differences in overall nest microbial assemblages. DFA was run using abundance data for all bacterial and fugal isolates, thus ensuring that both between-nest presence and within-nest abundance data could be utilised. A full DFA was calculated using all isolates that occurred in more than one nest (n = 39). The classification power of this DFA was ascertained using a jackknife cross-validation procedure, such that the model was repeatedly calculated with the omission of a different single case, which was then classified [49]. In this way, power was tested using a different datapoint to those that generated the model. This procedure was used as the comparatively small sample size precluded use of the preferred split-sample validation process [35]. It should be noted that the results of this full DFA should be interpreted with caution as the recommended case:variable ratio of 3:1 was exceeded given the large number of predictors [57].

To determine which isolates were the most important in creating community-level differences, multiple DFAs were constructed and examined using an Information-Theoretic (IT) approach based on Akaike's Information Criterion (AIC) [2, 9]. This post-hoc model selection process is based upon combining model fit (based on log-likelihood, which is related to the Kullback-Leibler distance) and parsimony (the number of explanatory variables in the model; K). Use of the IT-AIC framework to determine the relative importance of different models created using different variables from a given dataset is considered by many to be superior to calculating models using a stepwise procedure because of the inconsistencies between different stepwise model selection algorithms [66].

Here, the AIC value of each model ($_i$) was computed using equation 1, after which the amount of empirical evidence (Δ) was calculated by comparing model AIC values using equation 2:

$$AIC = -2(\text{log-likelihood}) + 2K \qquad \textit{(equation 1)}$$

$$\Delta_i = AIC_i - AIC_{min} \qquad \textit{(equation 2)}$$

Models that had low Δ values were considered superior to those with high Δ values using a relative scoring system [9]: Δ of 0-2 = very strong support; 3 = strong support; 5-9 = considerably less support; > 10 = essentially no support. The classification power of DFA models with low Δ values was calculated and a Multivariate Analysis of Variance (MANOVA) was undertaken to establish whether differences between the groups were significant. Only models that had 16 variables or fewer were assessed to ensure that a maximum case:variable ratio of 3:1 was maintained. In all cases, the assumption of homogeneity in the variance-covariance matrix was tested using Box's *M*-test: this was always met.

Results

Potentially confounding factors

There was no difference in the height of nestboxes above the ground or the decay status of the tree between the blue tit and great tit datasets (mean height of nestboxes used by blue tits = 3.01m, height of nestboxes used by great tits = 3.06m, independent *t*-test $t = -0.455$, d.f. = 38, $P = 0.658$; decay rank of trees supporting all boxes used by blue or great tits in the study year = 2). Other potentially confounding variables were accounted for in the paired study design (see Methods).

Nest analysis

There was no significant difference between the weights of nests between blue tits and great tits (means 50.76g and 54.85g, respectively; $t = -1.239$, d.f. = 38, $P = 0.223$). All nests were dominated by wood hair moss (*Polytricum formosum*) and contained abundant Tamarisk moss (*Thuidium tamariscinum*) and ordinary moss (*Brachythecium rutabulum*). All nests contained a small amount of badger (*Meles meles*) hair. Two thirds of the nests contained abundant sheep's wool. There was no significant difference in the prevalence of sheep's wool (the only material to vary between nests in either presence or abundance) between the two species (blue tit = 14 nests, great tit = 12 nests; $\chi^2 = 0.154$, d.f. = 1; $P = 0.695$).

Microbial species

In total, 32 culturable bacterial species and 13 culturable fungal species were isolated from the nests (blue tit = 23 bacteria and 12 fungi; great tit = 28 bacteria and 11 fungi). Nests of both species contained bacteria belonging to the genera *Bacillus, Enterobacter, Escherichia, Pseudomonas* and *Staphylococcus,* while great tit nests also supported *Aeromonas, Paenibacillus* and *Roseomonas* bacteria (Fig. 1). Both blue and great tit nests were dominated by *Pseudomonas fluorescens* biotype B (70% and 65% of nests, respectively), *P. putida* biotype B (45% and 40% of nests, respectively) and the potential pathogen *Staphylococcus hyicus* (40% and 25% of nests, respectively). Fungi isolated from nests of both species belonged to the genera *Arthrinium, Aspergillus*, *Candida*, *Cladosporium, Epicoccum*, *Microsporum* and *Trichoderma* (all Ascomycetes) as well as *Mucor* (a Zygomycete). *Chrysosporium* fungi were also found in great tit nests (Fig. 2). The nests of both species were dominated by *Epicoccum purpurascens* (formerly *E. nigrum*) (95% of nests) and *Cladosporium herbarum* (40% of nests). This is apparently the first time that many of the bacteria and fungi isolated have been reported in avian nesting environments (Figs 1 and 2).

Differences in nest microbial load between avian species

Although there was considerable variability between individual nests of the same species (Figs 1 and 2), blue tit nests had a higher average microbial load than great tit nests (blue tit mean = 4.4×10^{12} CFUs per nest, great tit mean = 2.5×10^{12} CFUs per nest; paired *t*-test $t = 2.194$, d.f. = 19, $P = 0.041$; Fig. 3). This can be attributed to a significantly higher culturable bacterial load in blue tit nests compared with

great tit nests (means = 3.2 x $10^{12}$and 1.2 x 10^{12}, respectively; paired *t*-test $t = 2.052$, d.f. = 19, $P = 0.045$). There was no significant difference in fungal load between the nests of blue and great tits (means = 1.3 x 10^{12} and 1.4 x 10^{12}, respectively; paired *t*-test $t = -0.548$, d.f. = 19, $P = 0.590$). There was no relationship between total microbial load and the number of chicks per nest (Pearson correlation: $r = .096$, $n = 40$, $P = 0.555$). As expected given the lack of interspecific difference in nest mass, all significant results reported here remained unchanged when absolute microbe abundance data was substituted for relative abundance data (i.e. number of CFUs divided by nest mass).

Differences in nest microbial assemblages between avian species

Four bacterial and two fungal species were found only in blue tit nests, while nine bacterial and one fungal species were found only in great tit nests (Figs 1 and 2). The majority of the microbes only found in the nests of one species were comparatively uncommon, occurring in 10% of cases or fewer. The exceptions were *Staphylococcus lentus* (which occurred in 25% of great tit nests and no blue tit nests) and *Pseudomonas aeruginosa* (which occurred in 30% of blue tit nests and no great tit nests). Of the microbes that occurred in the nests of both species, there was a significantly higher prevalence of two bacteria (*Enterobacter cloacae and Pseudomonas agarici*) in blue tit nests compared to great tit nests and a significantly higher prevalence of one bacterium (*P. varonii*) and one fungus (*Cladosporium cladosporioides*) in great tit nests compared to blue tit nests (Table 1).

There were no differences in the abundance of any bacterial species between blue and great tit nests, despite significantly higher overall bacterial loads in the former. However, one fungal species, *C. cladosporioides*, occurred in significantly higher abundances in great tit nests (mean abundance in blue tit nestboxes = 37 x 10^9, mean abundance in great tit nests = 333 x 10^9; paired *t*-test $t = -2.871$, d.f. = 19, $P = 0.010$). This was calculated only using the nests inhabited by *C. cladosporioides*, such that the test was not skewed by the unequal prevalence of this fungus (see above).

DFA proved an extremely useful method of assigning nests to the correct avian species according to their microbial community. A full DFA, run using all bacterial and fungal species, classified unknown cases to the correct avian species with 100% accuracy. However, this full DFA model was far from parsimonious, as reflected by very high AIC and Δ values (Table 2). Of a multitude of partial DFAs, nine were created that had low Δ values (≤ 4) and that were separated from all other models by a Δ difference of > 14, each of which contained between one and eight microbial variables. Of these nine models, seven had good classification accuracy (> 70%) and significant MANOVAs, which suggested that the interspecific differences were highly significant ($P < 0.003$ in all cases) (the remaining two models with low Δ values only just classified more cases correctly than expected by random chance (52.5% and 55%) and were

associated with non-significant MANOVAs: see Table 2). The significance of the MANOVAs reported here remained unchanged when Bonferroni corrections were applied to account for non-independence of the tests between the models (family-wise error). The trade-off that exists in AIC between model fit and parsimony is evident in Table 2, with the best (lowest) Δ value coming at the mid-point between classification accuracy and the number of variables in the model (Model 5).

Relating microbial data to avian reproductive success

The extent to which avian reproductive success could be related to nest microbial data was extremely limited as the breeding success during the study was very high. Total chick mortality (i.e. the loss of an entire brood) occurred at one (blue tit) nest following parental predation. Partial chick mortality occurred in just one (great tit) nest. This nest had a higher than average microbial load (CFU = 2.7×10^{12} compared to a mean of 1.5×10^{12}) and the highest abundance of the allergenic fungus *Ep. purpurascens* (CFU = 150×10^{10} compared to a mean of 80×10^{10}).

Discussion

The most commonly occurring bacterial genus isolated from the nests of both species was *Pseudomonas*, which occurred in high numbers in all nests, followed by species of the genera *Bacillus* and *Staphylococcus*. These genera have previously been associated with birds or their nesting environments [4, 5, 36, 38] and were the genera most often associated with house wren (*Troglodytes aedon*) nests in Illinois, USA [55]. The most abundant *Pseudomonas* species were *P. fluorescens* biotype B and *P. putida* biotype B. This is seemingly the first time that *Pseudomonas* isolates from avian nesting environments have been identified to species level. However, *P. fluorescens* has been isolated from feathers of the Eastern bluebird (*Sialia sialis*) in America [51], from pharyngeal swabs of alpine accentors (*Prunella collaris*) in Slovakia [28] and from fæces of wild birds in Wisconsin, USA [7]. It is interesting to note that *Pseudomonas* spp. dominated the nest samples, since *Bacillus* spp. has been found to dominate feather samples using similar culture techniques to those used here [51].

Associations between wild birds and fungi are not well researched [12]. This is surprising as this study indicates that fungi are a substantial part of all nest microbes, comprising around 38% of all microbial colonies. Moreover, the fungal community was diverse: 13 different species were identified, such that more than a quarter of the 45 microbial species isolated from blue and great tit nests were fungi. Of the dominant fungal species, *Epicoccum purpurascens* and *Cladosporium herbarum*, only the latter has been found previously in bird nests [26].

The microbial diversity of the nests probably stems from the number of different sources of microorganisms. Some microbes are likely to have originated from the birds themselves. These include plumage fungi such as *Chrysosporium tropicum* and *Microsporum gallinae* [27] and gut bacteria including *Enterobacter cloacae* [1]. The moss in the nest would probably have been a large source of microbes; for example *Pseudomonas syringae* is normally isolated from vegetation [60]. Introduction of non-vegetative material into the nest could also have an impact. For example, *Staphylococcus lentus*, which is often isolated from sheep [18], was found only in great tit nests containing sheep's wool. Microbes might also occur in association with the nestbox (e.g. *Ep. purpurascens*, which often grows on wood: [37]). Information on the origins of microbes within overall avian nesting environments is an important avenue for future research that would further understanding of bird-microbe interactions.

It is important to note that this study has identified microbial species through culture-based methods and thus the microbes, and particularly the bacteria, discussed here will only be a subset of those actually present [3]. Given how few studies have been undertaken, it is not known what the ratio of culturable to nonculturable microbes is in avian nest material. However, culture-based and culture-independent techniques have previously revealed different microbial communities on bird feather samples [51].

Accordingly, the current study should not be taken as a full per-nest microbial profile, simply a profile of culturable species. It should also be noted that of the culturable species present, only those that could initiate and sustain growth on the generalist media used here (see methods), excluding strict anærobes, would be recorded. As noted above, the potential for underestimation of fungal species capable of growing following mycelial fragmentation cannot be discounted. Although these limitations and potential biases are important to note, most are of limited importance when comparing relative microbial load and assemblages interspecifically since they would likely affect all nests equally.

Pathogenic microbes

Pathogens in the nesting environment can have a significant impact on offspring survival at embryonic and nestling stages [15, 46]. The most prevalent pathogenic bacteria in the nests of both avian species were *S. hyicus* and *E. cloacae.* The former can cause conjunctivitis in poultry [53], while the latter is a common pathogen of black-bellied whistling ducks (*Dendrocygna autumnalis*) [1]. *Escherichia coli* was also found in nests of both parid species. Three common pathogenic fungi were found: *M. gallinae*, which can cause favus (ringworm) in poultry [19]; *Ca. albicans*, which can cause candidiasis and death in young sparrows (*Passer domesticus* and *P. montanus*) [22, 46]; and *A. flavous,* an important cause of avian aspergillosis and aflatoxicosis [68].

Keratinolytic microbes

Four keratinolytic microbes were isolated; two bacteria (*Pseudomonas stutzeri* and *Bacillus subtilis*) and two fungi (*Ch. tropicum* and *M. gallinae*) [27, 50, 65]. By digesting the β-keratin in avian plumage, feather-degrading microbes can potentially decrease bird fitness. Keratinolytic microbes could thus potentially have an important affect on young birds during feather production (i.e. the time when alticial birds are in the nest). *B. subtilis* has been also implicated in embryo mortality in the eggs of house and tree sparrows [46].

Differences in nest microbial load between avian species

The bacterial load (and thus total microbial load) of blue tit nests was significantly higher than that of great tit nests. This was not as a result of differences in the amount or type of nesting material used since these factors did not differ between species. The number of chicks per nest is also unlikely to have influenced this since: (1) this was controlled for in the sampling, with boxes containing a given number of young being included equally as often in the great tit sample as the blue tit sample; and (2) there was no relationship between microbial load and number of offspring. This suggests that blue tits either introduce more bacteria into the nest (e.g. on their feathers) than great tits or that bacterial accumulation within the nest during the course of breeding occurs to a greater extent. The latter could occur with differences in nest microclimate, particularly temperature, arising from differences in external environment or brooding behaviour. Less effective nest sanitization, particularly in the removal of fæcal sacks, is also possible. The

abundance of ectoparasites in the nest structure could also influence bacterial load as parasites have their own microflora [61]. Both birds are sedentary and have similar life-history traits, although diet is more generalist in blue tit populations when co-occurrence with great tit populations occurs (see below), such that they might be exposed to a higher microbial load in their diets. Attempting to disentangle these influences – for example by swabbing nests *in-situ* prior to egg laying and again post-fledging – would be a useful avenue for future research.

Differences in nest microbial assemblages between avian species

The microbial assemblages in blue and great tit nests differed substantially; varying in terms of presence, prevalence and per-nest abundance. Of particular interest are differences in two potential pathogenic bacteria; *E. cloacae* (more prevalent in blue tit nests) and *P. aeruginosa* (only present in blue tit nests). The difference in *E. cloacae* prevalence could be due to differences in diet. Diet is a key factor in the assemblage of gut bacteria, both within and between avian species [6, 23]. Differences in diet were also hypothesised as the reason why cloacal bacterial communities differed between blue and great tit chicks raised in their own nest [33]. Although both parids feed their young predominantly on caterpillars, the actual species can differ. Moreover, in sympatric populations (as here), blue tits become more generalist foragers and feed nestlings prey such as spiders [59]. The difference in the presence of *P. aeruginosa*, which was only found (in high prevalence) in blue tit nests is harder to explain. However, as this species thrives in moist conditions, it could relate to the moisture content of the nests that may be higher in blue tit nests as parents frequently add fresh plant material frequently throughout the nesting progress [45], an activity that has not been documented for great tits. *Pseudomonas aeruginosa* can penetrate eggshells causing embryo death, and can cause localized or systemic disease in newly-hatched chicks, as well as sinusitis and conjunctivas in adults [14, 53]. Virulent strains can cause dehydration, dyspnea, septicæmia and death [64]. Depending on the relative susceptibility of blue and great tit chicks to diseases caused by *E. cloacae* and *P. aeruginosa,* higher prevalence might cause higher rates of nestling morbidity/mortality. Although there is no evidence for this here given the very high reproductive success during this study, it could be a condition-dependant effect, such that increased morbidity or mortality would become evident in seasons with poor weather or prey abundance. Such condition-dependant effects have been found previously with regard to parasitism [e.g. 54]. Alternatively, it is possible that exposure to potential pathogens at an early age might allow individuals to develop antibodies to reduce the incidence of microbial-induced disease in adulthood. The same is true for increased exposure to the allergenic *Cladosporium cladosporioides* fungus, which found in greater prevalence and abundance in great tit nests. It would be interesting to compare levels of Immunoglobulin E for blue and great tit chicks with reference to exposure to this fungus, and also for *Ep. purpurascens,* which was found in particularly high numbers in the only nest that suffered partial brood mortality.

The differences in the overall microbial community allowed the nests to be classified according to avian species with 100% accuracy on the basis of the presence and abundance of all microbial species present in at least one nest (Table 2). This demonstrates that in addition to variation in the presence, prevalence and abundance of individual microbes, there are community-level interspecific differences too. The partial DFAs reveal that these community-level differences are based on a few key microbes, including *P. agarici*, which seem to be indicative of the overall microbial assemblage. The difference in microbial community blue and great tit nests parallels the situation for bacterial differences in the cloæae of blue and great tit chicks [33]. However, whether this difference is caused by the same bacteria cannot be determined, since the methods used to profile microbial communities in the aforementioned study did not allow identification of individual species.

Implications of this study

This study suggests that despite substantial intraspectific variation in the microbial load and assemblage of individual nests, there are still significant interspecific systematic differences, even when the avian species concerned belong to the same taxonomic family, are sympatric, are ecologically-similar, and construct similar nests that do not vary significantly in mass or materials. The evolutionary reasons for such distinct microbial patterns, both at the level of the individual microbe and the level of the complete microbial community, and the affect these differences have on life history traits such as reproductive success need to be investigated more fully as part of the evolving research area of ornithological microbiology.

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Table 1: Microbial species that differ in prevalence significantly between blue tit and great tit nests.

Species	**Prevalence in blue tit nests**	**Prevalence in great tit nests**	**Significance of difference (Fisher's exact test)**
Enterobacter cloacae	35%	5%	*P* = 0.044
Pseudomonas agarici	55%	10%	*P* = 0.006
Pseudomonas varonii	5%	40%	*P* = 0.020
Cladosporium cladosporioides	75%	20%	*P* = 0.010

Table 2: Ten discriminant function analysis models created to classify nests to the correct avian species (blue tit or great tit) on the basis of microbial communities. The classification accuracy of the model was determined on the basis of cross-validation following jackknifing (see methods). The significance of model is reported using a MANOVA.

Model	Species included in model	AIC[a]	Δ_i [b]	Cross-validated cases classified correctly	MANOVA
Complete[c]	All	80.000	72.287	100.0%	P < 0.0001
Partial (low Δ, but also low number (< 70%) of cross-validated cases classified correctly and non-significant MANOVAs)					
1	*Pseudomonas chlororaphis*	8.100	0.387	55.0%	*P* = 0.154
2	*Pseudomonas fluorescens* (biotype D)	8.978	1.265	52.5%	*P = 0.156*
Partial (low Δ, high number (>70%) of cross-validated cases classified correctly and highly significant MANOVAs)					
3	*Pseudomonas agarici*	10.286	2.573	72.5%	*P* = 0.003
4	*P. agarici* *P. chlororaphis* *Cladosporium cladosporioides*	11.221	3.498	80.0%	*P* < 0.001
5	*P. agarici* *P. chlororaphis* *C. cladosporioides* *P. fluorescens* (biotype D)	7.713	- - - -	85.0%	*P* < 0.001
6	*P. agarici* *P. chlororaphis* *C. cladosporioides* *P. fluorescens* (biotype D) *Candida albicans*	8.713	1.000	87.5%	*P* < 0.001
7	*P. agarici* *P chlororaphis* *C. cladosporioides* *P. fluorescens* (biotype D) *Ca. albicans* *Staphylococcus lentus*	9.003	1.290	90.0%	*P* < 0.001
8	*P. agarici* *P. chlororaphis* *C. cladosporioides* *P. fluorescens* (biotype D) *Ca. albicans* *S. lentus* *P. aeruginosa*	10.713	3.000	92.5%	*P* < 0.001
9	*P. agarici* *P. chlororaphis* *C. cladosporioides* *P. fluorescens* (biotype D) *Ca. albicans* *S. lentus* *P. aeruginosa* *Bacillus* sp.	11.713	4.000	95%	*P* < 0.001

[a, b] See methods for full details and calculation equations

[c] Should be interpreted with caution as the minimum recommended case:variable ratio was exceeded

Figure legends

Figure 1: Bacterial species isolated from blue and great tit nests. Abundance is presented on the box plots (vertical black bar = mean, horizontal bar = range). Prevalence is given as a percentage of nests in each species group (*n* = 20) containing each bacterial species. [a,b] Bacteria seemingly not previously identified in avian nesting at genus or species level, respectively, are indicted by a plus sign. This is based on information synthesized from [4, 36, 38, 55, 64] and reviews [8, 67]

Figure 2: Fungal species isolated from blue and great tit nests. Abundance is presented on the box plots (vertical black bar = mean, horizontal bar = range). Prevalence is given as a percentage of nests in each species group (*n* = 20) containing each fungal species. [a,b] Fungi seemingly not previously identified in avian nesting at genus or species level, respectively, are indicted by a plus sign. This is based on information synthesized from reviews [26, 27, 67].

Figure 3: Differences in the mean number of colony forming units (CFUs) between blue tit and great tit nests for overall microbial load, bacterial load and fungal load (error bars = standard error of the mean).