Running head: Antimicrobial activity in avian nests

Antimicrobial activity of nest-lining feathers is enhanced by breeding activity
in avian nests

Cristina Ruiz-Castellano¹, Magdalena Ruiz-Rodríguez², Gustavo Tomás¹ and Juan José
Soler¹

¹Departamento de Ecología Funcional y Evolutiva, Estación Experimental de Zonas Áridas (CSIC), Ctra. Sacramento s/n, La Cañada de San Urbano, E-04120 Almería, Spain.

²Biologie Integrative des Organismes Marins, Observatoire Océanologique, Sorbonne Universités, Avenue du Fontaulé, 66650, Banyuls-Sur-Mer, France.

* For correspondence: e-mail: ruizcastellano.c@gmail.com

Keywords: Antimicrobial activity, eggshell bacterial load, feather nest material, incubation behavior, keratinolytic bacteria, Spotless Starling.

Abstract

The use of feathers as nest material has been proposed as a kind of self-medication strategy because antimicrobial-producing microorganisms living on feathers may defend offspring against pathogenic infections. In this case, it is expected that density of antimicrobial-producing bacteria, and their antimicrobial effects, are higher in feathers that line the nests than in eggshells. Moreover, we know that feather pigmentation and breeding activity may influence density and antimicrobial production of bacteria. To test these predictions, we analyzed bacterial densities and antimicrobial activity of bacterial colonies isolated from bird eggshells and nest-lining feathers against bacterial strains comprising potential pathogens. Samples were collected from spotless starling (*Sturnus unicolor*) nests, and from artificial nests to isolate the effects of breeding activity on bacterial communities. The composition of feathers lining the nests was experimentally manipulated to create groups of nests with pigmented feathers, with unpigmented feathers, with both types of feathers or without feathers. Although we did not detect an effect of experimental feather treatments, we found that bacterial colonies isolated from feathers were more active against the tested bacterial strains than those isolated from eggshells. Moreover, bacterial density on feathers, keratinolytic bacteria on eggshells and antimicrobial activity of colonies isolated were higher in starling nests than in artificial nests. These results suggest that antimicrobial activity of bacteria growing on nest-lining feathers would be one of the mechanisms explaining the previously detected antimicrobial effects of this material in avian nests, and that breeding activity results in nest bacterial communities with higher antimicrobial activity.

Introduction

Animals are infected by numerous parasites of different taxa including arthropods, helminths, bacteria and fungi (Loye and Zuk 1999; Goater *et al.* 2001; Benskin *et al.* 2009). By definition, parasites reduce survival prospects and fitness of their hosts (Price 1980) and select for a plethora of defensive strategies to reduce the probability of infection and/or the negative effects of parasitism (Schmid-Hempel 2011). One of those strategies is the use of compounds produced by other organisms for protection against pathogens, called self-medication (sensus Clayton and Wolfe 1993). This strategy is widespread in nature and has recently received special attention, not only because of its relevance on the evolution of parasites and hosts, but also because of its promising implications regarding animal and human health (De Roode *et al.* 2013).

Self-medication behaviors have prophylactic and/or therapeutic functions (Lozano 1998; Hart 2005; De Roode *et al.* 2013). Green plants are one of the materials more commonly used by animals for self-medication, mainly due to the abundance and diversity of secondary metabolites, including volatile compounds that repel and/or kill parasites (Jacobson 1982; Clark and Mason 1985; Scott-Baumann and Morgan 2015). Birds use green materials in their nests to reduce infections by ectoparasites (Dubiec *et al.* 2013) and microorganisms (Clark and Mason 1985; Gwinner and Berger 2005; Mennerat *et al.* 2009). They also use antimicrobial-producing microorganisms. This is for instance the case of common hoopoes (*Upupa epops*) that host antibiotic-producing bacteria in their uropygial gland, which prevent trans-shell bacterial infection of their eggs (Soler *et al.* 2008; Martín-Vivaldi *et al.* 2010; 2014) and degradation of their feathers by pathogenic microorganisms (Ruiz-Rodríguez *et al.* 2009).

The use of antimicrobial-producing bacteria may be widespread in birds, for instance by using nest materials that differentially favors growth of microorganisms with antimicrobial properties (Soler *et al.* 2010). Particular attention has been given to the possible antibacterial function of feathers in avian nests (Peralta-Sánchez *et al.* 2010; 2011; 2014; Ruiz-Castellano *et al.* 2016; Soler *et al.* 2017), which was predicted due to known antimicrobial properties of most bacterial strains that are able to degrade keratin (Soler *et al.* 2010), the main component of feathers. The microbiome of feathers is relatively complex and depends on several environmental conditions (Shawkey *et al.* 2005; Bisson *et al.* 2007; Dille *et al.* 2016). Some of the bacteria found in feathers of wild birds are those belonging to the genus *Bacillus* (Burt and Ichida 1999; Whitaker *et al.* 2005; Kent and Burt 2016) and *Pseudomonas* (Grizard *et al.* 2015), for which antimicrobial properties are well known (Abriouel *et al.* 2011; Kumari 2014). Thus, by using feathers as nest lining material, birds could influence bacterial environments where offspring develop due to antagonistic activity of keratinolytic microbiota, which could be directed towards potentially pathogenic bacteria (Soler *et al.* 2010). There is experimental and correlational evidence of beneficial effects of the use of feathers as nest-lining material in terms of reduction of bacterial loads in eggshells of barn swallows (*Hirundo rustica*) (Peralta-Sánchez *et al.* 2010; 2011) and spotless starlings (*Sturnus unicolor*) (Ruiz-Castellano *et al.* 2016). However, antimicrobial activity of bacteria growing in nest-feathers is not the only hypothetical mechanism explaining such results. Differential investment in nest sanitation activity in relation to the experimental addition of attractive feathers to avian nests might also account for the detected effects on eggshell bacterial loads (Veiga and Polo 2016). Although these two hypothetical mechanisms are not mutually exclusive, we here explore exclusive predictions of the hypothesis that particular antimicrobial activity of bacteria is one of the underlying mechanisms (see below).

We analyzed antimicrobial activity of bacterial colonies isolated from eggshells and feathers in bird nests, both with and without experimental feathers added, with the expectation that bacteria from experimental feathers should demonstrate higher antimicrobial activity than those from eggshells. The reasoning is that feathers harbor keratinolytic bacteria at a higher density than eggshells, and keratinolytic bacteria have great antimicrobial activity (Soler *et al.* 2010).

Furthermore, it is known from laboratory studies that growth rates of keratinolytic bacteria are lower in pigmented than in unpigmented feathers (Goldstein *et al.* 2004; Gunderson *et al.* 2008) which, in theory, may result in different bacterial communities. Consequently, different selective pressures due to bacterial interference (Riley and Wertz 2002) could be expected, which will originate different antimicrobial activity depending on feather pigmentation. Actually, antimicrobial effects of bacterial colonies isolated from nest-lining feathers in barn swallow nests differ among unpigmented and pigmented ones (Peralta-Sánchez *et al.* 2014).

Here, to explore these effects, we manipulated the composition of nest-lining feathers by adding to nests: (i) unpigmented feathers, (ii) pigmented feathers, or (iii) both. All these feathers were previously cleaned from bacteria and, thus, analyzed bacterial colonies come from the nest environment. Finally, there was a group of nests (iv) with no feathers added. Even in the case that antimicrobial activity of bacteria growing on feathers was particularly high, some other environmental factors are likely modulating it such as the strength of bacterial interference (Riley and Wertz 2002). Particularly interesting are the environmental conditions linked to breeding activity. For instance, incubation reduce eggshell humidity (D'Alba *et al.* 2010; Ruiz-de-Castañeda *et al.* 2011) and might favor the growth of bacterial colonies with antimicrobial properties on the eggshells (Grizard *et al.* 2014), which would

explain the reduced microbial load and diversity on eggshells in natural conditions (Cook *et al.* 2005a; 2005b; Shawkey *et al.* 2009; Ruiz-de-Castañeda *et al.* 2012). Interestingly, the production and activity of secondary metabolites of bacteria depends on temperature and nutrient availability in the nest (Biswas *et al.* 1991; Mataragas *et al.* 2003; 2004) and, thus, breeding activity could also affect antimicrobial activity of growing bacteria. Here, we explore this hypothesis by quantifying the bacterial density and the antimicrobial activity of colonies isolated from experimental nest-feathers and eggshells in nests with and without breeding activity. For this purpose, we used active nests of spotless starlings and artificial nests in nest-boxes containing experimental eggs.

Summarizing, we predict that 1) nests with experimental feathers added have higher antimicrobial activity than nests without feathers added, and nests with unpigmented feathers have the highest bacterial density and antimicrobial activity. We also expect that 2) independently of their color, feathers have more keratinolytic bacterial density and antimicrobial activity than eggshells. Finally, we predict that 3) natural nests have higher keratinolytic bacterial density and antimicrobial activity of colonies in eggshells and feathers than artificial nests due to the effects of breeding activity.

Materials and Methods

Study area and species

The study was performed during the breeding season of 2014 in the Hoya de Guadix, southeast Spain (37°18'N, 3°11'W), a high-altitude plateau 1000 m a.s.l, with a semi-arid climate. There were 80 cork-made nest-boxes (internal height * width * depth: 350 * 180 * 210 mm, bottom-to-hole height: 240 mm) available for spotless starlings (hereafter starlings)

attached to tree trunks or walls at 3-4 m above ground. Starlings in our population usually commence to build their nests in March, with straw, green plants and feathers as main nest materials. Egg laying is quite synchronous in our population and starts at mid-April (Soler *et al.* 2008). Clutches are of 4-5 eggs on average, with incubation typically starting with the penultimate egg, mainly performed by females (Navarro *et al.* 2011; Veiga and Polo 2016).

Field work and experimental procedures

Natural nests

Starting from April 10th (2014), approximately one week before the start of egg laying, we visited nest-boxes every three days. Once a nest was detected as complete, we removed any feather that starlings had in their nests and randomly assigned each nest to one of the four experimental treatments (Fig. 1). Treatments consisted of allocating to the nest (i) 20 pigmented feathers (i.e., close to the average number of feathers found in starling nests in previous years in the study area (Ruiz-Castellano *et al.* 2018) ($n = 5$ nests), (ii) 20 unpigmented feathers ($n = 6$), (iii) 10 pigmented plus 10 unpigmented feathers (hereafter mixed nests) ($n = 6$) (see details on feather preparation below), or (iv) leave the nest without feathers ($n = 6$). Seven days later (i.e., the day with three or less eggs in the nest, before incubation started) we refreshed feather treatments by removing feathers incorporated by adults and adding new experimental feathers when necessary. Ten days later (i.e., at the end of incubation), we measured length and width of all eggs with a digital caliper to the nearest 0.01 mm. On this day, we also sampled bacteria on the eggshells (see below) and collected one experimental feather of the same color that the experimental treatment from nests under pigmented and unpigmented feathers experimental treatments. From nests under the mixed experimental treatment, we collected one pigmented and one unpigmented experimental

feather, and from nests without experimental feathers, we collected one pigmented and one unpigmented non-experimental feather if present (Fig. 1).

Experimental feathers added to nests were from chickens that grew in small farms close to the study area. We selected white (i.e., unpigmented) and black (i.e., pigmented) body feathers of similar size (8-10 cm) as those used by starlings from our population as lining material. Experimental feathers were marked on the quill with a permanent marker allowing distinguishing them from those carried by adults to the nests. Subsequently, feathers were sterilized in the laboratory using a UV sterilizer chamber (Burdinola BV-100) during 10 minutes on each feather side (Ruiz-Rodríguez et al. 2009). Afterwards, we stored 10 pigmented or unpigmented feathers in separate hermetic bags and stored them at 4 °C until its use in experimental nests.

Artificial nests

The experimental treatments for artificial nests were the same than for natural ones. Forty new nest-boxes were erected one beside the other in the study area for this experiment (10 nest-boxes per treatment, assigned alternatively to the four treatments). The entrance of these nest-boxes was closed with a plastic mesh to prevent animals' access. On the same day (hereafter day 0), they were filled to one fourth of the volume with UV-sterilized polyester fiberfill, on top of which experimental feathers were placed in a hollow simulating a nest cup. Seven days later, one quail egg (Huevos Guillén S.L., Quart de Poblet, Valencia, Spain) previously cleaned with disinfectant wipes (Aseptonet, Laboratoires Sarbec, Cod.998077-51EN) was experimentally deposited on top of the nest material. Afterwards, nest-boxes were visited every second day to gently move the eggs ensuring contact of the whole eggshell

surface with nest-lining material. Ten days after placing the eggs in nests, we sampled bacteria from eggshells and collected one feather per nest (from mixed nests we collected one pigmented and one unpigmented feather, see Fig. 1).

The use of quail eggs in artificial nests is an approach that has been successfully employed in previous studies comparing bacterial loads of eggshells under different experimental treatments in artificial and natural nests of magpies (*Pica pica*) (Soler *et al.* 2015).

Bacterial sampling

For each nest visit and sampling, we wore new gloves sterilized with 96% ethanol to prevent contamination of eggshell bacterial samples among nests. Eggshells were sampled by cleaning the complete egg surface with a sterile rayon swab (EUROTUBO® DeltaLab) slightly wet with sterile sodium phosphate buffer (0.2 M; pH = 7.2). After cleaning, we introduced the swab in an Eppendorf tube with the buffer solution to preserve it. Feather samples were collected in individual hermetic bags. Eggshell and feather samples were preserved at 4-6 °C in a refrigerator until being processed in the laboratory within 24 h and a month after collection, respectively.

The capacity of bacterial strains to produce antimicrobial compounds is genetically regulated. Even after a period in which they have a low metabolic activity, as is the case of being kept in the fridge or freezer, they maintain their antimicrobial production when they recover the normal activity having the optimal conditions, as they have in our experiments (see below) (Ruiz-Rodríguez *et al.* 2012; 2013).

Laboratory work

Cultivation of eggshell and feather samples

Feather samples collected from nests were processed in the sterilizer chamber. From each feather, 1 cm² was cut with sterilized scissors and placed in an Eppendorf tube previously weighed in a precision balance (Metler Toledo, AB135-S/FACT Classic Plus; precision 0.00001 g). Before cutting each piece of feather, scissors were sterilized with alcohol and burned. Tubes containing feather samples were weighed again to obtain the feather mass. Subsequently, 1 mL of sterile sodium phosphate buffer (0.2 M; pH 7.2) was added.

From now on, eggshell and feather samples were treated in the same way. Tubes containing feather pieces or swabs were vigorously shaken in a vortex (Boeco V1 Plus) for at least one minute, favoring bacterial release from feathers or swabs. It is worth to mention here that although sonication is more appropriate to dislodge the attached bacteria (Bisson *et al.* 2007; Saag *et al.* 2011), it may also result in the loss of cellular membrane integrity (Foladori *et al.* 2007). Thus, because we need viable bacteria to grow and to estimate their antimicrobial activity, we did not sonicate feathers or swabs.

100 µl of the buffer of each sample were used for cultivation of aliquots of serial dilutions (until 10⁻⁴) in the following three different solid media: Tryptic Soy Agar (TSA), a broadly used general medium to grow aerobic mesophilic bacteria (Scharlau Chemie S.A., Barcelona), and two specific media for keratinolytic bacteria: feather meal agar made with unpigmented feathers (UP-FMA) and with pigmented feathers (P-FMA). These two last media mainly contain keratin. FMA media was made with 15 g L⁻¹ feather meal, 0.5 g NaCl, 0.30 g L⁻¹ K₂HPO₄, 0.40 g L⁻¹ KH₂PO₄, 15 g L⁻¹ agar (Sangali and Brandelli 2000) and 0.1 mg L⁻¹ cycloheximid to avoid fungi growth (Shawkey *et al.* 2005; Møller *et al.* 2009; Czirják *et al.* 2010). The rationale for the use of UP-FMA and P-FMA was that it is possible that experimental treatment effects were more easily detected in P-FMA or UP-FMA, due to the

different growth of keratinolytic bacteria on pigmented and unpigmented feathers (See Introduction). Plates were incubated at 37 °C, and after 72 h, the number of colonies on each plate was counted.

Eggshell bacterial density was estimated by standardization of the number of colonies per cm² of sampled eggshell (CFU, Colony Forming Units). Eggshell surface was estimated following Narushin (2005) from length and width of each egg: $S = (3.155 - 0.0136 * L + 0.0115 * W) * L * W$, where S is the egg surface in cm², W is the egg width and L is the egg length. Feather bacterial density was estimated as the number of colonies per mg of feather.

Antimicrobial activity of colonies isolated from eggshells and feathers

From each cultivated plate, a maximum of five colonies of different morphology were isolated and transferred to 6 mL of Brain Heart Infusion (BHI, Scharlau Chemie S.A., Barcelona), and incubated at 37 °C during 24 h. Then, BHI containing bacteria was spread onto TSA plates by streaking technique, incubated at 37 °C during 24 h, and preserved at 4 °C until the antimicrobial assay within the following 72 h. This step was repeated twice to minimize possibilities of contamination of each isolate. For antimicrobial assays, each colony was replicated by spotting onto 18 TSA plates (35 colonies per plate), and incubated for 24 h at 37 °C. After growing, plates were covered with 6 mL of soft agar (Brain Heart Infusion added 0.8% agar, Scharlau Chemie S.A., Barcelona), inoculated with 150 µL of a 12 h at 37 °C culture of the indicator strains, and then incubated for 24 h at 37 °C. The antimicrobial activity of each isolated colony was revealed by the presence of clear growth-inhibition halos around the spot of isolates (for more details see Ruiz-Rodríguez *et al.* 2012).

Tests of antimicrobial activity were performed against 18 typified bacterial strains of a wide range of bacterial taxa, including bacteria that could be potentially pathogenic for

birds (Pinowski *et al.* 1994; Hubálek 2004; Benskin *et al.* 2009). Bacteria strains were collected from the Spanish Type Culture Collection (CECT) and from our laboratory collection. We used *Bacillus licheniformis* D13, *Bacillus megatherium*, *Bacillus thuringiensis*, *Enterococcus faecalis* MRR-103, *Enterococcus faecium* UJA34, *Escherichia coli* CECT774, *Klebsiella* sp., *Lactobacillus lactis lactis* LM2301, *Lactobacillus paracasei* 11-2, *Lactobacillus plantarum* CECT784, *Listeria innocua* CECT340, *Listeria monocytogenes* CECT4032, *Micrococcus luteus* 241, *Mycobacterium* sp., *Pseudomonas putida*, *Proteus* sp., *Salmonella choleraesuis* CECT443 and *Staphylococcus aureus* CECT240.

We performed a total of 9,719 tests of antimicrobial activity against these 18 indicator bacterial strains. Of these tests, 5,145 were performed with bacterial colonies isolated from feathers (N = 285), and 4,574 with colonies isolated from eggshells (N = 253).

Statistical analyses

Estimated bacterial densities were log₁₀ transformed to approach Gaussian distributions. We used MCMCglmm models as implemented in R (R Core Team 2015) with the appropriate libraries (“MCMCglmm” (Hadfield 2010), “MASS” (Venables and Ripley 2002) and “mvtnorm” (Genz and Bretz 2009)) to explore predictors of bacterial loads in feathers and eggshells and antimicrobial activity of bacterial colonies. Models explaining eggshell bacterial load included density of mesophilic and of keratinolytic (both UP-FMA and P-FMA) bacteria as dependent variables, and experimental treatments (i.e., four levels of feather manipulations) and nest type (i.e., natural or artificial) as fixed factors. MCMCglmm models exploring bacterial density of feathers also included feather color as independent fixed factor. Because feathers’ color and two of the experimental treatments provided the

same information (i.e., in experimental nests with only unpigmented or pigmented feathers we could sample just one type of feather of the same color than the experimental treatment), experimental feather treatment in these models refers to unicolor (nests with only pigmented or unpigmented experimental feathers) or mixed color (nests with both pigmented and unpigmented experimental feathers). However, since two feathers were sampled in nests with mixed treatments, we included nest identity as random factor in these models. It should be noted here that estimated bacterial density of feathers and of eggshells cannot be compared because the former is relative to feather mass and the latter to eggshell surface.

Frequencies of presence or absence of antimicrobial activity in colonies isolated from feathers and eggs were included in the statistical models as a multi-response variable. Thus, whether or not antimicrobial activity was detected against each indicator strain, for each isolated colony, was used as binary response in separate models by assuming multinomial distribution of our statistical MCMCglmm models. In a first model we explored the effects of bacterial origin (i.e., feathers or eggshells), nest type (i.e., artificial or natural) and experimental treatments (four levels) on the antimicrobial activity of isolates. This model also included nest identity as random factor because more than one sample was collected from some of the experimental nests (see above). Furthermore, we explored the effect of feather color from which bacteria were isolated on the antimicrobial activity. To do this, we separately analyzed microbial activity of colonies isolated from feathers in models that included nest type (natural *vs* artificial), experimental treatment (unicolor or mixed) and feather color as fixed factors, and nest identity as random effect. Full models included interactions, but main effect probabilities were estimated in models without interactions. Models were reduced starting from the higher order interaction by removing one by one

independent factors with higher probability until there were no variables with $P > 0.1$. Results from full and reduced models are shown.

Used non-informative priors for models that did or did not include random factors were [prior1 = list(R = list(V = 1, nu = 0.002), G = list(G1 = list(V = 1, nu = 0.002)))] and [prior2 = list(R = list(V = 1, nu = 0.002))], respectively. Estimates and probabilities were calculated with 100000 interactions (nitt=), thinning intervals (thin=) of 10, and with a burn in period (burnin=) of 10000. Inferences were based on posterior means of the estimate (post.mean) and 95% credibility intervals as well as on the probability of parameters being different from zero (pMCMC).

Results

Bacterial loads in feathers and eggshells

Feathers from natural nests harbored bacteria at a higher density than feathers from artificial nests, while experimental treatments, feather pigmentation, and the interaction among factors did not explain additional variance (Table 1 and 2). This was the case independently of using culture medium for mesophilic bacteria or restrictive media for keratinolytic bacteria (Fig. 2). However, mesophilic bacterial loads of eggshells in natural nests were similar to that of artificial nests and neither the experimental treatment (Table 2) nor the interaction with artificial-natural nests explained additional variance in bacterial loads significantly (Table 1). Interestingly, and independently of experimental treatment, eggshells in natural nests harbored keratinolytic bacteria at a higher density than eggshells in artificial nests (Table 1, Fig. 2).

Finally, we explored the relationship between bacterial loads (mesophilic and keratinolytic bacteria) of feathers and of eggshells in both artificial and natural nests, and we found no significant relationship (Pearson correlation $P > 0.137$).

Antimicrobial activity of bacteria isolated from artificial and natural starling nests

Antimicrobial activity (Table 3) tended to be more frequently detected in colonies from feathers than in those from eggshells. Moreover, colonies from feathers and eggshells of natural starling nests demonstrated higher antimicrobial activity than those from artificial nests. Finally, the effect of nest type (artificial *vs* natural) did not depend on the origin of the bacterial colonies (feathers *vs* eggshells) (see interaction in Table 4, Fig. 3).

We separately explored the effects of experimental treatments on antimicrobial activity of bacteria isolated from feathers and from eggshells. We did not detect an effect of experimental treatments (Table 2 and 4), but we found that feather bacteria from natural nests demonstrated higher antimicrobial activity than those from artificial nests (Table 4). Likewise, eggshell bacteria from natural nests demonstrated higher antimicrobial activity than eggshell bacteria from artificial nests (Table 4).

Discussion

Our main aim was to explore predictions of one of the mechanisms potentially explaining the detected antimicrobial effects of feathers in avian nests (Peralta-Sánchez *et al.* 2010; Ruiz-Castellano *et al.* 2016), which deals with the antimicrobials production of bacteria growing on nest feather material. Although we did not detect an effect of feather treatments on density and antimicrobial activity of bacteria, we found that colonies isolated from feathers were

more frequently active against indicator bacterial strains than those isolated from eggshells. Moreover, densities of keratinolytic bacteria on feathers and eggshells were higher in natural than in artificial nests, and colonies isolated from natural nests demonstrated higher antimicrobial capabilities than those from artificial nests, independently of whether colonies were isolated from feathers or eggshells. Below we discuss possible explanations, biases of our experimental approach, and the importance of nest building behavior, as well as incubation, in determining bacterial environments and risk of infection for developing offspring.

In our study, we did not detect the effects of experimental feather treatments, both in natural and artificial nests, on bacterial density and on antimicrobial activity of isolated colonies from eggshells and feathers. It is possible that larger sample sizes are needed to detect the experimental effects (Table 2). We have previously detected antimicrobial beneficial effects of unpigmented feathers in barn swallow (Peralta-Sánchez *et al.* 2014) and of pigmented feathers in starlings (Ruiz-Castellano *et al.* 2016; Soler *et al.* 2017). The effects of breeding activity were explored by using quail eggs in artificial nests. We found that density of mesophilic bacteria in eggshells did not differ between natural and artificial nests, which could validate the usage of quail eggs. Moreover, since eggshells of different species may vary in their antimicrobial defenses, and therefore in probability of keratinolytic bacteria colonization and growth (Wellman-Labadie *et al.* 2008a; 2008b; 2010; Soler *et al.* 2011, D'Alba *et al.* 2014; Martín-Vivaldi *et al.* 2014), comparisons of the effects of experimental treatments on quail and starling eggs may suffer from some kind of bias. This possibility however would hardly explain the detected higher keratinolytic bacterial loads on starling eggshells in natural nests because the same effects were detected in experimental feathers (all of them were prepared in our laboratory). Moreover, although it is known that incubation

reduces eggshell bacterial loads and diversity (Cook *et al.* 2005a; Shawkey *et al.* 2009; Soler *et al.* 2015; Ruiz-Castellano *et al.* 2016), density of keratinolytic bacteria on the eggshells in natural nests with incubation activity is usually higher than in artificial nests (Ruiz-Castellano *et al.* 2016; Soler *et al.* 2015). Thus, it is likely that bacteria already present in nests, or carried by parents, and environmental factors linked to breeding activity such as temperature and humidity, were responsible for the higher bacterial density detected in sampled material from natural nests.

In natural nests, apart from experimental feathers, parents carried feathers to the nests with their own bacteria. Moreover, parents are also a source of bacteria for feather material and eggshells that do not exist in artificial nests. Thus, although keratinolytic bacteria are not the only ones that feathers harbor (Shawkey *et al.* 2005), the above scenarios possibly explain the higher density of keratinolytic bacteria found in eggshells of natural nests. Interestingly, previous research has shown that incubation differentially favors growth of some bacterial strains (Grizard *et al.* 2014) such as keratinolytic bacteria of the genus *Pseudomonas* or *Bacillus* (Wang *et al.* 2011; Potter *et al.* 2013; Grizard *et al.* 2015). Because of the antimicrobial properties of some of these bacteria, these results open the possibility that avian incubation behavior selects for beneficial antimicrobial-producing bacteria on the eggshell that would protect embryos from pathogenic infection (Wang *et al.* 2011). In accordance with these studies, even though parents could be responsible for the keratinolytic bacteria in natural nests, we found that the expected higher bacterial density on eggs from natural nests, with incubation activity, was only true for keratinolytic bacteria.

In addition, we also found that antimicrobial activity of colonies isolated from natural nests was higher than that of colonies isolated from artificial nests. We know that bacterial production of secondary metabolites depends on several environmental factors like

temperature or nutrient availability (Biswas *et al.* 1991; Mataragas *et al.* 2003; 2004). Thus, it is possible that breeding activity, by the modification of nest environmental conditions (Cook *et al.* 2005b; Ruiz-de-Castañeda *et al.* 2011) and nutrients for bacterial growth (Ibáñez-Álamo *et al.* 2014), has an effect on bacteria, enhancing segregation of antimicrobial secondary metabolites (see Introduction for rationale of this expected association).

Independently of the effects of breeding activity, and in accordance with the hypothesis tested, antimicrobial activity was more frequently detected in colonies isolated from feathers than from eggshells. We did not find the predicted differential effects of feather color on the antimicrobial activity of the colonies isolated from them. In a previous study, colonies isolated from unpigmented feathers of barn swallow nests had higher antimicrobial potential than those from pigmented feathers (Peralta-Sánchez *et al.* 2014). However, in starlings, we detected stronger antimicrobial beneficial effects for pigmented feathers (Ruiz-Castellano *et al.* 2016), and the higher antimicrobial activity of colonies isolated from unpigmented feathers detected here did not reach statistical significance. A larger sample size may be necessary to detect a differential effect of feather pigmentation on antimicrobial activity of bacteria growing on feathers. In any case, the detected higher effects of colonies from feathers over those coming from eggshells suggest that this nest material can be seen as antimicrobial source that influence bacterial environment of avian nests. Thus, these results support the hypothesis that one of the mechanisms explaining the detected antimicrobial effects of nest-lining feathers could be related to the antimicrobial properties of bacterial colonies growing on this material.

It could be argued that an additional mechanism to account for our results involves the role of feathers as sexual display between adult birds (see Introduction), which may induce, for instance, differential investment in nest sanitation behaviors and originate

differences in the nest microbiome (e.g., Azcárate-García *et al.* 2019). However, although our experimental approach does not allow estimating relative importance of sexual selection and antimicrobial properties of feathers explaining nest bacterial environment, our results point out independent effects of feathers. This is because antimicrobial activities of colonies from feathers resulted higher than those from eggshells, in natural but also in artificial nests.

Conclusions

In support of the hypothesis that feathers in nests of birds should be important sources of antimicrobial-producing bacteria, we detected broad antimicrobial activities for colonies isolated from experimental nest-lining feathers in natural and artificial nests. Consequently, antimicrobial activity of bacteria growing on nest-lining feathers would be one of the mechanisms underlying the previously detected antimicrobial effects of this lining-material in avian nests. However, more studies are necessary to identify bacteria growing on feathers and their functional role in the nest environment. Furthermore, we show that antimicrobial activity of colonies from artificial nests was lower than that of colonies from natural nests, which suggests that breeding activity might differentially favor growth of antimicrobial-producing bacteria. These results urge molecular characterization of the microbiome associated with breeding activity and nest materials that enhance growth of especially active antimicrobial-producing bacteria in bird nests, and therefore deserve further investigation.

Ethical statement

The study was performed according to relevant Spanish national (Decreto 105/2011, 19 de Abril) and regional guidelines. The protocol was approved by ethics committee of Spanish National Research Council (CSIC) and all necessary permits for nest and egg manipulations

were obtained from Consejería de Medio Ambiente de la Junta de Andalucía, Spain (Ref: SGYB/FOA/AFR/CFS and SGMN/GyB/JMIF). This study did not involve endangered or protected species.

Funding

This work was supported by the currently named Ministerio de Ciencia, Innovación y Universidades and European funds (FEDER) (CGL2013-48193-C3-1-P, CGL2017-83103-P, CGL2017-89063-P). MRR received a postdoc from the programme “JAE-Doc” and CRC had a predoctoral grant from the Spanish Government. GT was supported by Ramón y Cajal programme (Spain).

Authors' contributions

JJS, MRR, and GT designed the study with considerable assistance from CRC. CRC and MRR performed all bacterial analyses. GT and CRC performed most of the field work with assistance by JJS. JJS and CRC performed all the statistical analyses and wrote the manuscript with substantial contribution from all authors.

Acknowledgments

We thank Estefanía López Hernández for assistance in laboratory work and Manuel Martín-Vivaldi and Eva Valdivia for comments on a previous version that greatly improved the quality of the article.

References

- Abriouel H, Franz CMAP, Omar NB *et al.* Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiol Rev* 2011; **35**: 201-232.
- Azcárate-García M, Ruiz-Rodríguez M, Díaz-Lora S *et al.* Experimentally broken faecal sacs affect nest bacterial environment, development and survival of spotless starling nestlings. *J Avian Biol* 2019; doi: 10.1111/jav.02044.
- Benskin CMH, Wilson K, Jones K *et al.* Bacterial pathogens in wild birds: A review of the frequency and effects of infection. *Biol Rev* 2009; **84**: 349-373.
- Bisson IA, Marra PP, Burt J *et al.* A molecular comparison of plumage and soil bacteria across biogeographic, ecological, and taxonomic scales. *Microb Ecol* 2007; **54**: 5-81.
- Biswas SR, Ray P, Johnson MC *et al.* Influence of growth conditions on the production of a bacteriocin, pediocin AcH, by *Pediococcus acidilactici* H. *Appl Environ Microbiol* 1991; **57**: 1265-1267.
- Burt EH, Ichida JM. Occurrence of feather-degrading bacilli in the plumage of birds. *Auk* 1999; **116**: 364-372.
- Clark L, Mason JR. Use of nest material as insecticidal and anti-pathogenic agents by the European Starling. *Oecologia* 1985; **67**: 69-176.
- Clayton DH, Wolfe ND. The adaptive significance of self-medication. *Trends Ecol Evol* 1993; **8**: 60-63.
- Cook MI, Beissinger SR, Toranzos GA *et al.* Incubation reduces microbial growth on eggshells and the opportunity for trans-shell infection. *Ecol Lett* 2005a; **8**: 532-537.
- Cook MI, Beissinger SR, Toranzos GA *et al.* Microbial infection affects egg viability and incubation behavior in a tropical passerine. *Behav Ecol* 2005b; **16**: 30-36.
- Czirják GA, Møller AP, Mousseau TA *et al.* Microorganisms associated with feathers of barn swallows in radioactively contaminated areas around Chernobyl. *Microb Ecol* 2010; **60**: 373-380.
- D'Alba L, Oborn A, Shawkey MD. Experimental evidence that keeping eggs dry is a mechanism for the antimicrobial effects of avian incubation. *Naturwissenschaften* 2010; **97**: 1089-1095.
- D'Alba L, Jones DN, Badawy HT *et al.* Antimicrobial properties of a nanostructured eggshell from a compost-nesting bird. *J Exp Biol* 2014; **217**: 1116-1121.
- De Roode JC, Lefèvre T, Hunter MD. Self-medication in animals. *Science* 2013; **340**: 150-151.

- Dille JW, Rogers CM, Schneegurt MA. Isolation and characterization of bacteria from the feathers of wild Dark-eyed Juncos (*Junco hyemalis*). *Auk* 2016; **133**: 155-167.
- Dubiec A, Gózd I, Mazgajski TD. Green plant material in avian nests. *Avian Biol Res* 2013; **6**: 133-146.
- Foladori P, Laura B, Gianni A *et al*. Effects of sonication on bacteria viability in wastewater treatment plants evaluated by flow cytometry—Fecal indicators, wastewater and activated sludge. *Water Res* 2007; **41**: 235-243.
- Genz A, Bretz F. Computation of Multivariate Normal and t Probabilities. Heidelberg: Springer-Verlag, 2009.
- Goater TM, Goater CP, Esch GW. Parasitism: the diversity and ecology of animal parasites. Cambridge University Press, 2001.
- Goldstein G, Flory KR, Browne BA *et al*. Bacterial degradation of black and white feathers. *Auk* 2004; **121**: 656-659.
- Grizard S, Dini-Andreote F, Tieleman BI *et al*. Dynamics of bacterial and fungal communities associated with eggshells during incubation. *Ecol Evol* 2014; **4**: 1140-1157.
- Grizard S, Versteegh MA, Ndithia HK *et al*. Shifts in bacterial communities of eggshells and antimicrobial activities in eggs during incubation in a ground-nesting passerine. *PLoS ONE* 2015; DOI: 10:e0121716.
- Gunderson AR, Frame AM, Swaddle JP *et al*. Resistance of melanized feathers to bacterial degradation: Is it really so black and white? *J Avian Biol* 2008; **39**: 539-545.
- Gwinner H, Berger S. European starlings: Nestling condition, parasites and green nest material during the breeding season. *J Ornithol* 2005; **146**: 365-371.
- Hadfield JD. MCMC methods for multi-response generalized linear mixed models: The MCMCglmm R package. *J Stat Softw* 2010; **33**: 1-22.
- Hart BL. The evolution of herbal medicine: Behavioural perspectives. *Anim Behav* 2005; **70**: 975-989.
- Hubálek Z. An annotated checklist of pathogenic microorganisms associated with migratory birds. *J Wildlife Dis* 2004; **40**: 639-659.
- Ibáñez-Álamo JD, Ruiz-Rodríguez M, Soler JJ. The mucous covering of fecal sacs prevents birds from infection with enteric bacteria. *J Avian Biol* 2014; **45**: 354-358.
- Jacobson M. Plants, insects, and man-their interrelationships. *Econ Bot* 1982; **36**: 346-354.
- Kent CM, Burt EH. Feather-degrading bacilli in the plumage of wild birds: Prevalence and relation to feather wear. *Auk* 2016; **133**: 583-592.

- Kumari VJ. Comparative analysis of bacteriocins produced by *Pseudomonas aeruginosa* and *Bacillus brevis*. *Asian J Microbiol Biotechnol Envir Sci* 2014; **16**: 295-298.
- Loye JE, Zuk M. Bird-parasite interactions: ecology, evolution and behaviour. Oxford: Oxford university press, 1999.
- Lozano GA. Parasitic stress and self-Medication in wild animals. In Møller AP, Milinski, M Slater PJB (ed.). *Advances in the Study of Behavior: Stress and Behavior*. Academic Press, 1998, 291-317.
- Martín-Vivaldi M, Peña A, Peralta-Sánchez JM *et al.* Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. *P Roy Soc B-Biol Sci* 2010; **277**: 123-130.
- Martín-Vivaldi M, Soler JJ, Peralta-Sánchez JM *et al.* Special structures of hoopoe eggshells enhance the adhesion of symbiont-carrying uropygial secretion that increase hatching success. *J Anim Ecol* 2014; **83**: 1289-1301.
- Mataragas M, Metaxopoulos J, Galiotou M *et al.* Influence of pH and temperature on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. *Meat Sci* 2003; **64**: 265-271.
- Mataragas M, Drosinos EH, Tsakalidou E *et al.* Influence of nutrients on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. *Int J Gen Mol Microbiol* 2004; **85**: 191-198.
- Mennerat A, Mirleau P, Blondel J *et al.* Aromatic plants in nests of the blue tit *Cyanistes caeruleus* protect chicks from bacteria. *Oecologia* 2009; **161**: 849-855.
- Møller AP, Czirjak GA, Heeb P. Feather micro-organisms and uropygial antimicrobial defences in a colonial passerine bird. *Funct Ecol* 2009; **23**: 1097-1102.
- Narushin VG. Egg geometry calculation using the measurements of length and breadth. *Poultry Sci* 2005; **84**: 482-484.
- Navarro C, Pérez-Contreras T, Avilés JM *et al.* Blue-green eggshell coloration reflects yolk antioxidant content in spotless starlings *Sturnus unicolor*. *J Avian Biol* 2011; **42**: 538-543.
- Peralta-Sánchez JM, Møller AP, Martín-Platero AM *et al.* Number and colour composition of nest lining feathers predict eggshell bacterial community in barn swallow nests: An experimental study. *Funct Ecol* 2010; **24**: 426-433.
- Peralta-Sánchez JM, Møller AP, Soler JJ. Colour composition of nest lining feathers affects hatching success of barn swallows, *Hirundo rustica* (Passeriformes: *Hirundinidae*). *Biol J Linn Soc* 2011; **102**: 67-74.

- Peralta-Sánchez JM, Soler JJ, Martín-Platero AM *et al.* Eggshell bacterial load is related to antimicrobial properties of feathers lining barn swallow nests. *Microb Ecol* 2014; **67**: 480-487.
- Pinowski J, Barkowska M, Kruszewicz AH *et al.* The causes of the mortality of eggs and nestlings of *Passer sp.* *J Bioscience* 1994; **19**: 441-451.
- Potter BA, Carlson BM, Adams AE *et al.* An assessment of the microbial diversity present on the surface of naturally incubated house wren eggs. *Open Ornithol J* 2013; **6**: 32-39.
- Price PW. Evolutionary biology of parasites. Princeton: Princeton University Press, 1980.
- Riley MA, Wertz JE. Bacteriocins: Evolution, ecology, and application. *Annu Rev Microbiol* 2002; **56**: 117-137.
- Ruiz-Castellano C, Tomás G, Ruiz-Rodríguez M *et al.* Nest material shapes eggs bacterial environment. *PLoS ONE* 2016; DOI: 11:e0148894.
- Ruiz-Castellano C, Tomás G, Ruiz-Rodríguez M *et al.* Nest material preferences by spotless starlings. *Behav Ecol* 2018; **29**: 137-144.
- Ruiz-De-Castañeda R, Vela AI, González-Braojos S *et al.* Drying eggs to inhibit bacteria: Incubation during laying in a cavity nesting passerine. *Behav Process* 2011; **88**: 142-148.
- Ruiz-De-Castañeda R, Vela AI, Lobato E *et al.* Early onset of incubation and eggshell bacterial loads in a temperate-zone cavity-nesting passerine. *Condor* 2012; **114**: 203-211.
- Ruiz-Rodríguez M, Martínez-Bueno M, Martín-Vivaldi M *et al.* Bacteriocins with a broader antimicrobial spectrum prevail in enterococcal symbionts isolated from the hoopoe's uropygial gland. *FEMS Microbiol Ecol* 2013; **85**: 495-502.
- Ruiz-Rodríguez M, Valdivia E, Soler JJ *et al.* Symbiotic bacteria living in the hoopoe's uropygial gland prevent feather degradation. *J Exp Biol* 2009; **212**: 3621-3626.
- Ruiz-Rodríguez M, Valdivia E, Martín-Vivaldi M *et al.* Antimicrobial activity and genetic profile of enterococci isolated from hoopoes uropygial gland. *PLoS ONE* 2012; DOI 7:e41843.
- Saag P, Tilgar V, Mänd R *et al.* Plumage bacterial assemblages in a breeding wild passerine: relationships with ecological factors and body condition. *Microb Ecol* 2011; **61**: 740-749.
- Sangali S, Brandelli A. Feather keratin hydrolysis by a *Vibrio sp.* strain kr2. *J Appl Microbiol* 2000; **89**: 735-743.

- Schmid-Hempel P. Evolutionary parasitology: The integrated study of infections, immunology, ecology and genetics. Oxford: Oxford University Press, 2011.
- Scott-Baumann JF, Morgan ER. A review of the nest protection hypothesis: Does inclusion of fresh green plant material in birds' nests reduce parasite infestation? *Parasitology* 2015; **142**: 1016-1023.
- Shawkey MD, Mills KL, Dale C *et al.* Microbial diversity of wild bird feathers revealed through culture-based and culture-independent techniques. *Microb Ecol* 2005; **50**: 40-47.
- Shawkey MD, Firestone MK, Brodie EL *et al.* Avian incubation inhibits growth and diversification of bacterial assemblages on eggs. *PLoS ONE* 2009; DOI: 4:e4522.
- Soler JJ, Martín-Vivaldi M, Ruiz-Rodríguez M *et al.* Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. *Funct Ecol* 2008a, **22**: 864-871.
- Soler JJ, Navarro C, Pérez-Contreras T *et al.* Sexually selected egg coloration in spotless starlings. *Am Nat* 2008b; **171**: 183-194.
- Soler JJ, Martín-Vivaldi M, Peralta-Sánchez JM *et al.* Antibiotic-producing bacteria as a possible defence of birds against pathogenic microorganisms. *Open Ornithol J* 2010; **3**: 93-100.
- Soler JJ, Peralta-Sánchez JM, Martínez-Bueno M *et al.* Brood parasitism is associated with increased bacterial contamination of host eggs: Bacterial loads of host and parasitic eggs. *Biol J Linn Soc* 2011; **103**: 836-848.
- Soler JJ, Ruiz-Castellano C, Figuerola J *et al.* Telomere length and dynamics of spotless starling nestlings depend on nest-building materials used by parents. *Anim Behav* 2017; **126**: 89-100.
- Soler JJ, Ruiz-Rodríguez M, Martín-Vivaldi M *et al.* Laying date, incubation and egg breakage as determinants of bacterial load on bird eggshells: experimental evidence. *Oecologia* 2015; **179**: 63-74.
- Veiga JP, Polo V. Feathers in the spotless starling nests: A sexually selected trait? *Behaviour* 2011; **148**: 1359-1375.
- Veiga JP, Polo V. Estornino Negro - *Sturnus unicolor*. In Salvador A, Morales MB (ed.). *Enciclopedia Virtual de los Vertebrados Españoles*. Madrid: Museo de Ciencias Naturales, 2016.
- Venables WN, Ripley BD. Modern applied statistic with S. New York: Springer, 2002.

- Wang JM, Firestone MK, Beissinger SR. Microbial and environmental effects on avian egg viability: Do tropical mechanisms act in a temperate environment? *Ecology* 2011; **92**: 1137-1145.
- Wellman-Labadie O, Picman J, Hincke MT. Antimicrobial activity of cuticle and outer eggshell protein extracts from three species of domestic birds. *Brit Poultry Sci* 2008a; **49**: 133-143.
- Wellman-Labadie O, Picman J, Hincke MT. Antimicrobial activity of the Anseriform outer eggshell and cuticle. *Comp Biochem Phys B* 2008b; **149**: 640-649.
- Wellman-Labadie O, Lemaire S, Mann K *et al.* Antimicrobial activity of lipophilic avian eggshell surface extracts. *J Agr Food Chem* 2010; **58**: 10156-10161.
- Whitaker JM, Cristol DA, Forsyth MH. Prevalence and genetic diversity of *Bacillus licheniformis* in avian plumage. *J Field Ornithol* 2005; **76**: 264-270.

Table 1: Results from MCMCglmm models explaining bacterial loads on feathers and eggshells cultured in TSA, UP-FMA and P-FMA media. We report posterior mean of the estimate (post.mean) and 95% credibility intervals as well as the probability of parameters being different from zero (pMCMC). Main effect probabilities were estimated in models without interaction and interaction probabilities in the complete models. M = Mixed colors; NF = No feathers; P = Pigmented feathers; F = Feathers; 2 = Two level feather treatments (unicolor or mixed color); 4 = Four level feather treatments (pigmented, unpigmented, mixed or control). See text for details. Associations with a $P < 0.05$ are in bold.

Table 2: Mean (SE)) of log10 bacterial density and antimicrobial activity of colonies isolated from eggshells and feathers of natural and artificial nests against each experimental treatment.

Table 3: Antimicrobial activity (Mean % (SE)) of colonies isolated from feathers and eggshells of natural and artificial nests against each strain tested.

Table 4: Results from MCMCglmm models explaining antimicrobial activity of bacterial colonies isolated from feathers and eggshells. Frequencies of antimicrobial activity detected against indicator strains were included in the models as multi-response variable. We report posterior mean of the estimate (post.mean) and 95% credibility intervals as well as the probability of parameters being different from zero (pMCMC). Main effect probabilities were estimated in models without interaction and interaction probabilities in the complete model. M = Mixed colors; NF = No feathers; P = Pigmented feathers; F = Feathers. 2 = Two

level feather treatments (unicolor or mixed color); 4 = Four level feather treatments (pigmented, unpigmented, mixed or control). See text for details Associations with a $P < 0.05$ are in bold.

Fig. 1: Schematic description of the experimental protocol.

Fig.2: Average log bacterial density $\pm$ CI 95% of feathers (colonies/mg of feather) (B, D, F) and eggshells (colonies/cm²) (A, C, E) estimated in culture media for mesophilic bacteria (TSA: Tryptic Soy Agar) (A, B)), and for keratinolytic bacteria in unpigmented (UP-FMA (C, D)) and pigmented (P-FMA (E, F)) feather meal agar. Bacterial density on eggshells in artificial (filled circles) and natural nests (open squares) under four different experimental feather treatments (mixed, Unpigm. = unpigmented, Pigm. = pigmented, and no feathers) are shown. Bacterial density on pigmented and unpigmented feathers that were in artificial or natural nests, with (mixed) or without (unicolor) feathers of other pigmentation are shown. Bacterial loads of keratinolytic bacteria on eggshells (UP-FMA ($p = 0.016$), P-FMA ($p = 0.027$)) and mesophilic and keratinolytic bacteria on feathers (UP-FMA ($p = 0.013$), P-FMA ($p = 0.003$) and TSA ($p = 0.001$)) were higher in natural than in artificial nests.

Fig. 3: Percentage of antimicrobial activity (%) of colonies isolated from feathers and eggshells of natural and artificial nests against bacteria strains.

Table 1:

	TSA		UnPigmented-FMA		Pigmented-FMA	
	post.mean (95% CI)	pMCM C	post.mean (95% CI)	pMCM C	post.mean (95% CI)	pMCMC
FEATHERS						
Complete model						
Nest type	0.647 (0.335-0.930)	<0.001	0.550 (0.147-0.947)	0.008	0.580 (0.215-0.930)	0.015
F color	-0.094 (-0.344- 0.152)	0.458	0.086 (-0.274-0.405)	0.618	0.048 (-0.245- 0.322)	0.737
F treatment (2)	-0.031 (-0.328-0.279)	0.836	0.216 (-0.186-0.623)	0.291	0.133 (-0.235- 0.495)	0.462
Interactions		> 0.230		> 0.069		> 0.151
Final model						
Nest type	0.655 (0.359-0.942)	0.001	0.503 (0.123-0.902)	0.013	0.551 (0.197-0.906)	0.003
EGGSHELLS						
Complete model						
Nest type	0.033 (-0.385-0.441)	0.866	0.167 (0.024-0.306)	0.025	0.097 (0.007-0.182)	0.029
F treatment (4) M	0.012 (-0.584-0.572)	0.975	0.045 (-0.148-0.235)	0.636	-0.050 (-0.167- 0.064)	0.398
F treatment (4) NF	0.165 (-0.399-0.720)	0.564	0.075 (-0.102-0.265)	0.418	-0.060 (-0.174- 0.052)	0.290
F treatment (4) P	0.014 (-0.536-0.588)	0.964	0.057 (-0.132-0.245)	0.548	0.050 (-0.062-0.167)	0.395
Interactions		> 0.120		>0.544		>0.440
Final model						
Nest type			0.169 (0.030-0.302)	0.016	0.097 (0.012-0.186)	0.027

1 Table 2:

Feather treatment		Artificial nests			Natural nests		
		Eggshell	Feather	N	Eggshell	Feather	N
Bacterial loads							
	Pigmented	1.27 (0.02)	1.42 (0.01)	10	1.23 (0.04)	1.95 (0.01)	5
	Unpigmented	1.05 (0.01)	1.19 (0.01)	10	1.58 (0.07)	1.96 (0.01)	6
	Mixed	1.15 (0.01)	1.39 (0.01)	10	1.78 (0.08)	2.39 (0.03)	6
	Control	1.70 (0.03)	0.00 (0.00)	10	1.10 (0.01)	1.70 (0.01)	6
Antimicrobial activity							
	Pigmented	16.33 (0.37)	27.24 (0.40)	10	28.41 (0.30)	24.71 (0.58)	5
	Unpigmented	10.97 (0.38)	18.64 (0.82)	10	21.92 (0.60)	30.05 (0.61)	6
	Mixed	13.49 (0.31)	19.97 (0.42)	10	22.18 (0.64)	28.61 (0.65)	6
	Control	17.02 (0.50)	00.00 (0.00)	10	21.71 (0.40)	33.86 (0.47)	6

2

3 Table 3:

Bacterial strain	Artificial nests				Starling nests			
	Eggs	N	Feathers	N	Eggs	N	Feathers	N
<i>B. licheniformis</i>	21.62 (3.59)	166	25.10 (4.40)	149	37.25 (5.17)	88	40.91 (4.65)	135
<i>B. megatherium</i>	26.75 (3.29)	166	26.47 (3.98)	149	50.00 (5.51)	88	40.59 (4.02)	137
<i>B. thuringiensis</i>	15.64 (2.98)	165	17.79 (3.94)	149	17.75 (3.62)	88	22.42 (3.97)	137
<i>E. faecalis</i>	14.49 (2.98)	162	17.06 (4.56)	149	19.92 (4.34)	86	25.32 (3.79)	137
<i>E. faecium</i>	16.28 (3.10)	167	20.54 (4.24)	149	18.75 (4.37)	88	26.13 (4.55)	138
<i>E. coli</i>	9.83 (2.32)	167	18.33 (3.84)	149	15.75 (3.47)	90	24.52 (3.46)	138
<i>Klebsiella sp.</i>	11.24 (2.49)	168	17.01 (3.26)	149	21.50 (3.70)	89	26.94 (4.13)	137
<i>L. lactis</i>	8.93 (2.01)	168	19.85 (3.97)	148	17.75 (3.85)	89	25.00 (3.78)	137
<i>L. paracasei</i>	16.67 (3.35)	166	25.44 (4.24)	149	21.00 (4.07)	87	25.81 (4.39)	137
<i>L. plantarum</i>	13.89 (3.27)	167	21.08 (3.70)	149	19.50 (4.18)	89	22.74 (4.32)	137
<i>L. inocua</i>	17.82 (2.69)	167	27.75 (4.72)	149	17.75 (3.78)	87	25.81 (4.19)	137
<i>L. monocytogenes</i>	17.91 (3.42)	167	17.60 (2.86)	149	24.00 (3.78)	89	25.32 (4.24)	136
<i>M. luteus</i>	9.32 (2.43)	164	19.31 (3.51)	148	25.67 (6.39)	83	25.97 (4.18)	137
<i>Mycobacterium sp.</i>	11.58 (2.94)	167	19.61 (4.12)	148	16.75 (6.39)	89	30.16 (4.14)	138
<i>Proteus sp.</i>	10.34 (2.51)	167	22.45 (3.97)	148	25.42 (4.19)	86	27.90 (4.02)	137
<i>P. putida</i>	8.08 (2.25)	167	15.10 (2.89)	149	17.67 (3.78)	86	22.26 (3.86)	137
<i>Salmonella sp.</i>	9.40 (2.36)	167	15.78 (3.64)	149	16.75 (2.84)	89	23.39 (3.60)	138
<i>S. aureus</i>	21.07 (3.53)	166	26.23 (4.57)	149	32.75 (5.60)	89	34.95 (4.11)	137

5 Table 4:

6

			post.mean (95% CI)	pMCMC
Bacteria from feathers and eggshells considered together				
	Complete model			
		Nest type	0.751 (0.202 - 1.341)	0.011
		Origin	0.436 (-0.069 - 0.901)	0.077
		Interaction	-0.191 (-0.962 - 0.533)	0.616
	Final model			
		Nest type	0.647 (0.242 - 1.031)	0.002
		Origin	0.357 (-0.010 - 0.729)	0.057
Bacteria from feathers				
	Complete model			
		Nest type	1.173 (0.071 - 2.313)	0.040
		F treatment (2)	0.428 (-0.809 - 1.616)	0.480
		Feather color	0.898 (-0.179 - 2.044)	0.110
		Interactions		> 0.354
	Final model			
		Nest type	0.596 (0.006 - 1.160)	0.045
		Feather color	0.466 (-0.100 - 1.021)	0.096
Bacteria from eggshells				
	Complete model			
		Nest type	0.766 (-0.521 - 2.142)	0.250
		F treatment (4) M	0.054 (-0.982 - 1.049)	0.913
		F treatment (4) NF	0.391 (-0.833 - 1.619)	0.519
		F treatment (4) P	0.731 (-0.421 - 1.836)	0.206
		Interactions		> 0.390
	Final model			
		Nest type	0.574 (0.020 - 1.158)	0.047

7 Fig 1:

8

9

10 Fig 2:

11

12

13 Fig 3:

14