



# Cohabitation with farm animals rather than breeding effort increases the infection with feather-associated bacteria in the barn swallow *Hirundo rustica*

Attila Fülöp, Csongor I. Vágási and Péter L. Pap

A. Fülöp (<http://orcid.org/0000-0001-5337-336X>) ([fafeldolgozo@gmail.com](mailto:fafeldolgozo@gmail.com)), C. I. Vágási and P. L. Pap, MTA-DE 'Lendület' Behavioural Ecology Research Group, Dept of Evolutionary Zoology and Human Biology, Univ. of Debrecen, Debrecen, Hungary, and Evolutionary Ecology Group, Hungarian Dept of Biology and Ecology, Babeş-Bolyai Univ., Cluj Napoca, Romania.

Feather-associated bacteria are widespread inhabitants of avian plumage. However, the determinants of the between-individual variation in plumage bacterial loads are less well understood. Infection intensities can be determined by ecological factors, such as breeding habitat, and can be actively regulated by hosts via preening. Preening, yet, is a resource intensive activity, and thus might be traded-off against reproductive investment in breeding birds. Here, we studied barn swallows *Hirundo rustica* to assess the bacterial cost of reproduction in relation to nesting site micro-habitats. Barn swallows prefer to breed in the company of large-sized farm animals, although the presence of mammalian livestock in barns assures a warm and humid micro-climate that favours bacterial proliferation. Thus, we experimentally manipulated brood sizes of birds breeding in barns with, or without, farm animals and measured total cultivable bacteria (TCB) and feather-degrading bacteria (FDB) from the plumage. We found that the abundance of feather-associated bacteria (i.e. both TCB and FDB) in females, but not males, breeding in barns with livestock were significantly higher than in conspecifics breeding in empty barns. Plumage bacterial loads, however, were not affected by brood size manipulations in either sex. In addition, we report a negative relationship between both TCB and FDB and hatching date in females, and several sex and seasonal differences in plumage bacterial abundances. Our study is the first to show that breeding micro-habitat (i.e. livestock co-tenancy) has consequences for the abundance of feather-associated bacteria.

Birds are covered by an organized layer of feathers that are colonized by a diverse spectrum of bacteria (Burt and Ichida 1999, Shawkey et al. 2003, 2005, Whitaker et al. 2005, Vereá et al. 2014, Dille et al. 2016, Ruiz-González et al. 2016). Of these bacteria, some are symbiotic and produce beneficial antimicrobial substances (e.g. *Enterococcus* spp.; Ruiz-Rodríguez et al. 2009, Martínez-García et al. 2015), while others are parasitic and either induce internal infections (i.e. bacterial pathogens; reviewed by Hubálek 2004, Benskin et al. 2009) or enzymatically digest the feather keratin matrix (i.e. keratinolytic bacteria; Ramnani et al. 2005). Degradation of feathers by keratinolytic bacteria has been detected both in vivo (Leclaire et al. 2014) and in vitro (Ichida et al. 2001, Ramnani et al. 2005, Ruiz-Rodríguez et al. 2009) and, thus, this group of bacteria is known as feather-degrading bacteria (FDB).

Infection by plumage bacteria (i.e. total cultivable bacteria (TCB), including FDB) has important fitness consequences for hosts (Møller et al. 2012). One recent study found that birds experimentally exposed to higher combined loads of TCB and FDB upregulate their immune responses, suggesting that plumage bacterial infection has important physiological costs (Leclaire et al. 2015). In addition, high

abundances of FDB also impair plumage functioning by causing structural damage to feathers (Fülöp et al. 2016), which may ultimately lead to a decrease in thermal insulation (Brush 1965, Booth et al. 1993), flight efficiency (Swaddle et al. 1996), and/or plumage signalling (Shawkey et al. 2007, 2009, Leclaire et al. 2014, Ruiz-Rodríguez et al. 2015; but see Jacob et al. 2014).

Plumage bacteria are common among birds, although infection intensities are highly variable both among, and within species (Burt and Ichida 1999, Møller et al. 2009, Cziráj et al. 2010, Saag et al. 2011, Fülöp et al. 2016, Kent and Burt 2016). These differences can be determined by the life history or ecology of the hosts (Burt and Ichida 1999, Saag et al. 2011, Kent and Burt 2016). Breeding habitat is an important ecological factor that can have a significant impact on feather bacterial diversity and density (Bisson et al. 2007, 2009, Peele et al. 2009, Saag et al. 2011), because reproduction implies various activities, assuring a direct exchange of bacterial communities between the environment and brood-caring parents. Adult birds may acquire some of their plumage bacteria from the soil (e.g. during feeding or when collecting nest material), which harbours a high variety of bacteria, some of them also capable of feather degradation

(Lucas et al. 2003, Bisson et al. 2007). During incubation, birds can get infected via direct contact with nesting material (e.g. through nest-lining feathers; Peralta-Sánchez et al. 2010, 2014). Breeding habitat can also determine plumage bacterial communities indirectly because the associated microclimatic conditions (e.g. temperature and humidity) affect rates of bacterial proliferation (Stolp 1988).

Birds actively regulate bacterial abundances in their plumage through various behavioural traits (reviewed by Gunderson 2008) and oily secretions from the uropygial gland, spread onto the plumage during preening (Jacob and Ziswiler 1982, Shawkey et al. 2003, Soler et al. 2008, Møller et al. 2009, Martín-Vivaldi et al. 2010). Preening, however, is a time- and energy-consuming activity (Møller 1991, Cotgreave and Clayton 1994, Viblanc et al. 2011), and during the breeding period birds prioritize allocation of their resources into reproduction. Thus, a trade-off between reproductive effort and self-maintenance can arise, which may lead to elevated feather bacterial loads in individuals with high reproductive effort (Alt et al. 2015). However, the trade-off between reproductive effort and self-maintenance and its relation with the nest micro- and/or macro-habitat is not well-understood (but see Alt et al. 2015). Here we experimentally explore this assumed trade-off and the influence of environmental conditions (i.e. habitat) on its resolution.

The barn swallow *Hirundo rustica* is a human-associated aerial insectivorous passerine that breeds inside buildings, typically in barns, sheds, and/or stables (Møller 1994a). The nest is made of mud pellets and the interior is lined with grass and feathers. Barn swallows prefer to nest within barns, where large-sized farm animals (cattle, horses and/or pigs) are present (Møller 1983, 2001, Ambrosini et al. 2002). Birds benefit from the company of livestock, which attract large numbers of flying insects (e.g. Coleoptera, Hymenoptera, Diptera, Hemiptera; Orłowski and Karg 2011, 2013) constituting the birds' main food (Møller 2001, Ambrosini et al. 2002). The presence of livestock also means a higher temperature and more constant microclimate inside barns (Ambrosini and Saino 2010). These two environmental factors combined have been shown to facilitate higher annual reproductive output in barn swallows (Ambrosini and Saino 2010, Grüebler et al. 2010). However, the downside of cohabitation with farm animals is a potentially elevated microbial pressure as a consequence of the warm and humid microclimate. Nevertheless, the possible fitness decrease due to cohabitation is thought to be exceeded by advantages, otherwise this environmental preference would no longer exist.

In this study, we investigated the cost of reproduction in terms of feather bacterial infection in relation to nest site selection in breeding barn swallows by experimentally manipulating the parental effort of birds in different nesting environments (i.e. barns with, or without, livestock). We aimed to test whether: 1) nest site micro-habitat influences plumage bacterial loads of adult birds; 2) an increased investment into reproduction increases the abundance of plumage bacteria; and if 3) the potential cost of nest site selection interacts with that of reproductive effort. Based on the assumptions that livestock presence assures an elevated bacterial pressure in barns, and active regulation of plumage bacterial abundances is a resource demanding activity, we predicted a higher plumage bacterial load in birds cohabiting

with mammalian livestock and in birds with experimentally increased brood size, and an additional cost of nesting with livestock to the bacterial costs associated with reproduction (i.e. bacterial loads predicted by the experimental manipulation).

## Methods

### Study site and period

This study was undertaken in Cojocna village (46°44'54"N, 23°50'0"E; central Transylvania, Romania) during the 2013 and 2014 breeding seasons. At this study site, barn swallows usually breed solitarily in stall buildings, although in some cases, several pairs aggregate into small loose colonies of up to ten pairs. In contrast with western European countries, traditional farming and animal housing still persist in this area, which allows the barn swallows to choose between stall buildings that are either populated or unpopulated by livestock as nesting sites.

### Field procedures and sampling

We captured adult female barn swallows with nest traps on day 1 (day 0 = hatching day) and both parents on day 13 to enable bacterial sampling and biometric measurements. In a few cases of inclement weather, adults were captured with a delay of one or two days (i.e. day 14 or 15); however, these delays did not cause any difference in sampling date between sexes, nesting site micro-habitats, or experimental groups (linear mixed-effects model with year as random factor, sex:  $\chi^2 = 0.00$ ,  $df = 1$ ,  $p = 0.978$ ; nesting site micro-habitat:  $\chi^2 = 2.67$ ,  $df = 1$ ,  $p = 0.102$ ; treatment group:  $\chi^2 = 0.33$ ,  $df = 2$ ,  $p = 0.846$ ).

Immediately after capture, we collected approximately five belly feathers following the method described by Czírják et al. (2013). We collected feather samples from just this region of the body, as it has been previously shown that bacterial abundance from different body regions of the same individual are significantly positively correlated (Gunderson et al. 2009; for some exceptions see Saag et al. 2012). Therefore, bacterial abundances obtained from this body region should offer an adequate representation of the general infestation level on an individual. Feather samples were collected using forceps that had been sterilized with ethanol (70%) and heat (flamed for at least 10 s) and placed in individually labelled sterile Eppendorf tubes. Because females were captured twice, handling during feather sampling and measurements was minimized and we washed our hands regularly with ethanol (70%) to avoid exogenous sample or plumage contamination. We detected no signs of contamination in the bacterial cultures obtained during laboratory analyses (see below), and therefore we concluded that our sampling and handling methods were appropriate. Subsequent to sampling, Eppendorf tubes were kept at ~4°C in a dark box and transported to the laboratory within ten hours, where samples were refrigerated at -20°C until microbiological analyses could be completed. Because all samples were stored without cryoprotectant, abundances of different bacterial groups can be underestimated; however, because all samples

were stored under the same conditions and for similar time periods, we consider that this possible effect did not produce a methodological bias (see also Cziráj et al. 2013).

After feather sampling, we marked all birds with a uniquely numbered aluminium ring, and we measured their body mass ( $\pm 0.1$  g with a Pesola spring balance), tarsus length ( $\pm 0.01$  mm with a digital calliper), wing, and tail length ( $\pm 0.5$  mm with a ruler). The sex of each individual was determined via visual examination of the presence, or absence, of a brood patch, as only females develop a brood patch.

For each nest we determined laying date (i.e. laying of the first egg), clutch size at the onset of incubation, and brood size on days 1 and 12. For each breeding location (i.e. stall and/or barn) we also recorded the presence, or absence, of livestock (dairy cattle, pigs and/or horses) (Ambrosini et al. 2002, 2006, Ambrosini and Saino 2010, Gruebler et al. 2010). Barns populated by mammals are warmer and more humid due to the heat production and evaporation by livestock in contrast with empty barns or with poultry only (unpubl.; Ambrosini and Saino 2010). Thus, we refer to this binary environmental trait as breeding site micro-habitat or micro-environment (*sensu* Ambrosini and Saino 2010). We provide a brief comparison of various reproductive traits of nests between nesting site micro-habitats in Supplementary material Appendix 1.

In 2013 and 2014 we also performed a brood size manipulation experiment using synchronous broods (i.e. in cases where hatching occurs on the same day), increasing or decreasing the original brood size by one nestling on day 1, and using asynchronous broods as controls. The laying date, clutch size, and brood size before manipulation did not differ between the nesting micro-habitats and experimental groups either in the case of females (all  $p > 0.062$ ) or males (all  $p > 0.104$ ), while sample sizes for the decreased, control, and enlarged groups were 26, 36, and 24, respectively, in barns with livestock, and 5, 6, and 5, respectively, in barns without livestock. Barn swallows prefer to breed in barns with farm animals, reflected by higher number of breeding pairs in the populated barns than in the unpopulated breeding locations. However, distribution of experimental groups was not affected by unbalanced numbers of breeding birds between populated and unpopulated barns. The body mass, wing and tail length of females (all measured on day 1) were similar between the nesting site micro-habitats (all  $p > 0.166$ ); however the tarsus was significantly longer in females breeding in unpopulated barns than in those breeding in populated barns (general linear mixed-effects model with year and individual ID as random factors,  $\chi^2 = 15.54$ ,  $df = 1$ ,  $p < 0.001$ ). None of the biometrical parameters measured in females on day 1 differed between experimental groups (all  $p > 0.065$ ). In males the body mass, tarsus, wing and tail lengths (all measured on day 13) were similar between nest site micro-habitats (all  $p > 0.105$ ). Body mass differed significantly between experimental groups, because males from the increased group had lower body mass than males from the control and decreased groups (general linear mixed-effects model with year and individual ID as random factors,  $\chi^2 = 9.74$ ,  $df = 2$ ,  $p = 0.007$ ); tarsus, wing and tail lengths of males were similar between experimental groups (all  $p > 0.132$ ).

## Microbiological analyses

The abundance (used interchangeably with load) of micro-organisms inhabiting the plumage of barn swallows was quantified under sterile conditions using microbiological (i.e. culture-based) techniques. Following the methods described by Cziráj et al. (2010) we measured the abundance of TCB and the abundance of FDB from plumage. To obtain both free-living and attached bacteria, feathers were sonicated using an ultrasonic cleaner (Elma and Co., Elmasonic S15) for 15 min in three cycles (5 min sonication followed by 5 min pause) in 0.8 ml sterile saline solution (0.90% w/v). After sonication, the samples were vortexed for 20 s and the bacterial suspension was transferred into sterile 1.5 ml Eppendorf tubes. Feathers were re-suspended in 0.5 ml sterile saline solution and vortexed again for 20 s. The bacterial suspension obtained was transferred in the same individually labelled Eppendorf tube as before, yielding ~1.3 ml bacterial suspension per individual. The feathers were then dried at 70°C for 24 h and their mass was measured using an analytical balance ( $\pm 0.1$  mg; Ohaus, USA, Ohaus Pioneer) in order to express bacterial loads per unit feather mass.

To quantify the abundance of TCB we used Tryptone Soya Agar (TSA; Biolab, #TSA20500). TSA is a rich, general-purpose (i.e. non-selective) medium that assures the growth of a wide range of both heterotrophic bacteria and fungi. However, in order to inhibit fungal growth on the medium we added 0.1 mg l<sup>-1</sup> cycloheximide (Sigma, #01810) to the TSA (TSAcy). To quantify overall FDB load we used feather-meal agar (FMA). FMA contains 15 g l<sup>-1</sup> feather meal (75% protein hydrolyzed chicken feather meal; Lotus Industrial, Guangzhou, China), 0.5 g l<sup>-1</sup> NaCl, 0.30 g l<sup>-1</sup> 1 K<sub>2</sub>HPO<sub>4</sub>, 0.40 g l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> and 15 g l<sup>-1</sup> agar. Fungal growth was also restrained by adding 0.1 mg l<sup>-1</sup> cycloheximide to the FMA (FMAcy). FMAcy is described to be a selective media for FDB because its single available carbon source is feather keratin (Sangali and Brandelli 2000). Thus, any bacteria growing on the FMAcy must be capable of hydrolyzing feathers using the keratinase enzyme and can be referred to as a 'feather-degrader' (*sensu* Shawkey et al. 2009; see also Gunderson et al. 2009, Møller et al. 2009, Cziráj et al. 2010, 2013, Giraudeau et al. 2013).

Bacterial abundances were measured by spreading 100 µl of the bacterial suspension on plates (two replicates per individual) and incubating at 25°C for three days (in the case of TSAcy), and 14 d (in the case of FMAcy), respectively. After incubation, the number of visible colony-forming units (CFU) on each plate was counted and replicates were averaged. Because accurate counting of colonies was possible in all cases, we used only one dilution. Finally, average CFU numbers were corrected for the total suspension volume and feather mass (unit: CFU/mg feather). All laboratory procedures and counts were performed blindly with respect to the identity of individual birds, nesting site micro-habitats and experimental groups by the same person (AF).

## Statistical analyses

We analysed feather bacterial loads separately for the two microorganism categories, TCB and FDB, throughout. We

tested for differences in bacterial load between birds using general linear mixed-effects models (GLMMs). An overview of the GLMM structures used are presented in Supplementary material Appendix 2; all models used the same random effect structure unless otherwise stated (the exception is model 4 in Supplementary material Appendix 2). To control for random variation in infection intensity between individuals and between study years we entered both terms as random factors. Some birds breed in small colonies, which may also affect infection levels (Møller et al. 2009, Cziráj et al. 2010). Our preliminary analyses (GLMMs with year, individual ID and barn identity nested within study year as random factors) indicated that colony size as a discrete variable was not related to plumage bacterial loads in females on day 1 (TCB:  $\chi^2 = 0.08$ ,  $df = 1$ ,  $p = 0.771$ ; FDB:  $\chi^2 = 0.02$ ,  $df = 1$ ,  $p = 0.876$ ) or on day 13 (TCB:  $\chi^2 = 0.09$ ,  $df = 1$ ,  $p = 0.762$ ; FDB:  $\chi^2 = 0.30$ ,  $df = 1$ ,  $p = 0.581$ ). In males on day 13 colony size was not related to TCB loads ( $\chi^2 = 0.09$ ,  $df = 1$ ,  $p = 0.762$ ); however, it was significantly positively related to FDB loads, but only group dependently (experimental group  $\times$  colony size:  $\chi^2 = 13.82$ ,  $df = 2$ ,  $p < 0.001$ ). Still, because colony size did not confound the effect of other predictors, and had only a weak overall effect on plumage bacterial loads, to simplify our models we did not enter barn identity as a discrete predictor in our models, instead we entered as a nested random factor within study year to control for the potential effect of colony on plumage bacterial loads in birds breeding in the same barn.

First, we tested for differences in bacterial load between females breeding in barns with, or without, livestock on day 1 also controlling for hatching date and individual variation in body condition (Supplementary material Appendix 2, model 1). Second, we ran the same analyses for bacterial loads measured on day 13 for both females and males with the exception that brood size manipulation was entered as fixed factor. Since pairs are non-independent samples, microbiological data for males and females measured on day 13 were analysed separately (Supplementary material Appendix 2, models 2, 3). Third, we tested for differences in bacterial load between females and males on day 13, by using a pooled data set of females and males, with the same model construction as before (i.e. second set of analyses), except that we included sex as an additional fixed effect. In this model we also controlled for the similarity in infection between males and females within pairs, as plumage bacteria can be transmitted between birds breeding in the same nest (Kulkarni and Heeb 2007, Gunderson et al. 2009). Thus, we entered nest ID nested within study year in the model as an additional random effect (Supplementary material Appendix 2, model 4). Fourth, we tested whether bacterial abundances differed between the two sampling events in females (Supplementary material Appendix 2, model 5). In this model, bacterial load was entered as dependent variable, while nesting site micro-habitat, experimental group, hatching date, and sampling event were entered as fixed terms. Finally, we tested whether the change in the bacterial abundances (either TCB or FDB) in females between the two sampling events was determined by nesting site micro-habitat and/or experimental group. To do this, we first created a variable indicating the change in the abundance of bacterial load (i.e.  $\Delta_{TCB}$  and  $\Delta_{FDB}$ ) by subtracting the abundance values of day

1 from those of day 13. Thus,  $\Delta_{TCB}$  and  $\Delta_{FDB}$  values were entered as dependent variable in the models, while nesting site micro-habitat, experimental group, hatching date and the change in body condition ( $\Delta_{SMI}$ ) were entered as fixed terms (Supplementary material Appendix 2, model 6).  $\Delta_{SMI}$  was calculated in a similar manner as the changes in bacterial abundances presented above. We tested all second-order interactions of the fixed effects in each model.

We built saturated models in all cases, that were simplified to minimum adequate models using the R function ‘drop1’ following a backward-stepwise elimination procedure dropping non-significant ( $p > 0.05$ ) predictors and/or interactions with the largest p-value. Exceptions were those non-significant main effects that figured in significant interactions. Prior to analyses, TCB and FDB loads were box-cox ( $x + 1$ ) transformed to reach residual normality in the models. Body condition was expressed as scaled mass index (SMI) following Peig and Green (2009). SMI is a size-corrected body condition index calculated by the function  $SMI_i = M_i \times (L_0/L_i)^{2.341}$ , where  $M_i$  and  $L_i$  is the body mass and tarsus length of individual  $i$ , respectively,  $L_0$  is the mean tarsus length of the sample, and the exponent 2.341 is the slope of the SMA regression between log-body mass and log-tarsus length calculated from a sample of  $n = 319$  individuals (137 males and 182 females) captured between 2013–2015 in the same population (R package ‘lmodel2’; Legendre 2014). All analyses were carried out in the R statistical environment ver. 3.2.3 (R Core Team). GLMMs were constructed using the R package ‘lme4’ (Bates et al. 2015) and type II analysis-of-variance statistics were obtained using Wald Chi-square tests as implemented in the R package ‘car’ (function ‘Anova’; Fox and Weisberg 2011).

## Data deposition

Data available from the Dryad Digital Repository: <<http://dx.doi.org/10.5061/dryad.pr7bk>> (Fülöp et al. 2017).

## Results

The abundance of bacteria associated with the feathers of female and male barn swallows was highly variable (Table 1). We found a significant positive correlation between TCB and FDB on day 1 in females (Spearman’s rank correlation

Table 1. Summary statistics for the abundance of different groups of feather-associated bacteria isolated from feathers of adult barn swallows *Hirundo rustica* from Cojocna, Romania. Values from the two study years (2013, 2014) are pooled; day 0 = hatching day; unit of measure is number of colony forming units/mg feather (CFU/mg feather).

| Group of bacteria          | Mean | SE   | Median | Range      | n  |
|----------------------------|------|------|--------|------------|----|
| Female at day 1            |      |      |        |            |    |
| Total cultivable bacteria  | 6.63 | 1.08 | 3.40   | 0.13–87.70 | 95 |
| Feather-degrading bacteria | 0.83 | 0.14 | 0.38   | 0.00–11.37 | 95 |
| Female at day 13           |      |      |        |            |    |
| Total cultivable bacteria  | 5.53 | 0.73 | 2.73   | 0.15–36.00 | 95 |
| Feather-degrading bacteria | 0.92 | 0.28 | 0.27   | 0.00–24.75 | 95 |
| Male at day 13             |      |      |        |            |    |
| Total cultivable bacteria  | 3.82 | 0.96 | 1.51   | 0.00–83.39 | 92 |
| Feather-degrading bacteria | 0.41 | 0.07 | 0.17   | 0.00–5.37  | 92 |

on raw data,  $r = 0.856$ ,  $n = 95$ ,  $p < 0.001$ ) and on day 13 in both sexes (females:  $r = 0.663$ ,  $n = 95$ ,  $p < 0.001$ ; males:  $r = 0.726$ ,  $n = 92$ ,  $p < 0.001$ ).

The abundance of both TCB and FDB measured on day 1 was significantly higher in females breeding in populated barns compared to birds breeding in unpopulated barns (Table 2, Fig. 1a, b). The abundance of TCB and FDB measured on day 13 was higher in females breeding in populated barns than in unpopulated sites, although this difference was non-significant (Table 2, Fig. 1c, d). Differences in bacterial abundances between breeding sites were independent of brood size manipulation (breeding site micro-habitat  $\times$  brood size manipulation for TCB:  $\chi^2 = 0.05$ ,  $df = 2$ ,  $p = 0.974$ ; for FDB:  $\chi^2 = 0.27$ ,  $df = 2$ ,  $p = 0.870$ ), and hatching date was significantly negatively related to the abundance of both TCB and FDB of females on day 13 (Table 2; TCB:  $\beta$  (SE) =  $-0.009$  (0.003); FDB:  $\beta$  (SE) =  $-0.003$  (0.000)). The abundance of TCB and FDB in males on day 13 were not predicted by any of the factors measured (Table 2, Fig. 1e, f), and the interaction between breeding site micro-habitat and experimental brood size manipulation was non-significant (TCB:  $\chi^2 = 3.26$ ,  $df = 2$ ,  $p = 0.195$ ; FDB:  $\chi^2 = 5.61$ ,  $df = 2$ ,  $p = 0.060$ ).

The abundance of TCB and FDB, respectively, measured on day 13 were significantly higher in females than in males (TCB:  $\chi^2 = 20.20$ ,  $df = 1$ ,  $p < 0.001$ ; FDB:  $\chi^2 = 12.02$ ,  $df = 1$ ,  $p < 0.001$ ), and there was a significant difference in the abundance of both on females between day 1 and day 13, bacterial abundances being higher on day 1 than on day 13 (TCB:  $\chi^2 = 10.89$ ,  $df = 1$ ,  $p < 0.001$ ; FDB:  $\chi^2 = 10.80$ ,  $df = 1$ ,  $p = 0.001$ ). Yet, this decrease was similar in birds breeding in populated and unpopulated barns (breeding site micro-habitat  $\times$  sampling; TCB:  $\chi^2 = 0.42$ ,  $df = 1$ ,  $p = 0.516$ ; FDB:  $\chi^2 = 1.30$ ,  $df = 1$ ,  $p = 0.254$ ) and between the experimental groups (brood size manipulation  $\times$  sampling; TCB:  $\chi^2 = 2.47$ ,  $df = 2$ ,  $p = 0.289$ ; for FDB:  $\chi^2 = 0.98$ ,  $df = 2$ ,  $p = 0.610$ ).

The change in the abundance of TCB ( $\Delta_{TCB}$ ) in females between day 1 and day 13 was similar between nesting site micro-habitats ( $\chi^2 = 2.92$ ,  $df = 1$ ,  $p = 0.087$ ) and between experimental groups ( $\chi^2 = 3.48$ ,  $df = 2$ ,  $p = 0.175$ ).

The non-significant interaction between breeding site micro-habitat and brood size manipulation ( $\chi^2 = 1.26$ ,  $df = 2$ ,  $p = 0.530$ ) shows that  $\Delta_{TCB}$  was similar in females from different experimental groups and from the different nesting micro-habitats. No difference in the change of the abundance of FDB ( $\Delta_{FDB}$ ) between females breeding in barns with, and without, farm animals was apparent ( $\chi^2 = 1.14$ ,  $df = 1$ ,  $p = 0.284$ ), and experimental groups also had similar values ( $\chi^2 = 0.91$ ,  $df = 2$ ,  $p = 0.631$ ). The interaction between breeding site micro-habitat  $\times$  brood size manipulation was also non-significant ( $\chi^2 = 4.62$ ,  $df = 2$ ,  $p = 0.099$ ).

## Discussion

### Feather bacterial loads, nest site micro-habitat and experimental treatment

We demonstrate that female barn swallows breeding in barns with farm animals harbour higher plumage bacterial loads than those breeding in unpopulated barns. This difference was apparent for both groups of bacteria in females at hatching, but not in males. The effect of breeding site micro-habitat on plumage bacteria in females can be explained in at least two ways. First, the presence of livestock in barns assures the occurrence of a high variety of microorganisms (e.g. via faeces, feedlots; Salanitro et al. 1977, Dowd et al. 2008, Durso et al. 2010, 2011, Costa et al. 2012, Rudi et al. 2012), which can be transmitted to birds through nesting material or through the air. In this case, direct exchange of bacterial communities may take place. Second, large-sized farm animals may affect the bacterial loads of cohabiting birds through the microclimate they establish within a restricted space, such as the barn. Heat production by mammalian livestock can be substantial (Holmes and Mount 1967, Gebremedhin et al. 2008), and the increased temperature and humidity of the nesting site may influence reproduction and transmission of plumage bacteria. No previous studies have examined this relationship in plumage bacteria, although research on the bacterial assemblages of eggs and nests does provide evidence that micro-climatic factors

Table 2. The change in the abundance of total cultivable bacteria and feather-degrading bacteria of barn swallows *Hirundo rustica* in relation to the presence of farm animals in the breeding site micro-habitat, brood size manipulation (treatment), hatching date, and year at day 1 (female) and at day 13 (female and male); day 0 = hatching day.

|                             |    | Total cultivable bacteria |                  | Feather-degrading bacteria |              |
|-----------------------------|----|---------------------------|------------------|----------------------------|--------------|
|                             | df | $\chi^2$                  | p                | $\chi^2$                   | p            |
| Female at day 1             |    |                           |                  |                            |              |
| Breeding site micro-habitat | 1  | 4.86                      | <b>0.027</b>     | 8.01                       | <b>0.004</b> |
| Hatching date               | 1  | 0.50                      | 0.476            | 2.61                       | 0.105        |
| Year                        | 1  | 11.40                     | <b>&lt;0.001</b> | 6.74                       | <b>0.009</b> |
| Female at day 13            |    |                           |                  |                            |              |
| Treatment                   | 2  | 2.66                      | 0.263            | 0.84                       | 0.654        |
| Breeding site micro-habitat | 1  | 2.22                      | 0.135            | 2.29                       | 0.130        |
| Hatching date               | 1  | 7.53                      | <b>0.006</b>     | 9.95                       | <b>0.001</b> |
| Year                        | 1  | 4.29                      | <b>0.038</b>     | 0.00                       | 1.000        |
| Male at day 13              |    |                           |                  |                            |              |
| Treatment                   | 2  | 5.12                      | 0.077            | 0.32                       | 0.848        |
| Breeding site micro-habitat | 1  | 1.35                      | 0.245            | 2.07                       | 0.149        |
| Hatching date               | 1  | 0.10                      | 0.742            | 0.42                       | 0.513        |
| Year                        | 1  | 10.65                     | <b>0.001</b>     | 0.90                       | 0.342        |

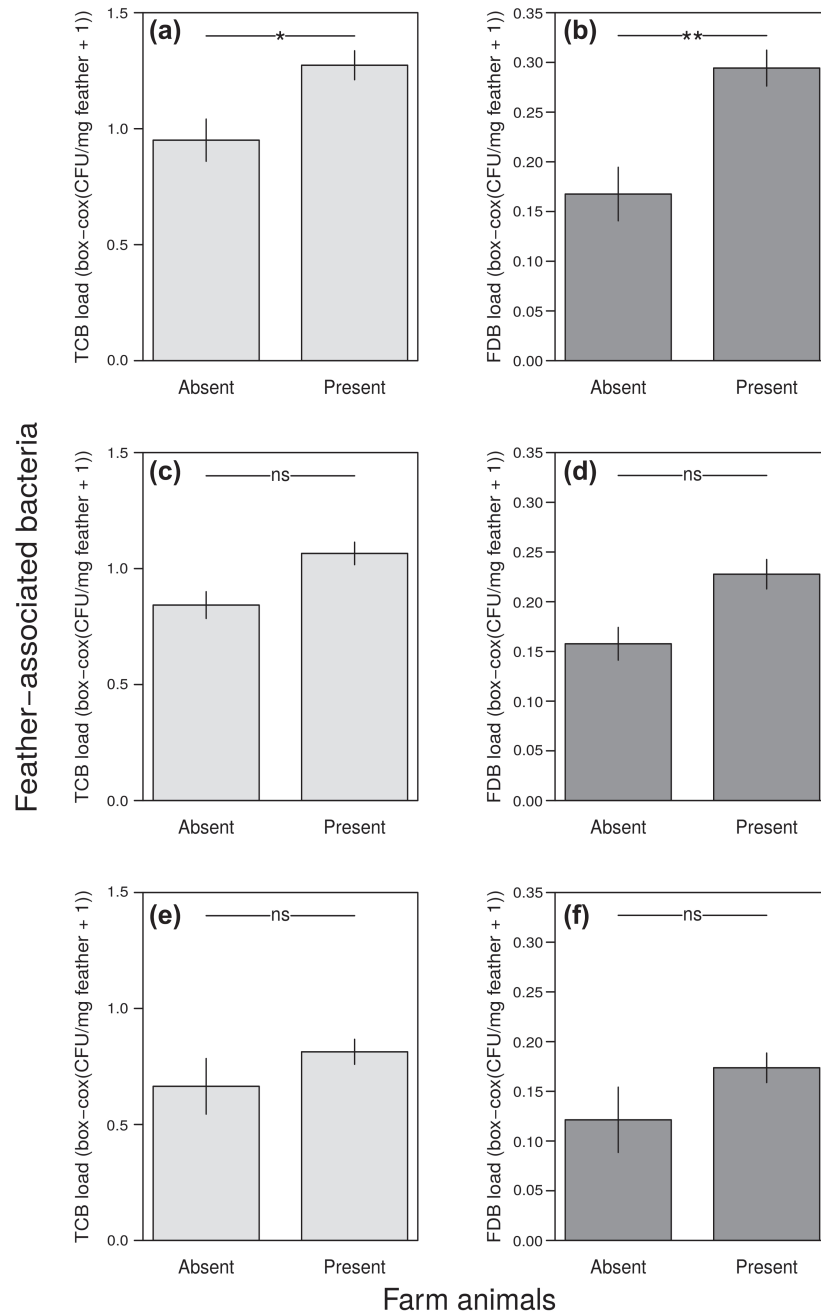


Figure 1. Abundance of different groups of feather-associated bacteria on barn swallows *Hirundo rustica* breeding in barns populated, or unpopulated, by farm animals. Barplots present the abundance of total cultivable bacteria (TCB) and that of the feather-degrading bacteria (FDB) for females at day 1 (a, b), females at day 13 (c, d) and males at day 13 (e, f). Mean + SE are presented. Asterisks denote significant differences between groups: \* $p < 0.05$ , \*\* $p < 0.01$ , ns – not significant.

influence bacterial infection intensities in nests through both temperature and humidity (Berger et al. 2003, Cook et al. 2005, D’Alba et al. 2010, Ruiz-de-Castañeda et al. 2011, Soler et al. 2015). Thus, while our results suggest that micro-environmental components are important in shaping the plumage bacterial abundance, underlying mechanisms still need to be clarified.

We have found limited support for the presence of a trade-off between plumage maintenance and reproductive effort in female barn swallows, because the change in bacterial abundances between day 1 and day 13 were similar

between experimental groups. Further, the costs associated with brood care in terms of feather bacterial infection were similar between different nest micro-environments (i.e. barns inhabited or not by livestock). Thus, our results do not support those in the studies of Lucas et al. (2003) and Alt et al. (2015), who showed that experimentally varied brood sizes influence the plumage bacterial densities of free-living bacteria, but not those of attached bacteria. Differences between our results and those of these two previous studies may be because earlier works used culture-independent techniques to quantify abundances of plumage bacteria, while we used

culture-based methods. Culture-based methods are precise, yet, less sensitive compared to direct cell counts (i.e. flow-cytometry; Pinder et al. 1990, Hoefel et al. 2003). Also, in our study, free-living and attached bacteria were quantified together (see Methods), thus, changes in bacterial abundances in one of the two groups could remain undetected. Another possible explanation for the lack of differences between experimental groups is the reduced cost of bacterial infection in our barn swallow population. This could be the result of multiple factors. First, compared to previous studies on the same species, we have found significantly lower bacterial infection intensities (cf. Møller et al. 2009, Cziráj et al. 2010), and therefore, we might suppose that birds can cope with these low levels of bacterial infection without compromising reproduction or self-maintenance. We note, though, that low bacterial intensities can be a result of the sample storage conditions as well, which may negatively influence bacterial viability (see Methods). Second, population and/or biogeographic differences might exist in the costs associated with plumage bacterial infection. However, we can only speculate on these differences since these issues have only been superficially investigated (Burt and Ichida 2004, Bisson et al. 2007).

### Feather bacterial loads and hatching date

Our results show a negative relationship between bacterial load (both TCB and FDB) and hatching date in females on day 13 which was not apparent on day 1. Our findings are in contrast with the results of Møller et al. (2015) and Soler et al. (2015), who both found positive associations between bacterial density and laying date in skin bacteria (i.e. beak and claws of goshawks *Accipiter gentilis*) and egg bacteria (i.e. in naturally incubated magpie *Pica pica* nests), respectively. However, Soler et al. (2015) found a negative relationship between egg bacterial density in magpie nests without incubation activity. This negative relationship between plumage bacterial load and hatching date in our study can most probably be explained by a significant increase of daylength during the breeding period (daylength on 1 April: ca 13 h, on 31 July: ca 15 h; data from <www.sci.fi/~benefon/sol.html>). As daylength increases, the sunlight exposure time of adult birds also increases proportionally. Sunlight inhibits the growth of feather bacteria (Saranathan and Burt 2007). Therefore, the elevated level to sunlight exposure of adult birds over the breeding season may negatively affect plumage bacterial abundances. This explanation might also sound plausible for the negative relationship found between egg bacterial loads and laying date in unincubated magpie nests by Soler et al. (2015), since sunlight could inhibit bacterial proliferation on exposed eggs in the nest, as well. A second possible explanation for the negative relationship between plumage bacterial load and hatching date we found may be that the birds that start to reproduce earlier in the season devote less time to self-maintenance because of a tighter schedule after arriving on the breeding grounds. However, we cannot test this assumption because we recorded only laying and hatching data, but not the arrival times of adult birds on the breeding grounds. Nevertheless, this second explanation seems intuitively less likely because arrival date, laying date, and hatching date are all strongly correlated

(Ninni et al. 2004), and because early breeding has known to have multiple fitness benefits in barn swallows (Møller 1994a, b).

### Sex and seasonal differences in feather bacterial loads

We have found sexual differences in plumage bacterial loads; females exhibit significantly higher overall infection levels (both TCB and FDB) than males on day 13. These higher infection levels in females can be explained by the differing sex roles in this species; just females incubate in European barn swallows and, at the same time, males usually stay out of the barn and only make short visits to the nest (Jones 1987, Turner and Rose 1989, Smith and Montgomerie 1992). Subsequent to hatching, during the first few days of chick development, females spend considerably more time in the nest than males (unpubl.; Møller 1994a), another behavioural difference that means that makes females more exposed to the microorganisms associated with the nest and/or nest site during incubation and chick-rearing periods. Our results regarding higher FDB infestation in females is in line with former studies on this species (Møller et al. 2009, Cziráj et al. 2010); however, in contrast, no previous studies have reported significant differences between the sexes in other cultivable bacteria groups than FDB.

We have shown that in female barn swallows, the abundance of TCB and FDB decreased significantly during the chick-rearing period, supporting former findings on wild birds infested with free-living and attached bacteria (Alt et al. 2015). Bacterial abundances from the plumage are actively regulated through several self-cleaning activities (Cziráj et al. 2013, Leclaire et al. 2014, but see Giraudeau et al. 2013). Yet, during incubation the behavioural limitation of incubating females to preen and to sunbath might result in an elevated level of bacterial infection at hatching, compared to the infestation intensities of adults later in the breeding season or to non-reproducing birds (as suggested by Alt et al. 2015). This explanation is supported in part also by a study on great tits *Parus major*, where Kilgas et al. (2012) found that the plumage bacterial loads increase significantly during the nest building period and peak when the nest is completed. Since both egg laying and incubation are activities which impose time limitations for self-maintenance in females, the elevated bacterial loads acquired during nest building might still persist until the end of incubation (i.e. hatching). Therefore, the decrease of the abundance of bacteria on females from day 1 to day 13 might mirror this phenomenon. This might also explain the lack of difference we found in bacterial loads between nesting site micro-habitats on day 12 in females, and the lack of relationship between bacterial densities and hatching date on day 1, in contrast with that from day 13.

### Conclusions

We show for the first time that the abundance of feather-associated bacteria reflect the micro-habitat characteristics of the breeding site. We report a negative relationship between bacterial load and hatching date, and several sex- and seasonal differences in plumage bacterial abundances. Yet, we

fail to detect a trade-off between reproduction and defence against plumage-dwelling bacteria, as well as an interaction between nesting site micro-habitat and reproductive effort. Our results suggest that in barn swallows the resource allocation costs associated with reproduction are minor at least in terms of infection by plumage bacteria. This observation is also supported by the preference of barn swallows for barns inhabited by livestock, even though that implies an elevated exposure to microbial infections.

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Supplementary material (Appendix JAV-01262 at <[www.avianbiology.org/appendix/jav-01262](http://www.avianbiology.org/appendix/jav-01262)>). Appendix 1–2.