Acquisition of uropygial gland microbiome by hoopoe nestlings

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Abstract

Mutualistic symbioses between animals and bacteria depend on acquisition of appropriate symbionts while avoiding exploitation by non-beneficial microbes. The mode of acquisition of symbionts would determine, not only the probability of encountering, but also evolutionary outcomes of mutualistic counterparts. The microbiome inhabiting the uropygial gland of the European hoopoe (Upupa epops) includes a variety of bacterial strains, some of them providing antimicrobial benefits. Here, the mode of acquisition and stability of this microbiome is analyzed by means of Automated rRNA Intergenic Spacer Analysis and two different experiments. The first experiment impeded mothers’ access to their glands, thus avoiding direct transmission of microorganisms from female to offspring secretions. The second experiment explored the stability of the microbiomes by inoculating glands with secretions from alien nests. The first experiment provoked a reduction in similarity of microbiomes of mother and nestlings. Interestingly, some bacterial strains were more often detected when females had not access to their glands, suggesting antagonistic effects among bacteria from different sources. The second experiment caused an increase in richness of the microbiome of receivers in terms of prevalence of Operational Taxonomic Units (OTUs) that reduced differences in microbiomes of donors and receivers. That occurred because OTUs that were present in donors but not in receivers incorporated to the microbiome of the latter, which provoked that cross-inoculated nestlings got similar final microbiomes that included the most prevalent OTUs. The results are therefore consistent with a central role of vertical transmission in bacterial acquisition by nestling hoopoes, and support the idea that the typical composition of the hoopoe gland microbiome is reached by the incorporation of some bacteria during the nestling period. This scenario suggests the existence of a coevolved core microbiome composed by a mix of specialized vertically transmitted strains and facultative symbionts able to coexist with them. The implications of this mixed mode of transmission for the evolution of the mutualism are discussed.
Introduction

The life of every animal is conditioned by interactions with an extremely high variety of ubiquitous bacteria that are able of colonizing and exploiting any nutritional resource in any chemical form [1]. Many of those potential partners are enemies, while some others do not affect the host, or even provide benefits. Evolutionary processes should therefore select for strategies that reduce encounters with parasitic microorganisms; and increase the probability of recruitment of the beneficial ones [2]. Benefits of the association with bacteria can have multiple forms: from nutritive resources to defensive products [3] or inactivation of toxic residues [4]. Hosts provide beneficial microorganisms with physical support and resources for growth, which could also be exploited by undesired parasitic-symbiont partners (e.g. [5]). Even though the risk of exploitation of host resources by non-mutualistic symbionts is considered a destabilizing force of mutualistic relationships (reviewed in [6]), mutualisms with bacteria are ubiquitous and remain during evolutionary time [4] indicating the existence of mechanisms preventing parasitic exploitation.

One of the main questions in evolutionary ecology is to disentangle mechanisms explaining mutualistic coevolutionary relationships while preventing the intromission of the surrounding potential exploiters. Several studies have modelled the stability of mutualisms and reviewed the current evidence for these systems to be stable [7-11]. In this sense, vertical transmission from parents would reduce the risk of exploitation and provide descendants with an adequate pool of collaborators [2,12-15]. However, hosts that acquire their mutualistic symbionts horizontally are more compromised by parasites' exploitation because they need to recruit appropriated symbionts from the surroundings, which depend upon availability [16,17]. Indeed, they need to collect beneficial strains while avoiding colonization by parasites ("partner choice" [18]). Such selection procedures involve recognition, attachment, regulation of immune responses, and control of nutrient release for the symbiont [17,19,20]. Horizontal transmission may also imply some advantages since it allows a plastic selection of cooperators and the best option may differ for different environmental conditions (e.g. [21]). For instance, bacterial symbionts producing more antimicrobials that benefit hosts can be established automatically within the symbiont community.
of hosts ("byproduct cooperation" [4,22]) and impede colonization of parasitic symbionts. Therefore, even complex communities resulting from horizontal transmission reach stability, as shown by the microbiome of rumen of mammals that has demonstrated high inertia and resilience after perturbations, including the inoculation of alien communities [23]. Some models have even suggested the possibility that mixed communities, including both vertically and horizontally acquired strains, would further guarantee the stability of the association [24]. The scenarios described above, therefore, indicate that knowing the mode of acquisition of symbionts is essential for understanding functioning, stability and evolution of mutualistic associations.

An interesting model for exploring the importance of mechanisms of symbiont acquisition is the system formed by the hole-nester European hoopoe (Upupa epops) and the bacteria living in its uropygial gland [25-27]. The uropygial secretion of Upupiformes (hoopoes and their relative woodhoopoes) is special (dark and odorous) due to the presence of symbiotic bacteria in the gland [28,29]. In the case of the hoopoes, their symbionts change the composition of the oil produced by the bird, generate metabolites with antimicrobial properties [30-33], and positively affect hatching success [27] when the female voluntarily impregnates their eggshell during incubation [34,35]. The special secretions with bacteria are only maintained during the stay within the nest-hole of females and nestlings [29] and, therefore, must be acquired every breeding season. Results of previous Automated rRNA Intergenic Spacer Analysis (ARISA) showed that both females and nestlings host a bacterial community formed by a group of eight highly prevalent strains accompanied by a long list of Operational Taxonomic Units (OTUs) in a range of frequencies of appearance from 50% to 10% [36]. Moreover, cross-fostering experiments suggested that nestlings are able to acquire new symbiont strains when moved to a different nest [36,37]. In this way, the stability of the bacterial community could depend on the possibility of encountering new strains and, thus, vertical and horizontal acquisition of symbionts may explain microbiomes of nestlings [36,37]. However, the factors affecting the dynamic of colonization of the gland by bacterial symbionts are not known, and differences in competitive ability of different bacterial strains reaching the uropygial gland are supposed to affect their success colonizing and growing in the uropygial secretion [38]. This
hypothesis can be experimentally tested by inoculating glands with secretions harboring bacteria coming from different nest environments [16,39]. Such experiment will also allow identifying particular bacterial strains able to colonize uropygial glands where they were not present.

In this context, glands of nestlings close to fledge were inoculated with secretion from alien nestlings that acquired their microbiome in different nests. Assuming some level of vertical transmission of symbionts, to increase differences among donor and receptor of inocula, cross-inoculations were performed between nestlings from nests with mothers that had full or experimentally restricted access to their uropygial gland. Impeding bird access to their glands by covering them has previously been used with success to test effects of uropygial secretions in several studies [34,35,40,41]. We have used here such an approach as a reversible alternative to the extirpation of glands (e.g. [42]). In this way, two crossed experimental treatments were performed: (1) restriction of gland access for females and (2) cross-inoculation of nestling secretions. Thus, only a fraction of experimentally inoculated nestlings had the possibility of acquiring bacterial strains from their mothers' glands. This experimental approach also allow testing the influence of vertical transmission (i.e. female treatment) on the effect of experimental inoculation of nestling uropygial glands.

With this experimental design, the following hypotheses and predictions were tested: (a) there is vertical transmission of bacteria directly from female to offspring glands if the composition of microbiomes of nestlings is affected by experimental covering of female glands. (b) If hoopoes harbor a particular co-evolved microbial assemblage in secretions, the cross-inoculation experiment should result in the colonization of glands by complementary bacterial strains, commonly detected in hoopoes. This should particularly be the case in nests with experimental females where the vertical transmission of commonly detected strains to uropigial gland of nestlings is restricted. This scenario also predicts an increase in microbiome similarity of secretions after the inoculation experiment. (c) On the other hand, if different communities are the result of different optimal microbiomes for different individuals (see [23]), there should be evidence of stability (i.e., no effects of cross-inoculation on similarity) and a limited probability of colonization by new bacterial
strains. (d) Finally if different strains differ in the ability to colonize and establishing in the
microbiome of the uropygial gland, we should find that the effects of experimental restriction of
vertical transmission or the inoculation experiment on particular microorganisms, will depend on
these abilities (i.e. identity).

Material and Methods

Study area

The study was performed during the 2010 and 2012 breeding seasons, in a captive population of
hoopoes maintained since 2008. The captive pairs were distributed in three localities with
appropriate facilities in southern Spain; the Faculty of Sciences of the Granada University
(37°18′N, 3°61′W, Granada province), the Hoya of Guadix (37°31′N, 3°12′W, Granada province),
and the Finca Experimental la Hoya of EEZA-CSIC (36°84′N, 2°47′W, Almería province). All
females and nestlings were ringed with numbered aluminum rings for individual recognition.
Breeding pairs were housed in independent cages of at least 3m x 2m x 2m installed in the open,
scattered and isolated to avoid interactions between pairs and ensure successful breeding. Cages
had access to soil and live food (crickets, fly larvae and meat (beef heart)) ad libitum. Each pair had
a nest-box made in cork (internal height * width * depth: 35 * 18 * 21 cm, bottom-to-hole height:
24 cm, entrance diameter 5.5 cm) with a two cm layer of thin pine bark pieces on the bottom. Cages
were visited and inspected daily from mid-February to the end of July.

Several studies have found that microbiomes of wild and captive populations of animals
differ (reviewed in [43]). We have previously shown that microbiomes of wild hoopoes are slighter
richer than those of captive hoopoes [44,45]. Such differences, however, only affected to a small
fraction of the microbiome bacterial strains and, thus, we believe that our experiments in captivity
allow to infer valid conclusions on the way hoopoes acquire their uropygial gland microbiome in
the wild.
Two experimental approaches were used; one to restrict the vertical transmission of bacterial communities from females to offspring, and the other to detect possible changes in the microbiome composition of the uropygial secretion of nestlings along their stay in the nest after inoculation with alien secretions.

In Experiment 1, females' access to the uropygial gland was manipulated from the start of laying until they finished brooding (Fig 1a, see [34] for details of the method for covering female glands). Thus, experimental females could not use the uropygial secretion to cover eggs [34,35] or to preen nestlings or themselves [36], and the direct or indirect transmission of bacteria from female gland to those of their nestlings was impeded. Two groups of control birds were established. Control I females were provided with a similar structure as experimental females, but not covering the gland entrance and thus not preventing normal access to secretions. A third group of breeding females (control II) were visited and handled at the same rate as those of experimental and control I groups. Females were assigned to a particular treatment in sequence by laying date, alternating experimental with one of the two control treatments, which were alternatively selected to get a similar sample size between experimental females and both control groups combined. Treatments were balanced within sub-populations. In order to test the effect of the experiment on the transmission of symbiotic bacteria from mother to offspring, the microbiomes of their uropygial glands were compared. Female glands were sampled on the day of start of laying, before manipulation, and those of nestlings on the 16th day of the nestling period (Fig. 1a).

Experiment 2 was performed with 16 days old chicks of mothers that were subject to the Experiment 1. After sampling secretions, half of the nestlings in each brood (Controls) were inoculated with 3 µl of their own secretion with a sterile micropipette within the papilla of the gland, while the other half of the brood (Experimentals) were inoculated with 3 µl of secretion proceeding from nestlings of a different nest. Except for the few cases in which wild broods were
used as donors of secretions, mothers of donor nestlings were under different experimental treatment than those of receivers. The 3 µl of secretion used for inoculation were pipetted from a sterile microfuge tube where the available secretion of each nestling was previously homogenized. Nests were paired by hatching date so that secretions were interchanged between nestlings of similar ages. Experimental and control treatments were assigned to nestlings in each brood alternating along the size hierarchy. The oldest nestlings of the two broods within a duplica were assigned the same treatments, but alternated between successive pairs of nests. To estimate the change in microbiomes caused by the experiment, nestlings were sampled again after seven days (Fig. 1b).

We only considered breeding attempts from captive females for which initial and final secretions of at least one control and one experimental nestling were obtained. Only one nestling per treatment (the oldest surviving on day 23) was included per nest. Complete information (initial and final samples of one control and one experimental nestling) was obtained for 18 nests, but the information on the communities of females and donors only for 17 and 15 nests respectively. For this reason, sample sizes differ among performed analyses. All broods were from different females, except for two of the same female in 2012 that received two different treatments (experimental and control II) which have been considered as independent information in the analyses.

**Sampling protocol**

Incubating females and nestlings were caught by hand and, after sampling uropygial secretions (see [27] for sampling method), were released again within the nest box. The secretion was transferred to a sterile microfuge tube, homogenized with a micropipette and stored at 4º C until used for inoculation in the following 2 hours. Other two aliquots of 5 µl were separated in different sterile microfuge tubes, one for estimation of the load of mesophilic bacteria by culture methods, and other was frozen for the molecular analysis of the microbiome composition.

**Bacterial load**
To estimate the load of cultivable bacteria, secretions were cultured in Petri dishes with Tryptic Soy Agar (TSA) medium. Plates were inoculated, within the same day of sampling, with 5 µl of serial dilutions of the secretions in sterile Phosphate Buffer Saline (PBS), and cultures were incubated aerobically at 37 °C for 24 h. Bacterial load was expressed as number of colony-forming units (CFUs) per µl of secretion.

**Molecular analyses**

Bacterial DNA was extracted from uropygial secretions with a commercial KIT (The FavorPrep™ Blood Genomic DNA Extraction Kit, Favorgen). Automated rRNA Intergenic Spacer Analysis (ARISA) was used to characterize the composition of bacterial communities (see Supplemental Material-I for details). The purpose here is not to describe the composition of hoopoe uropygial microbiomes, but explore influence of mothers (i.e. vertical transmission) and of later colonization (i.e. inoculation) on the microbiome of nestling hoopoes, with particular emphasis on bacteria of known beneficial effects (i.e. enterococci). Thus, the use of ARISA is appropriate here. Furthermore, particular OTUs from ARISA have previously been suggested to interact with the life history characteristics of hoopoes [36,46,47], which will allow interpretation of results of particular OTUs in the performed experiment in relation to what is already known of their role in these communities. Given that it is possible that different fragment lengths came from identical bacterial strains or species, except when indicated we use them as Operational Taxonomic Units (OTUs) of unknown taxonomic affiliation. The generated presence-absence matrix was used in all analyses.

The hoopoe nestlings involved in the inoculation experiment harbored a total of 82 OTUs, 49 of which were present only in up to three individuals before the experimental inoculation of their glands. For the calculation of richness only the 33 OTUs present in at least four of those samples were considered [47]. Moreover, to understand the dynamic of the main assemblages naturally encountered in hoopoes, and for analyses considering differences in composition among bacterial communities, only those with at least 50% prevalence were considered (for this calculation we only included samples of females and the first available for nestlings of control females). There were 16
OTUs fulfilling this criterion, which are named by the length of their ITS fragment (OTUs 182, 242, 254, 278, 306, 310, 326, 330, 346, 350, 406, 422, 466, 474, 534 and 566), which hereafter are referred as the “core microbiome” of secretions. The main effects of both experiments on microbiome composition did not change if we use the 33 OTUs with prevalence >3 instead.

Statistical analyses

Three different estimates have been used characterizing the microbiome of the uropygial secretion of nestlings: richness (number of OTUs), composition (matrices of similarities among microbiomes using Jaccard index) and cultivable bacterial load (CFUs/µl). Both richness and bacterial load approached normal distributions after log transformation (Kolmogorov-Smirnov tests p > 0.2) and were thus used in General Linear Models (GLMs hereafter).

To detect possible opposed effects of experiments on different components of the microbial communities, sample positions in the multiscale Jaccard distance space were decomposed in two or three first axes of Principal Coordinates Analyses (PCo). The coordinates (i.e. scores) of each sample were used as dependent variables and the statistical tests performed with Primer7.

Before the inoculation treatments, bacterial load, richness and composition of microbiomes of the secretion of nestlings in nests of females under the two control treatments did not differ significantly (GLMs, after controlling for nest identity nested within experimental treatment as random factor, bacterial load: F(1, 9) = 0.001, p = 0.97; richness: F(1, 9) = 1.44, p = 0.269; microbiome composition PCo1: F(1, 9) = 0.019, p = 0.894; PCo2: F(1, 9) = 0.958, p = 0.360). Thus, data of the broods of control-I and control-II females were pooled in a single group of control nests in subsequent analyses.

The effect of female treatment on similarity between nestling and female microbiomes, as well as the effect of the inoculation experiment on microbiomes of initial and final nestling samples were explored in repeated measures ANOVAs. The fixe effect of treatment was included as
between-factor (Experimental/Control female for Experiment 1; Cross-inoculated/Control nestlings for Experiment 2), and type of individual (i.e., nestling or female) and time of sampling, respectively, as the within fixed factors. For analyses related to Experiment 1, the repeated factor tests for differences between females and nestlings in PCo scores. For those related to Experiment 2, the repeated factor compared PCo scores between the initial and final samples (those obtained one week after inoculation) of nestlings.

To estimate the dispersion of samples within the groups of cross-inoculated and control nestlings in the PCo space, differences in the deviations from the median of each group were calculated with PermDisp. We have also calculated average similarity of samples within groups and probability of occurrence of the most frequent OTUs with Simper analyses. These analyses let testing how much did microbiomes of nestlings within each experimental group converged after inoculation. All these tools have been used in Primer7.

Bacterial loads of secretion of 16 days old nestlings did not differ between years (GLM after controlling for nest identity nested within year (GLM, F(1,17) = 2.58, p = 0.126), nor did PCo1 scores (GLM, F(1,17) = 1.32, p = 0.268), although the effect of year on PCo2 scores approached significance (GLM, F(1,17) = 3.63, p = 0.076). Since results for PCo scores are qualitatively identical independent of inclusion of year as an additional independent factor, only results of models that do not include year identity are shown. However, richness of bacterial community of nestlings at this age were lower in 2012 than in 2010 (GLM, F(1,17) = 6.18, p = 0.024) and, thus, year identity was included in the analyses explaining variation in richness.

Neither richness, nor bacterial load (CFUs/µl), or PCo scores differed among subpopulations (GLMs, all p>0.05) and treatments were balanced within them. Thus, subpopulation identity was not included in the analyses. Moreover, nestlings sharing the same nest have identical values for female related variables (e.g. bacterial load in CFUs/µl) and, to account for non-independence of
Finally, for analyses exploring similar association for different OTUs, the false-discovery-rate (FDR) correction was applied to establish the appropriate $q$ values, which were the calculated $p$ values after the FDR correction [48].

**Results**

**Bacterial richness**

The hoopoe nestlings involved in the inoculation experiment on average harbored 13.4 ± 6.0 (SD) OTUs per sample ($N = 72$ samples, including together those obtained before and a week after the inoculation treatment). Females had less OTUs than nestlings (using only nestling samples before inoculation; GLM controlling for the random effect of nest identity, comparison female-nestling: $F(1,34) = 17.57$, $p < 0.001$; females: mean ± SD = 6.29 ± 2.59, $N = 17$; nestlings: mean ± SD = 12.56 ± 5.78, $N = 36$; nest identity: $F(17, 34) = 1.30$, $p = 0.248$).

Manipulation of female access to their glands did not significantly affect the number of OTUs present in nestlings' glands (GLM controlling for year, $F(1,14) = 0.22$, $p = 0.646$). Moreover, richness of nestlings' gland communities did not depend on that of their mothers at the beginning of incubation (GLM controlling for year, $F(1,14) = 1.24$, $p = 0.284$).

The inoculation experiment produced a significant increase in the number of OTUs present in experimental nestlings in comparison with their control brothers that were inoculated their own secretion (Table 1, Fig. 2).

The detected effect of the inoculation experiment on community richness resulted from an increase in the prevalence of most OTUs in experimental nestlings, while such prevalence remained stable in control nestlings (Fig. 3, Repeated measures ANOVA for the prevalence of each OTU, interaction between type of nestling and within factor: $(F(1,64) = 22.9$, $p < 0.0001$).
The composition of nestlings' core microbiomes differed from those of their mothers (repeated measures MANOVA with PCo1 and PCo2 scores: R1, Wilks = 0.194, F (2, 15) = 64.4, p < 0.0001). In addition, microbiomes were more similar between control females and their nestlings than between experimental females and their nestlings (repeated measures MANOVA: R1* female treatment, Wilks = 0.781, F (2, 15) = 4.33, p = 0.033). Mosty, these effects were due to differences related to PCo2 (Table 2, Fig. 4a). Scores of this PCo explained 19.1% of total variance of the microbiome composition of samples (Fig. 4b).

The inoculation experiment also affected the composition of the microbiome present in nestling glands (repeated measures MANOVA with PCo1, PCo2 and PCo3 scores: R1 (paired comparison before-after inoculation), Wilks = 0.645, F (3, 32) = 5.88, p = 0.003). The microbiomes of experimental nestlings (cross-inoculated with secretions from a different nest) experienced larger changes than those of control nestlings (repeated measures MANOVA: R1* nestling treatment, Wilks = 0.781, F (3, 32) = 4.44, p = 0.010). The effects of the inoculation experiment were due to differences in PCo1 and PCo2 scores (Table 3, Fig. 5). Moreover, final among-individuals variation was higher for control (deviations from the median of each group mean(SE) = 46.7(3.8)) than for experimental (mean(SE) = 20.2(5.0)) nestlings (PermDisp, F (1, 34) = 17.70, p < 0.001). The microbiome composition of the final samples of cross-inoculated nestlings showed a higher level of similarity among them (Simper, average similarity = 77.46 %, with 10 OTUs of the core microbiome with a probability of presence > 80%) than among those of control nestlings (average similarity 44.84 %, only 4 OTUs with a probability of occurrence > 60 %). Indeed, samples of cross-inoculated nestlings appeared close to each other in the PCo space (Fig. 5), in the area that correlates with a higher number of OTUs (Fig. 4 in Supplemental material-II).

For the subsample of nests with information on the microbiome of donors and receivers, differences in their PCo1 scores positively correlated with the changes in microbiome composition caused by the inoculation experiment (only experimental nestlings, Linear Regression F(1, 13) =
15.17, p = 0.0018, R^2 = 0.539). This relationship did not reach statistical significance for PCo2 scores (F(1, 13) = 4.56, p = 0.052, R^2 = 0.260).

**Cultivable bacterial density**

Bacterial density of nestling secretions at 16 days of age was positively correlated with that of the secretions of their mothers at the beginning of incubation (Table 4, Fig. 6). Interestingly, this association occurs in nests of control but not in those of experimental females with covered uropygial glands (Table 4, Fig. 6b). In addition, we found that nestlings of experimental females harbored in their secretions more cultivable bacteria than those of females with access to their glands (Fig. 6a).

The density of cultivable bacteria in the secretions of nestlings one week after the inoculation experiment did not differ from their initial bacterial density (Repeated measures ANOVA, R1: F(1,33) = 0.06, p = 0.816). It was the case for nestlings inoculated with either own or alien secretions (R1* Nestling type: F(1,33) = 0.15, p = 0.702).

**Experimental effects on particular OTUs**

Six OTUs of nestling secretions were significantly affected by the experiments. The covering of female glands affected the presence of four OTUs in the secretion of nestlings. In nestlings of mothers that had not access to their uropygial gland, prevalence of OTUs 346 and 466 decreased, while that of OTUs 306 and 406 increased (Table 5). Moreover, the presence of the OTU566 in nestlings was not affected by female manipulation, but was positively associated with its detection in their mothers, either experimental or control (Table 5). For all these OTUs, but also for most in the core microbiome, the effect of the inoculation experiment depended on microbiome composition of donors and receivers. Whenever an OTU was present in the donor and not in the receiver, the latter incorporated to the nestling secretion. In addition, there was an association between the cultivable bacterial density in secretions and the presence of particular OTUs in their
microbiome. It was higher for secretions harboring OTU306, while presence of OTUs 242, 346 and 566 was negatively related to cultivable bacterial density (summary of results in Table 5, analyses in Supplemental Material-II).

Discussion

Experimental results support for the first time the hypothesis that the microbiome hosted in the uropygial gland of nestling hoopoes is in part transmitted vertically from mother to offspring, but also that nestling microbiomes maintain their ability to incorporate new strains during the entire nesting period. These results have important consequences for the understanding of the dynamic and evolution of the relationships between hoopoes and their uropygial gland symbionts.

The detected effects of covering female glands support the influence of female uropygial secretion conforming the microbiome of that of their offspring. Differences between nestlings and mothers in microbiome composition and cultivable bacterial density were significantly larger for nests of females with impeded access to their glands. Previous studies performing cross-fostering experiments already showed the existence of genetic effects (i.e. nest of origin) on the composition of the microbiome of hoopoe nestlings [36,37]. However, similarities due to nest of origin might be due to direct transmission from females to offspring or common acquisition by mothers and nestlings, and cross-fostering experiments of nestlings do not allow to distinguish between these two possibilities. Results presented here definitely show that vertical transmission is in fact responsible of the previously detected effect of nest of origin, at least partially. This is an important result, because vertical transmission of those strains assures that part of the microbial community that nestlings harbor in their glands come from an individual (their mother), which has already been successful in surviving and breeding, thus transferring an optimized microbiota adapted to their particular environment [2,13,14]. It is well established that the evolution of vertically transmitted symbionts selects for characteristics that benefit both hosts and symbionts [15]. Moreover, when hosts benefits are mediated by antibiotic production, as it occurs in hoopoes [27,31-34], the vertical
transmission of one antibiotic producing strain would constraint the recruitment of non-resistant ones and favor recruitment of other antibiotic producers [24]. The existence of several bacterial strains co-transmitted vertically from mother to offspring could also imply the evolution of tolerance, co-operation or resource dependence among them [49]. Vertical transmission also affects the evolution of the symbiotic relationship among counterparts [2,15]. Whenever symbionts complete their life cycle within a host species, and their fitness become close related to that of their host, the coevolutionary process provokes symbiont specialization on hosts as well as genetic barriers preventing gene flow among close relatives living in different hosts. Thus, similarly to what has been described in other systems [2], such process would lead to separated evolution among isolated populations of hosts, therefore predicting the existence of different microbial symbiont strains in different hoopoe populations, subspecies or species. However, particularities of the hoopoe-bacteria system may affect specialization and speciation processes of hoopoe symbionts. For instance, secretion of females drastically change during the non-breeding season (see below), which may imply either adaptations of the symbionts to resist that period, specialized body reservoirs in females, or ability to change of microhabitat within the female body. Future studies should explore such possibilities.

Interestingly, there were clear inter-nest differences in the microbiomes harbored by nestlings. This could be caused by a selective adaptive acquisition of bacteria [23], but also might be the consequence of differences in availability in nest environments. In accordance with the latter possibility, the experimental inoculation of secretions from a different nest provoked marked changes in richness and composition of microbiomes of nestlings' secretions after a particular community had been established along two thirds of their nestling cycle. Detected changes in the microbiome of nestlings due to inoculation were mainly explained by acquisition of strains present in the donor individual that were absent in the receiver secretion. Thus, after the experimental inoculations, communities of experimental nestlings from different nests became more similar to each other than those of control nestlings. These results suggest that there is a group of OTUs that are commonly found in hoopoe nestlings secretions and that conform to the typical microbiome. A
similar effect was found for enterococci strains differing in bacteriocin production that varied in their presence among hoopoe nests [38].

The experimental approaches and results allow discussing the origin of particular OTUs of nestling secretions. When females could not use their secretions, many nestlings failed in harboring OTU346 and OTU466, suggesting female secretion is the main source of these two OTUs. Moreover, although detection of OTU566 in offspring was positively associated with its presence in females, the manipulation of female access to glands did not affect its acquisition by nestlings. Therefore, either, environmental conditions shared by nestlings and mother are responsible for the recruitment of this OTU in their uropygial secretions, or there is transmission from female to offspring by other via different of the female gland. We know from previous work [47] that prevalence of OTU566 in glands and cloacae of females as well as on the eggshells are very similar (55%, 45%, 35% respectively). Something similar occurs with OTU306, which is especially common in female cloaca and on the eggshells (70% and 57% respectively, [47]) in comparison with prevalence in female uropygial gland (25%, 35% in the present study). OTU306 (together with OTU406) characterize the microbiomes of offspring of females without access to their glands. All this evidence suggests that the cloaca of mothers is the most probable source of these bacteria (at least of the OTU566 and OTU306) for nestlings. We know that Enterococcus faecalis, by far the most prevalent cultivable bacteria species growing from hoopoe secretions [27,32,38], produces an ARISA peak at 306 (Antonio Martín-Platero, pers. comm.). Interestingly, OTU306 was the only OTU that was positively related to density of cultivable bacteria in the secretion of nestling hoopoes. Previous studies have also shown that E. faecalis, and some others Enterococcus strains isolated from hoopoe secretions, produce bacteriocins with an ample antimicrobial spectrum [30,32,38] and that their abundance in the secretion of female hoopoes is positively related to hatching success [34]. Thus, detecting the possible source of this OTU for hoopoe secretions is of particular interest and, casting light on this subject, the experimental results suggest that hoopoes recruit it from the cloacae to uropygial glands.
Several results suggest that colonization of nestlings’ secretions by particular OTUs depends on the presence of other bacteria. First, the experimental covering of female glands resulted in nestling microbiomes with increased prevalence of OTU306 and OTU406 and this suggests that particular OTUs transmitted vertically prevent their establishment in the uropygium of nestlings.

Second, this experiment also affected the mother-offspring relationship in the density of cultivable bacteria. The abundance of cultivable bacteria in mother and offspring was related only for control females, while when the experiment impeded vertical transmission (experimental females), nestlings harbored greater loads of cultivable bacteria, and those loads were not predicted by that in mother glands. This suggests that the bacteria growing from nestlings’ secretions of control females are a subset of those growing from nestlings’ secretions in which vertical transmission has been impeded. Probably they are those with the potential to live in hoopoe secretions in presence of OTUs normally transmitted from female glands, while nestlings from experimental females harbor also less competitive strains able to grow in this experimental scenario of reduced competence. Given that OTU306 is the main predictor of cultivable bacteria load in secretions, this result can be interpreted in terms of abundance of OTU306. Since OTU306 probably represents mainly Enterococcus faecalis (see above), this possibility implies the existence of mechanisms selecting for particular Enterococci strains (i.e. those with higher antimicrobial potential).

Only three bird species are known to host symbiotic bacteria inside the uropygial gland in healthy individuals: the red-billed woodhoopoe (Phoeniculus purpureus), the European hoopoe and the turkey (Meleagris gallopavo, [50]). While in the turkey the influence of the symbionts (Corynebacterium uropygiale) has not yet been studied, in both Upupiform species, symbionts are responsible of several properties of the secretions including their defensive function [29,51,52]. In the red-billed woodhoopoe, all individuals maintain the symbiosis in the uropygial gland throughout the year, what has driven the evolution of a specific Enterococcus symbiont (E. phoeniculicula, [28]), although the whole community has never been studied by molecular methods in this system. In the case of European hoopoes, the association with bacteria in the gland is not
continuous, but cyclic [27,29]. This probably has prevented to some extent the specialization of the
symbiotic relationship [53,54]. Thus, the enterococci found in hoopoes are common in several
environments [32] and from the results in the present study they probably are recruited from the
cloaca. The microbiome of the uropygial secretion of hoopoes is more complex than that usually
established in animal glands specialized in hosting symbionts (e.g. single actinobacteria species
protecting fungus growing ants' gardens [55,56]). It includes a combination of 8 to 27 more
frequent OTUs accompanied by up to 124 scarce OTUs with reduced prevalence [44], and results
here suggest different sources for them. A few are inherited from mother to nestling gland, others
may come either from female or nestling cloaca, and many others can apparently be obtained from
environmental sources accessible within the nest-hole [36,37,45].

Despite such apparently complex microbiome, a more or less stable combination of OTUs is
typical (core microbiome), and results suggest that the competitive ability of strains vertically
transmitted from mothers gland differentially promote or restrict the establishment of others that are
able of colonizing the nestling gland from other sources (see [24]). Previously, it was shown that
the prevalence of different enterococci strains in the glands was related to their ability to produce
antimicrobial bacteriocins [38]. All these results suggest that a mutualism based in byproduct
cooperation [4,22] determine the microbial composition of hoopoe uropygial gland microbiome.

Summarizing, hoopoe nestling uropygial glands harbor core microbiomes that to some degree
vary in their composition, but, when experimentally put in contact, converge to the same
combination of OTUs. The experiments further demonstrate the importance of vertical transmission
determining the core microbiome of nestling glands, and suggest the existence of cloaca or gut
reservoirs for particular OTUs. Further advance in the understanding of evolution of this system
should address the determination of the taxonomic affiliation of these most common OTUs, in
order to study their particular function in the symbiotic community, and finding the inter-annual
reservoir for the vertically transmitted symbionts.
Ethical approval

We performed the study following relevant Spanish national (Decreto 105/2011, April 19) and regional guidelines. The ethics committee of the Spanish National Research Council (CSIC) approved the protocol, and the Consejería de Medio Ambiente de la Junta de Andalucía, Spain, provided all necessary permits for nest and nestling manipulations (Ref: SGYB/FOA/AFR/CFS) as good as the establishment and maintenance of the captive breeding population (Resolution of April 14 2008).

Conflict of Interest

The authors declare that they have no conflict of interest.

Data accessibility

Data used in this paper can be found in the CSIC Institutional Repository. (Accession numbers still not available)

Authors' contributions

MM-V, JJS, AMG, MRR and MMB conceived the ideas and designed methodology; AMG, LA and NJG-P collected the data with considerable help from MM-V and JJS; MM-V and JJS analyzed the data; MM-V led the writing of the manuscript with considerable help by JJS. All authors contributed critically to the drafts and gave final approval for publication.

References


uropygial gland hosts a bacterial community influenced by the living conditions of the bird.

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Table 1. Results of a Repeated Measures ANOVA analyzing the effect of the inoculation of uropygial secretion, collected from their own gland or from a nestling of a different nest (nestling type), on the number of Operational Taxonomic Units (OTUs) detected in nestling secretions before and one week after inoculation (R: repeated measures). The model also included the effect of year and the random effect of nest identity nested within study year. Significant effects are marked in bold.

<table>
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<tr>
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<th>p</th>
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<td>3.27</td>
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<tr>
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<td>7.46</td>
<td><strong>0.011</strong></td>
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<tr>
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<td>0.07</td>
<td>0.796</td>
</tr>
<tr>
<td>R1 x Nest (Year)</td>
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<td>1.45</td>
<td>0.227</td>
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<tr>
<td>Error</td>
<td></td>
<td>17</td>
<td></td>
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</tbody>
</table>
Table 2. Effects of the covering of female glands (Female treatment) on the similarities between mothers and their nestlings (repeated measure R1) in the composition of the core microbiome of their uropygial secretions. Females’ samples were collected before the experiment covering their glands and those of nestlings when they were 16 days old. Each repeated factor (R1) compares PCo scores of nestling and female. Results for R1 did not qualitatively change when not including the interaction with female treatment in the model. DFs adjusted to the number of nests. Significant p-values are in bold.

<table>
<thead>
<tr>
<th></th>
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<th>p</th>
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<tr>
<td><strong>PCo2</strong></td>
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<td>112.85</td>
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<tr>
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<td>5.65</td>
</tr>
<tr>
<td>Error</td>
<td></td>
<td>16</td>
<td></td>
</tr>
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Table 3. Results from Repeated measures ANOVAs exploring the effects of the experimental inoculation of nestlings glands with own uropygial secretion or that of a foreign nestling (nestling type) on the change of the microbiome composition after the inoculation experiment (repeated measure R1). Each repeated factor (R1) compares PCo scores of nestlings before and after inoculation. Results for R1 did not change when not including the interaction with type of nestling in the model. Significant p-values are in bold.

<table>
<thead>
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<th></th>
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<th>F</th>
<th>p</th>
</tr>
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<td>Error</td>
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<td></td>
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</table>
Table 4. Results of a General Linear Model (GLM) exploring the association between density of cultivable bacteria in nestling secretions (dependent variable, (cfu/µl)) and that of females. Whether or not uropygial gland of females was experimentally covered (female treatment) as well as its interaction with the bacterial density of female secretion were included in the model (1*2). The main effects remained statistically significant when removing the interaction. Degrees of freedom were adjusted to number of nests. Significant p-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>df</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
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<td>8.99</td>
<td><strong>0.011</strong></td>
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<tr>
<td>2) Log(CFUs/µl) female secretion</td>
<td>1</td>
<td>5.30</td>
<td><strong>0.038</strong></td>
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<tr>
<td>3) 1 * 2</td>
<td>1</td>
<td>3.91</td>
<td>0.069</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td></td>
<td></td>
</tr>
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</table>
Table 5. Summary of effects for the Operational Taxonomic Units (OTUs) with significant contributions to properties of the microbiome of hoopoe nestling secretions (see analyses in Supplemental Material-II).

<table>
<thead>
<tr>
<th>OTU</th>
<th>Affected by covering female gland</th>
<th>Related with presence in female gland</th>
<th>Effect of presence in donor</th>
<th>Relation with TSA growing</th>
<th>Probable source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>242</td>
<td></td>
<td>Yes</td>
<td>Negative</td>
<td></td>
<td>External</td>
</tr>
<tr>
<td>306</td>
<td>Positive</td>
<td>Yes</td>
<td>Positive</td>
<td></td>
<td>Gut</td>
</tr>
<tr>
<td>346</td>
<td>Negative</td>
<td>Yes</td>
<td>Negative</td>
<td></td>
<td>Female gland</td>
</tr>
<tr>
<td>406</td>
<td>Positive</td>
<td>Yes</td>
<td>Negative</td>
<td></td>
<td>External/Gut</td>
</tr>
<tr>
<td>466</td>
<td>Negative</td>
<td>Yes**</td>
<td>Yes</td>
<td></td>
<td>Female gland</td>
</tr>
<tr>
<td>566</td>
<td>Yes</td>
<td>Yes</td>
<td>Negative</td>
<td></td>
<td>Gut</td>
</tr>
</tbody>
</table>

* See explanations in Discussion

** Present in all females except in one experimental
Fig. 1 Schematic representation of the design and steps used for the two experiments. a) Experiment 1: manipulation of female access to the uropygial gland, to test the direct transmission of bacteria from female to offspring glands. Green arrows represent the hypothesized vertical transmission of bacteria. b) Experiment 2: cross-inoculation of secretions among nestlings from different nests, to test the stability of microbiomes and strain ability in colonizing a gland

Fig. 2 Effect of the experiment inoculating uropygial glands of hoopoe nestlings with secretions from the same nestling (control) or from a nestling of a different nest (experimental) on richness of the bacterial community hosted in their gland

Fig. 3 Prevalence of Operational Taxonomic Units (OTUs) found in uropygial secretions of nestling hoopoes before (black bars) and a week after (white bars) the inoculation of their glands with (a) their own secretion or (b) the secretion from a nestling of a different nest

Fig. 4 (a) Influence of manipulating females’ access to their uropygial gland on the differences between hoopoe females and their nestlings in PCo 2 scores. Means and 95% confidence intervals calculated for the number of nests are presented. (b) Differences in the composition of the hoopoe uropygial gland core microbiome, among control, experimental females and their nestlings, as reflected for PCo1 and PCo2

Fig. 5 PCo plot representing, by their proximity, the resemblance in composition of the microbiome among samples of uropygial secretions from nestlings before (initial) and a week after (final) the inoculation experiment. Control nestlings were inoculated their own secretion, while another nestling from each nest (cross-inoculated) was inoculated with secretion taken from a different brood

Fig. 6 Effects of manipulating female’s access to their uropygial gland on (a) the abundance of cultivable bacteria in nestling secretions and (b) the relationship between the bacterial growth from female and nestling secretions
Fig 1

**a**

Control (2 types)

Incubation | Nestling period
---|---

Experimental

Day 1:
- Sampling
- Female manipulation

Day 16:
- Nestling sampling

**b**

Control (2 types)

Incubation | Nestling period
---|---

Experimental

Day 16:
- Sampling
- Cross-inoculation

Day 23:
- Sampling
Fig. 2

Control nestlings
Experimental nestlings

Relative to inoculation

No. OTUs

Before
After

0
10
20
30
40
Fig 3.

(a) and (b) show the prevalence of OTUs 186 to 638. The x-axis represents the OTU numbers, and the y-axis represents the prevalence. The black bars indicate the prevalence of specific OTUs.
Fig 5

Nestling sample
- Initial control
- Final control
- Initial cross-inoculated
- Final cross-inoculated

PCO1 (37.4% of total variation)

PCO2 (18.5% of total variation)
Fig. 6a

![Graph showing the log of CFUs/µL in nesting secretions for experimental and control females. The graph indicates a decrease in log CFUs/µL for experimental females compared to control females.]

Fig 6b

![Graph showing the correlation between log (cfu/µL) in female secretions and log (cfu/µL) in nesting secretions. The graph includes data points for both experimental and control females, with a trend line showing a positive correlation for experimental females and a negative correlation for control females.]]
Acquisition of uropygial gland microbiome by hoopoe nestlings

Microbial Ecology

M. Martín-Vivaldi\textsuperscript{a,b,*}, J. J. Soler, Ángela Martínez-García, L. Arco, N. Juárez-Garcia-Pelayo, M. Ruiz-Rodríguez, M. Martínez-Bueno

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b) Estación Experimental de Zonas Áridas (CSIC) E-04120 Almería, Spain.

SUPPLEMENTAL MATERIAL -I. ARISA methodology

ARISA (Automated rRNA Intergenic Spacer Analysis, Fisher & Triplett 1999) amplifies an intergenic transcribed spacer (ITS) region between the prokaryotic 16S and 23S rDNA. The ITS was amplified using the primer pair ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) (Cardinale \textit{et al.} 2004). The primer ITSReub was labelled fluorescently with 6-FAM. Amplifications were performed in 50 µl reaction volumes containing ultrapure H2O, 1x 5 PRIME MasterMix including 1.5 mM Magnesium, 200 mM dNTPs, 1.25 U Taq polymerase (5 PRIME, Hamburg, Germany), 0.2 mM of primers and 5µl of diluted DNA 1:10. PCRs were carried out in Eppendorf Mastercycler Nexus Family. Fragments were amplified under the following conditions: initial denaturation at 94 °C 2 min, followed by 30 cycles with denaturation at 94 °C 45 s, annealing at 52 °C 45 s and extension at 72 °C 1 min, with a final extension at 72 °C 5 min. Amplified PCR products were diluted 1:10 and denatured by heating in formamide. Fragment lengths were determined by mean of automated fluorescent capillary electrophoresis on 3130 Genetic Analyzer. Electropherogram peak values were calculated after interpolation with an internal size standard named GeneScan™ 1200 LIZ dye Size Standard (both Applied Biosystems).
Peak Scanner v 1.0 (Applied Biosystems) was used to determine fragment lengths identifying different bacterial Operational Taxonomic Units (OTUs) within each sample. Scripts in R-environment [http://cran.r-project.org/]) available at http://www.ecology-research.com, were used for binning DNA fragment lengths from different samples. Binning exercise was performed by establishing a window size of 4 pair of bases and a distance of two consecutive binning frames (i.e. shift) of 0.1. Only peaks with values of relative intensity of fluorescence larger than 0.09% and fragments above a threshold of 50 fluorescence units that ranged between 100 and 1,000 bp (Ramette 2009) have been considered. Molecular fingerprinting techniques are highly reproducible, robust, and have been proven useful for comparative analysis of microbial community structure (Loisel et al. 2006; Bent & Forney 2008).

References


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Microbial Ecology

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* Correspondence author. e-mail: mmv@ugr.es

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b) Estación Experimental de Zonas Áridas (CSIC) E-04120 Almería, Spain.

SUPPLEMENTAL MATERIAL-II. Importance of particular OTUs

In order to know which of the OTUs detected in secretions by ARISA analyses were responsible of the detected experimental effects on cultivable bacterial load and composition of bacterial communities, we explored the association of the presence of each OTU of the core microbiome with these effects.

Relationship with cultivable bacterial load

The best GLZ models (those with lower AIC values differing in less than two unities) explaining bacterial load of secretions (considering all secretions from nestlings and females) included combinations of eight OTUs (Table 1) with positive and negative associations. The more clear association was detected for the OTU306 (Fig. 1) suggesting that it is the main component of the microbiome of hoopoe uropygial secretions able of growing in TSA medium in aerobic conditions. The detection of both positive and negative associations between presence of some OTUs and cultivable bacterial density may be the consequence of direct antagonistic effects or competence between both groups of OTUs.

<table>
<thead>
<tr>
<th>Model</th>
<th>OTUs</th>
<th>df</th>
<th>AIC</th>
</tr>
</thead>
</table>

Table 1. Best subsets of OTUs present in hoopoe uropygial secretions explaining bacterial growth in TSA general medium in a GLZ model with logit link function and a normal distribution.
Figure 1. Relationship between the presence of OTUs in hoopoe secretions and their cultivable bacterial load. Only the OTUs from the best subsets in table 2 with a significant Wald value in the whole effects model (* p<0.05, *** p<0.001) are presented. Whiskers show marginal means and 95% confident intervals.
The effect of covering female glands on the similarity of the microbiomes of nestlings and females was specially associated to the presence in secretions of three OTUs. Two of them correlated to the space occupied by the microbiomes of nestlings of experimental females (OTU306 and OTU406, Fig. 2) and another correlated to the position of the microbiomes of nestlings of control females (OTU466, Fig. 2). In accordance with this interpretation, the presence of these three OTUs in nestlings was affected by manipulation of female access to its gland (Table 2). In addition, prevalence of the OTU346 in nestlings was significantly associated with female experimental treatments when considering only females with the OTU in their glands (GLZ, Wald= 4.78, p = 0.029, q = 0.046). For two additional OTUs (OTU466 and OTU346) the manipulation of female access to their glands caused a reduction in the percentage of nestlings that harbored the OTU (Fig. 3). On the other hand, two OTUs (306 and 406) were more frequent in nestlings from experimental than from control females (Fig. 3).

Figure 2. PCO plot showing the resemblance between samples of the experiment manipulating female access
to its gland. The influence of each of the OTUs of the core microbiome is represented by the green lines being their length the value of the Spearman correlation coefficient.
Table 2. Influence of (1) the presence of an OTU in female secretions and (2) the experimental manipulation of female access to its gland (female treatment), on the occurrence of the same OTU in nestlings uropygial gland secretions. The table shows the results only for the four OTUs of the core microbiome with a significant effect of these factors. The best models according to the AIC criterion for each OTU in a GLZ design with both factors as predictors are presented. We applied the false discovery rate correction for multiple tests (the 14 tests for the OTUs of the core microbiome present in females) to calculate the q-values (adjusted p-values). The factors of each model with a significant effect are indicated in bold.

<table>
<thead>
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<th>Model</th>
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<td>1</td>
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<td>&lt;0,0001</td>
<td>&lt;0,001</td>
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* Given that OTU466 was present in all control females, we excluded the factor "OTU presence in female" from this analysis.
Figure 3. Influence of the experimental covering of female glands (Fem. treat.) and the presence of an OTU in females at the beginning of incubation (Presence in fem.) on its occurrence in their nestlings at 16 days of age. Graphs show only the effect for the five OTUs for which female manipulation or its presence in females significantly influenced presence in nestling secretions. CON = Control females. EXP = Experimental females. For OTU346, only the nests with its presence in the female (9 females and 18 nestlings) are considered (see text), for the remaining OTUs all nests with available information on the microbiome of female secretion are included (17 females and 34 nestlings). The OTU566 was present in nine females and absent in eight females.

Occurrence of the OTU566 in the microbiome of nestlings and their mothers were positively associated (Fig. 3) independently of female experimental treatment (Wald = 0.077, p = 0.782). This result suggests independent acquisition of nestlings and females within the nest environment, not dependent on transmission to nestlings from the mother’s gland after female manipulation.

Microbiome enrichment by cross-inoculation

Most cross-inoculated nestlings acquired new OTUs. Indeed, the final samples of cross-inoculated nestlings clearly congregated in the corner of the PCO space correlating with a greater number of OTUs (Fig. 4). The two OTUS whose prevalence in nestlings was reduced by the experiment covering female glands (OTU346 and OTU466) were closely associated with this section of the PCO space (Fig. 4) suggesting that they are among the components of the community that explain the detected experimental effects of cross-inoculation.
Moreover, for 10 out of the 14 OTUs of the core microbiome present in females (all except OTU278, OTU310, OTU330, OTU350 and OTU474), there was a significant relationship of the initial differences between receiver and donor and the change in harboring a particular OTU after inoculation (Pearson Chi-squares, after FDR correction for multiple tests, all q < 0.05). For these ten OTUs, in 92.1 % of the cases in which they were present in a donor and not in the receiver, the inoculated nestling incorporated the OTU to its microbiome.
Figure 4. PCO plot showing the resemblance between initial and final samples of nestlings for the two groups of the inoculation experiment. The vectors of the correlations of all OTUs with PCO axes are drawn in green.