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Gut Microbiota Colonization and Transmission in the Burying Beetle *Nicrophorus vespilloides* throughout Development

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ABSTRACT Carrion beetles in the genus *Nicrophorus* rear their offspring on decomposing carcasses where larvae are exposed to a diverse community of decomposer bacteria. Parents coat the carcass with antimicrobial secretions prior to egg hatch (defined as prehatch care) and also feed regurgitated food, and potentially bacteria, to larvae throughout development (defined as full care). Here, we partition the roles of prehatch and posthatch parental care in the transmission and persistence of culturable symbiotic bacteria to larvae. Using three treatment groups (full care, prehatch care only, and no care), we found that larvae receiving full care are predominantly colonized by bacteria resident in the maternal gut while larvae receiving no care are colonized with bacteria from the carcass. More importantly, larvae receiving only prehatch care were also predominantly colonized by maternal bacteria; this result indicates that parental treatment of the carcass, including application of bacteria to the carcass surface, is sufficient to ensure symbiont transfer even in the absence of direct larval feeding. Later in development, we found striking evidence that pupae undergo an aposymbiotic stage, after which they are recolonized at eclosion with bacteria similar to those found on the molted larval cuticle and on the wall of the pupal chamber. Our results clarify the importance of prehatch parental care for symbiont transmission in *Nicrophorus vespilloides* and suggest that these bacteria successfully outcompete decomposer bacteria during larval and pupal gut colonization.

IMPORTANCE Here, we examine the origin and persistence of the culturable gut microbiota of larvae in the burying beetle *Nicrophorus vespilloides*. This insect is particularly interesting for this study because larvae are reared on decomposing vertebrate carcasses, where they are exposed to high densities of carrion-decomposing microbes. Larvae also receive extensive parental care in the form of carcass preservation and direct larval feeding. We find that parents transmit their gut bacteria to larvae both directly, through regurgitation, and indirectly via their effects on the carcass. In addition, we find that larvae become aposymbiotic during pupation but are recolonized apparently from bacteria shed onto the insect cuticle before adult eclosion. Our results highlight the diverse interactions between insect behavior and development on microbiota composition. They further suggest that competitive interactions mediate the bacterial composition of *Nicrophorus* larvae together with or apart from the influence of beetle immunity, suggesting that the bacterial communities of these insects may be highly coevolved with those of their host species.

KEYWORDS *Nicrophorus*, parental care, symbiosis, microbiota, transmission

Animals are colonized by a diverse community of bacterial symbionts that play crucial roles in their ecology and evolution (1–3). This has been especially well studied in insects, whose bacterial symbionts can influence traits ranging from mate

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and diet choice (4, 5) to susceptibility to natural enemies (6, 7). Bacterial symbionts can also differ in the fidelity of their associations with their insect hosts. Endosymbionts like *Buchnera* in aphids, that serve obligate functions for their insect hosts by overcoming host nutritional deficiencies, are highly specific and have been associated with aphids for millions of years (8). At the opposite extreme, insects can retain transient associations with bacteria whose effects are more variable (5, 9–11). Although different factors may underlie the divergent influences of bacterial symbionts on insect hosts, one key component is the way that bacteria are transmitted between insect generations (12). Whereas obligate symbionts are always transmitted vertically, often via direct passage through eggs, more transient associations, typical of the gut microbiota, involve an external stage where bacteria are reacquired horizontally each generation via ingestion (13, 14).

Distinguishing symbionts on the basis of transmission mode (vertical versus horizontal) has been extremely useful in focusing attention on how this can align the fitness interests of symbionts and hosts (15, 16). However, many associations between insects and their microbial symbionts fall somewhere in the middle of these strict extremes. Among diverse possibilities, trophallaxis and coprophagy occur when bacteria are passed horizontally between individuals via oral-oral/anal contact or fecal consumption (17–19). Similarly, horizontal symbiont transmission can take place via ingestion of the bacteria-smeared egg coat or via consumption of bacteria-rich capsules (20, 21). While these methods of transfer can effectively vertically transmit symbionts from parent to offspring (13), the presence of an environmental component implies that young and developing insects can be simultaneously colonized by beneficial symbionts as well as environmental bacteria that can harm the host (21, 22). In these cases, the establishment of the inherited microbiota will be partly dependent on the ability of inherited symbionts to competitively exclude environmental bacteria as well as the timing and manner of their acquisition (23, 24). Additionally, especially for holometabolous insects that undergo a complete metamorphosis, the manner of acquisition can change markedly throughout development, at one stage occurring from the mother while at later stages occurring potentially through alternative transmission routes (12, 13).

Here, we examine the mechanisms of transmission and the stability of the culturable gut microbiota of the carrion beetle, *Nicrophorus vespilloides*, throughout its development. This system is particularly interesting for addressing these questions given the peculiar life history of these organisms. *Nicrophorus* beetles are reared on decomposing carrion where they encounter and ingest high densities of microbes (25–29). Eggs are laid in the soil near the carcass (25). Upon hatching, larvae migrate to the carcass where they both self-feed and are fed regurgitated material from the caring parents (25, 30, 31). Next, following an ~6- to 7-day feeding period upon the carcass, larvae cease feeding and disperse into the surrounding environment where they eventually pupate individually in underground chambers. Finally, pupae eclose into adults and emerge from the pupal chambers to commence feeding (25, 32).

N. vespilloides larvae may be exposed to a varied microbiota throughout development, and this will likely be influenced by the presence of parents and the stage of development (26–29, 33). First, parents may modify the carcass microbiota by coating it in antimicrobial secretions throughout the period of parental care (22, 27, 34). Notably, these secretions are not sterile and contain significant numbers of bacteria that can proliferate on the carcass (29). Second, parents feed larvae with regurgitated food, which may facilitate the transfer of the parental gut microbiota to offspring (posthatch care) (31). Finally, following dispersal, larvae cease feeding, thereby preventing continued colonization from diet-borne bacteria; then, during metamorphosis, they shed the larval gut (25). At present, there is no understanding of the dynamics of these gut bacterial communities through time.

There is little knowledge of the colonization dynamics of *Nicrophorus* gut bacteria or the extent to which colonization is influenced by parental care, a hallmark of this system. To examine these questions, we manipulated *N. vespilloides* parental care and used a culture-based approach to monitor the dynamics of symbiont colonization and

stability through development. Although culturing can underestimate bacterial densities compared to total cell counts or sequence-based approaches (see Fig. 1C), this approach allowed us to examine the largest set of experimental conditions while also identifying the bacterial groups that can be experimentally manipulated to understand mechanisms of colonization and community assembly of the microbiota using the *Nicrophorus* model system. Briefly, our results provide strong evidence that beetle parents play a defining role in the establishment of the bacteria residing in *Nicrophorus* larval guts; however, continuous parental care and feeding is not essential for the stable maintenance of this microbiota. We also find that pupae undergo an aposymbiotic stage, after which they are recolonized by bacteria in the pupal chamber. We discuss these results in the context of the role of the *Nicrophorus* microbiota for beetle fitness.

(This article was submitted to an online preprint archive [35].)

RESULTS

Bacterial CFU vary throughout development and as a function of parental care. The CFU of intestinal bacteria were quantified throughout development for three treatment groups corresponding to different levels of parental care. Following hatching from sterile eggs, larvae from all treatments rapidly acquire high bacterial densities within their guts. Bacterial densities vary significantly through time (general linear model [GLM] analyses, df = 10 and $P < 0.001$) and as a function of treatment (GLM, df = 2 and $P = 0.006$) and vary across nearly 6 orders of magnitude as a function of developmental stage. During larval feeding on the carcass, bacterial densities increase in all treatments, reaching densities of $\sim 10^6$ to 10^7/larva. In contrast, following dispersal, bacterial populations precipitously decline until, during pupation, bacteria were undetectable. Finally, as pupae eclose and reemerge from pupal chambers, they reacquire a high-density bacterial population within their guts (Fig. 1B and C). It is notable that this recovery occurs prior to feeding and before emergence from the pupal chamber, indicating that recolonization takes place from bacteria resident within the pupal chamber. The dynamics of colonization are broadly insensitive to experimental methods, as estimates of density based on total microscopic counts perfectly mirror those determined by plate counting (Fig. 1C), although CFU-based estimates consistently underestimate live counts (see Table S2 in the supplemental material). Minor differences were observed between total and live cell counts; however, these are only significantly different at day 3 and day 12, accounting for 43% and 28% of total cell numbers, respectively (paired *t* tests, df = 4; day 3, $P = 0.039$; day 12, $P = 0.019$).

Composition of *N. vespilloides* larval symbionts. Although bacterial densities differ across parental care treatments, there is broad overlap in the dynamics of CFU change through time. Despite these similarities, the composition and diversity (see Table S1 in the supplemental material) of these communities may vary. To understand these differences and to illuminate transmission dynamics from mothers to larvae, we tracked community composition of gut bacteria within larvae throughout development (Fig. 2) using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and compared these to the maternal samples. The maternal microbiota was dominated by four bacterial genera that together comprised >65% of recovered CFU, including *Providencia*, *Morganella*, *Vagococcus*, and *Proteus*, with several other genera appearing in lower frequencies (Fig. 2). We next examined genus level composition across the three larval treatment groups (full care, FC; prehatch parental care, PPC; no care, NC [g, samples taken from the gut; c, samples from the cuticle and chamber wall]) by analysis of similarity (ANOSIM). As anticipated, if transmission occurs via parents, we observed significant overlap in the bacterial communities of parental and larval gut communities from larvae receiving parental care throughout development ($R_{\text{FC-g vs mother}} = 0.277$; $P = 0.028$) (Table 1), as *R* values of <0.25 correspond to "barely separable" groups (36). Similarly, although to a lesser degree, there is concordance between the maternal microbiota and those of larvae receiving prehatch care only ($R_{\text{PPC-g vs mother}} = 0.331$; $P = 0.066$) (Table 1). In contrast, larvae reared in the absence of parental care are highly diverged from the parental microbiota

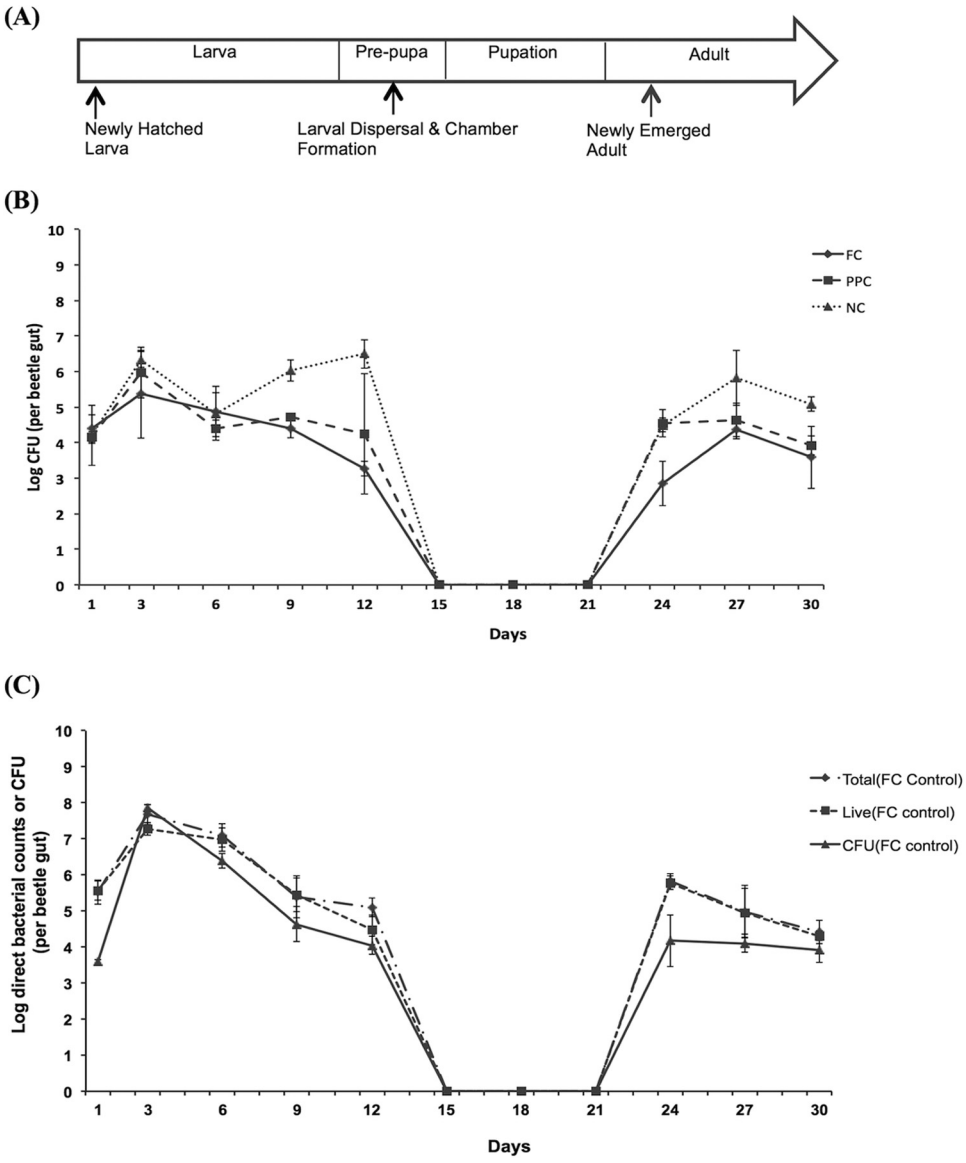

FIG 1 Change in total cell, live cell, and CFU of *Nicrophorus vespilloides* gut bacteria throughout development. (A) Overview of the time course of beetle developmental. (B) Change in CFU of host gut contents through time (means ± standard deviation (SD); $n = 3$/time point). FC corresponds to larval gut samples from full parental care broods, PPC corresponds to gut samples from preparental care broods, and NC corresponds to larval gut samples from no care broods. (C) Counts of total cells, live cells, and CFU in samples from FC broods (means ± SD; $n = 5$/time point). The limit of detection is ~10 cells/gut sample.

($R_{\text{NC-g vs mother}} = 1$; $P = 0.007$) (Table 1) (Fig. 3A and B). In particular, the gut community of NC larvae was shifted toward bacterial groups likely acquired from either the soil or the carcass (Fig. 2 and 3C), e.g., *Escherichia coli* (23.5%), *Serratia* (20.4%), and *Staphylococcus* (19.2%).

In comparing the larval microbiota of the three treatment groups, ANOSIM illustrated clear differences between the treatment groups overall (global test, $R = 0.815$ and $P = 0.001$), and although there are differences between the FC and PPC larvae, there is much greater similarity between the two groups with parental care ($R_{\text{FC-g vs PPC-g}} = 0.665$; $P = 0.001$) than between either care group and the no care larvae ($R_{\text{FC-g vs NC-g}} = 0.956$; $P = 0.001$ and $R_{\text{PPC-g vs NC-g}} = 0.994$; $P = 0.001$) (Table 1; see also Fig. S1 in the supplemental material). This is also apparent in the Venn diagrams in Fig. 3A, focusing on the presence/absence of specific bacterial groups. Together, these results indicate that transmission of the beetle microbiota occurs

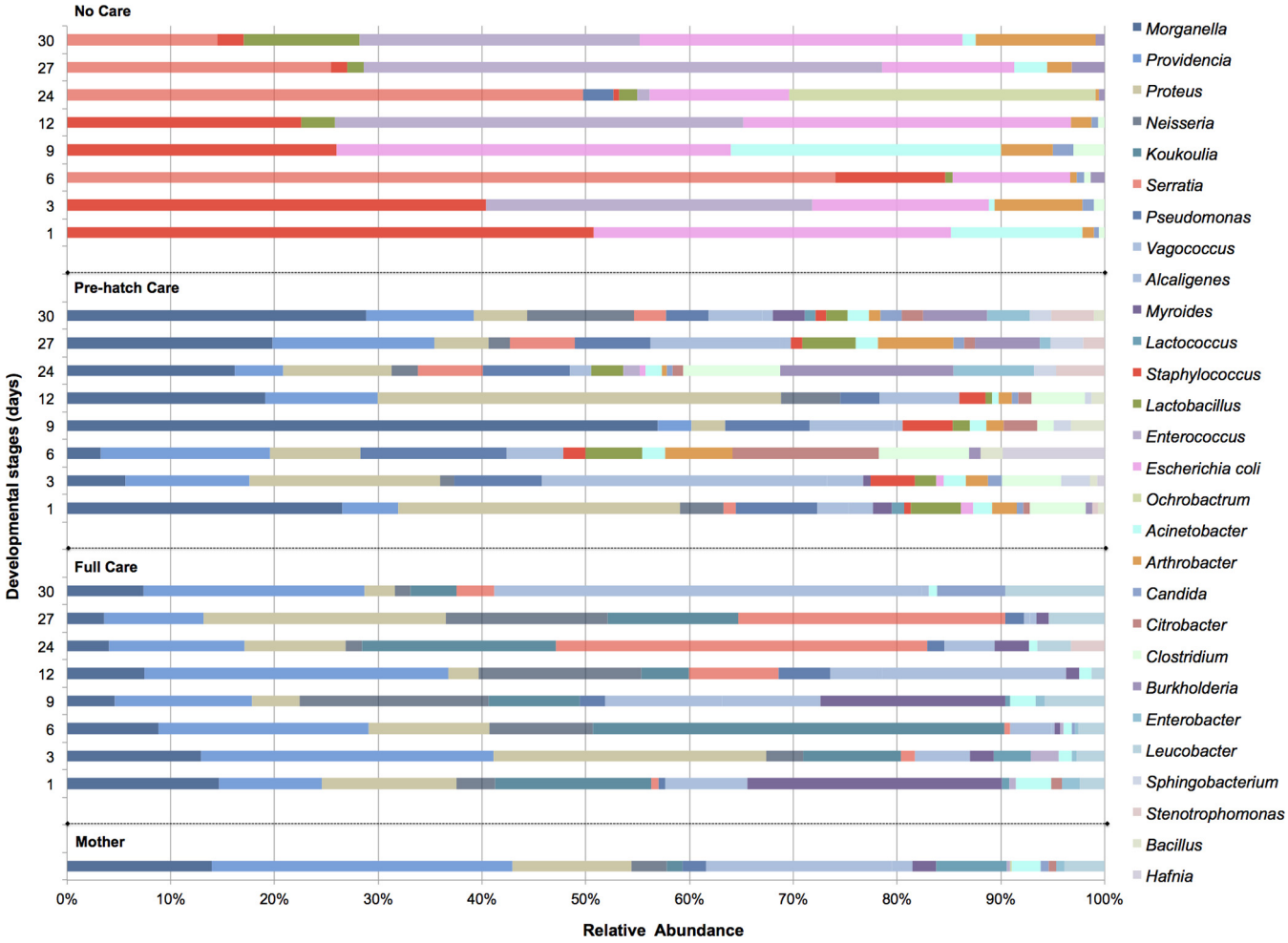

FIG 2 Composition of *N. vespilloides* gut microbiota throughout development. The maternal gut microbiota is shown at the bottom while treatment designations are the same as in Fig. 1. No CFU were detectable between days 15 to 21 of larval development, corresponding to the duration of pupation. Three individual larvae were independently analyzed for each time point. The *y* axis of day 1 to day 9 refers to the larval stage, day 12 corresponds to the prepupal stages, and day 24 to day 30 refers to adult formation.

predominantly from parents to offspring. However, they also reveal that continued replenishment of bacteria from parent to offspring via feeding is unnecessary to establish the endogenous microbiota. Instead, transmission can occur indirectly via deposition of the maternal bacteria on to the carcass by the mother during carcass preparation and subsequent colonization of larva via self-feeding.

Recolonization of *N. vespilloides* symbionts. An important result from these analyses is the aposymbiotic stage occurring during pupation followed by recolonization from within the pupal chamber. Notably, this result based on CFU was further confirmed by direct microscopic counts (Fig. 1C). To assess the source of recolonization, we sampled bacterial populations from the pupal cuticle and the wall of the pupal chamber, together with samples from the bulk soil in which pupal chambers were constructed. Treatment designations are defined above, with the addition of subscripts corresponding to each sampling site. For example, FC-g refers to samples taken from the guts of larvae receiving full care while FC-c represents samples from the cuticle and chamber wall of these same larvae. These analyses showed that the *N. vespilloides* pupal cuticle and chamber soil had very similar compositions (by pairwise test of ANOSIM, FC-g, FC-c, $R = 0.32$ and $P = 0.068$; PPC-g, PPC-c, $R = 0.02$ and $P = 0.052$; NC-g, NC-c, $R = 0.03$ and $P = 0.0397$) (Table 1) and that these were diverged compared to the bulk soil (by pairwise test of ANOSIM, FC-c, soil, $R = 0.89$ and $P = 0.094$; PPC-c, soil, $R = 1$ and $P = 0.114$; NC-c, soil, $R = 1$ and $P = 0.099$) (Table 1). Importantly, many bacterial

TABLE 1 ANOSIM on bacterial community dissimilarity

Groups[a]	*R* statistic	*P* value	No. of permutations	No. of observed	Test model
FC-g, PPC-g, NC-g	0.8152	0.001	999	352	Global
FC-g, PPC-g, NC-g, mother	0.741	0.001	999	264	Global
FC-c, PPC-c, NC-c, soil	0.7493	0.001	999	814	Global
FC-g, NC-g	0.9556	0.001	999	144	Pairwise
PPC-g, NC-g	0.9939	0.001	999	64	Pairwise
FC-g, PPC-g	0.6651	0.001	999	144	Pairwise
FC-g, mother	0.2769	0.028	999	24	Pairwise
PPC-g, mother	0.3306	0.066	999	24	Pairwise
NC-g, mother	1	0.007	999	24	Pairwise
FC-g, FC-c	0.3177	0.068	999	54	Pairwise
PPC-g, PPC-c	0.0242	0.052	999	54	Pairwise
NC-g, NC-c	0.0134	0.397	999	54	Pairwise
FC-c, soil	0.8889	0.094	720	9	Pairwise
PPC-c, soil	1	0.114	720	9	Pairwise
NC-c, soil	1	0.099	720	9	Pairwise

[a]Subscripts correspond to the site of isolation, e.g., FC-g corresponds to gut samples, FC-c corresponds to the pupal carapace and the wall of the pupal chamber, and soil corresponds to bulk soil outside the pupal chamber.

genera, irrespective of treatment, were found in the prepupal gut and the cuticle but infrequently or not at all in the soil. For example, the most common bacterial groups in FC larvae contained *Providencia* (FC-g, 18.3% versus FC-c, 17.1%), *Morganella* (FC-g, 10.0% versus FC-c, 8.7%), *Proteus* (FC-g, 14.0% versus FC-c, 3.9%), *Vagococcus* (FC-g, 7.0% versus FC-c, 6.1%), *Neisseria* (FC-g, 8.3% versus FC-c, 4.7%), and *Koukoulia* (FC-g, 13.2% versus FC-c, 8.9%) while these were undetected in soil. Similarly, the most abundant genera in NC beetles were only found in NC-g and NC-c: *Escherichia coli* (NC-g, 23.5% versus NC-c, 23.8%) and *Enterococcus* (NC-g, 18.5% versus NC-c, 18.8%) (Fig. 4A and B). These results indicate that the core components of previously colonized gut bacteria can successfully recolonize the host intestinal system after the aposymbiotic stage characteristic of pupation. Thus, although transmission and recolonization to larvae may occur via the environment, the bacterial species that recolonize the newly eclosing adult are highly biased toward bacterial species that were already present in the prepupal gut and that were originally acquired from the mother.

DISCUSSION

Animal symbionts can be passed to offspring through different mechanisms that vary in their reliability of transmission (12). While strict endosymbionts of animals are typically transmitted vertically via eggs, other mechanisms that include an environmental component may also reliably transmit bacteria between generations (15, 20). Here, we examined the mechanisms of bacterial transmission from *Nicrophorus vespilloides* mothers to offspring. *Nicrophorus* larvae are exposed to and consume high densities of bacteria throughout their development on decomposing carrion (26–29, 33). In earlier studies, we and others showed that parental care, including preservation of the carcass through secretion of lysozyme, a broad-spectrum antibacterial with greater specificity toward Gram-positive bacteria, and potentially other antimicrobials, is essential for maintaining larval fitness (22, 27, 28, 37). Additionally, preliminary metagenomic analyses from our own lab (our unpublished data) and published studies from others (29) have found that parental beetles significantly modify the bacterial composition of decomposing carrion, thereby potentially influencing the bacteria that larvae are exposed to and ingest.

To examine the influence of parental care on the transmission of bacteria from parents to offspring, we manipulated the level of care that parents provided to their larvae. With full parental care, parents apply oral and anal secretions to the carcass both before larvae hatch and throughout larval development (22, 27, 28); they also regurgitate food to larvae during the first 3 to 4 days of development (25, 30). As expected, given the continuous direct and indirect exposure to parental bacteria, larvae in this

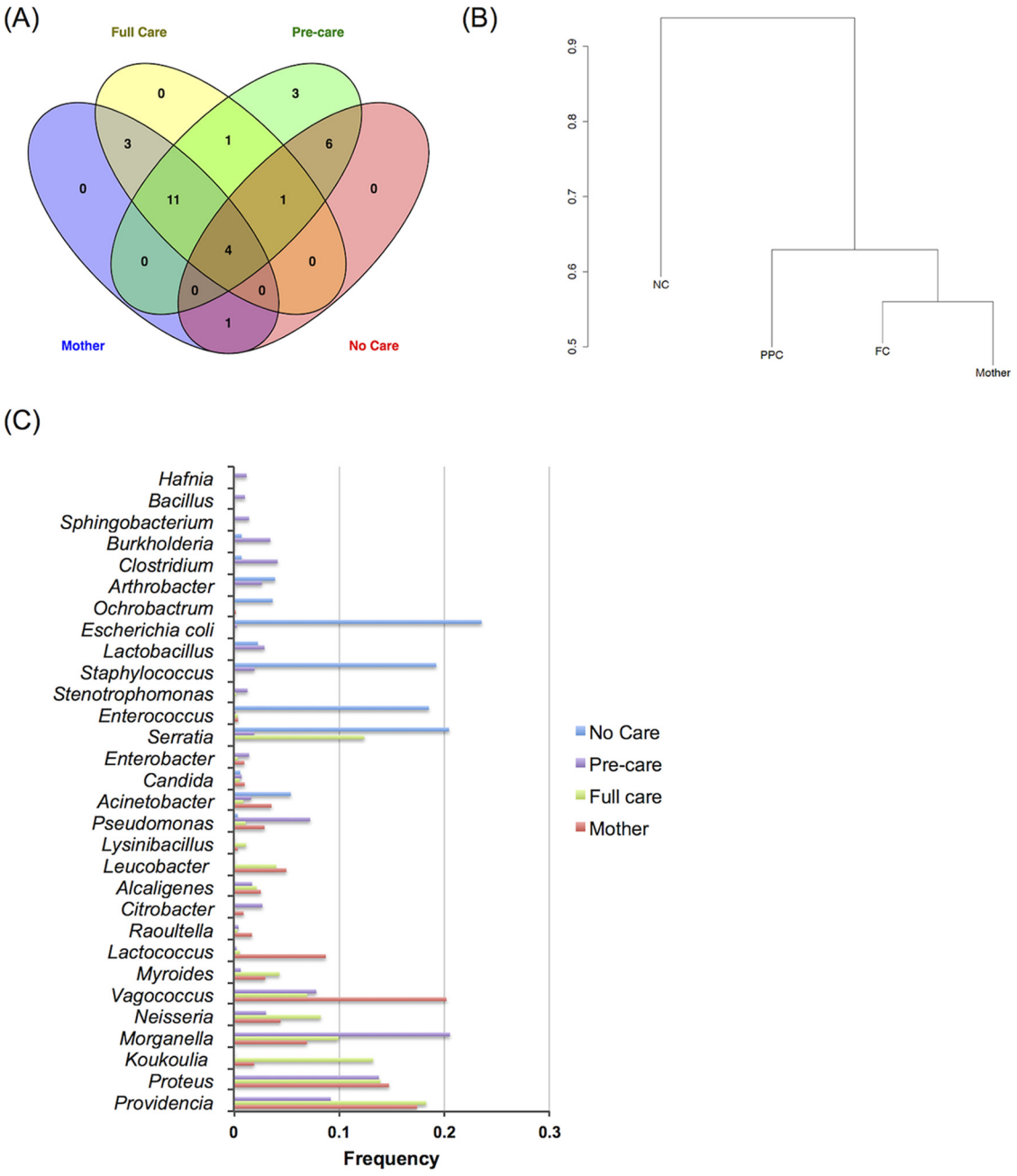

FIG 3 Frequencies of bacteria from gut communities across parental care treatments. (A) Shared and unique genera between treatment groups. Strains with a minimum frequency of 1% were included. (B) Hierarchical clustering on mean similarity of gut microbiota between treatment groups. (C) Overall composition of gut communities across treatments. Strains with frequencies lower than 1% across all communities were excluded from plots.

treatment were colonized predominantly with parental symbionts (Fig. 2); importantly, despite limitations associated with a CFU-based approach, we observed broad overlap between the dominant bacterial species that we cultured and those identified using sequence-based approaches (e.g., *Providencia*, *Morganella*, *Vagococcus*, *Proteus*, *Koukoulia*, and *Serratia*) (33). However, with full care treatment alone, it cannot be determined if larvae require constant replenishment of the parental species for these to be maintained in the larval gut, given other research showing that gut bacteria may be transient without continuous parental provisions (24, 38, 39). One possibility, for example, is that the dominant bacteria from the carcass may outcompete endogenous beetle bacteria within the larval gut; this may be driven actively if the bacteria on the

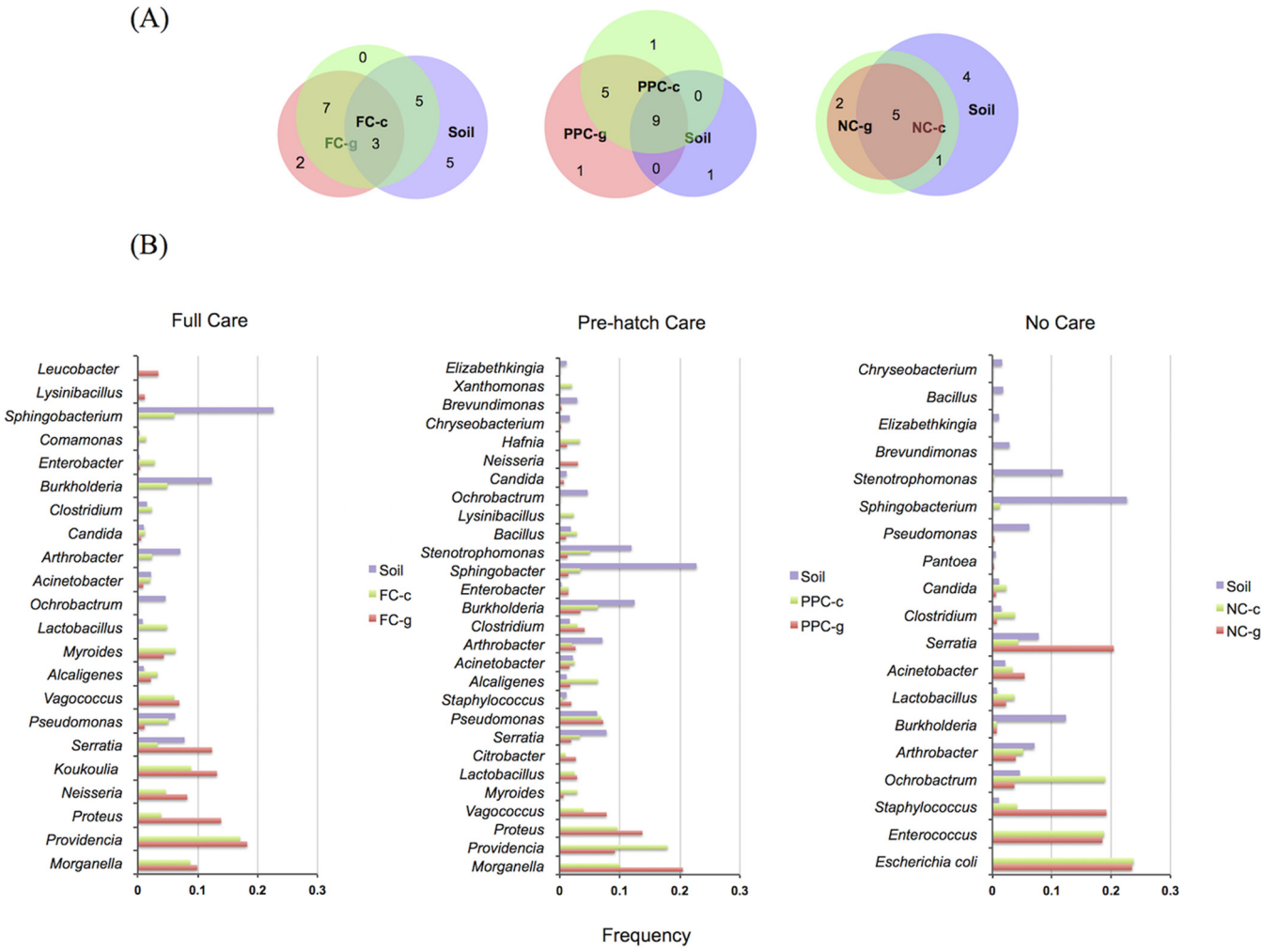

FIG 4 Recolonization of bacterial communities through pupations. (A) Shared and unique genera among treatment groups. Subscripts correspond to the site of isolation, e.g., FC-g corresponds to gut samples, FC-c corresponds to the pupal carapace in the wall of the pupal chamber, and Soil corresponds to bulk soil outside the pupal chamber. (B) Comparison of gut bacterial communities from each sample site and treatment. Strains with frequencies lower than 1% across all communities were excluded from plots.

carcass are particularly good colonizers or passively since larval exposure to carcass bacteria is continuous. To address this question, we established broods that only received prehatch care. In this treatment, parents have no direct exposure to larvae and can only influence larval exposure to bacteria indirectly through their influence on the carcass. It is important to note that because eggs are sterile, transmission is also prevented through this route (40). As with the full care treatment, larvae receiving only prehatch care were also predominantly colonized by maternal bacteria (Fig. 2 and 3). This was not due to an inability of bacteria from the gut to colonize larvae, as larvae in the no care treatment were also colonized by a high-density bacterial microbiota. Also, bacteria in the prehatch groups were partially colonized by carcass-derived bacteria (Fig. 2 and 3C), leading to higher bacterial diversity overall in this group (see Table S1 in the supplemental material) and indicating the capacity for carcass-derived bacteria to establish themselves within the larval gut. Rather, we interpret this result to indicate that "endogenous" bacteria from the mother are able to outcompete the carrion-associated microbes. Furthermore, this effect is long lasting and can persist entirely in the absence of direct maternal feeding. Although this interpretation is consistent with our data, this hypothesis will require experimental testing using the culturable species that we have now established in our collection of *N. vespilloides* symbionts.

At present, we understand relatively few of the mechanisms used by parents to

manipulate the carcass bacteria. However, several factors are likely to be important. First, when parents locate a carcass, they strip it of fur while simultaneously coating the carcass surface with oral and anal secretions. The composition of these secretions has only been partially characterized, but a key component is lysozyme (22, 27). Additionally, oral secretions contain bacteria that can serve as an inoculum to feeding larvae (our unpublished results). In addition to these behaviors, we have also observed parents opening the carcass and removing the mouse gut, behaviors that could potentially have a dramatic influence on larval bacterial exposure by introducing oxygen that may bias the bacterial community toward aerobic species or more simply by directly reducing the overall density of bacteria to which larvae are exposed. Following gut removal, parents continue to coat the carcass in secretions and then bury the balled up carrion underground (25, 41), which may influence moisture or temperature levels. Both behaviors may bias the persisting microbial species, potentially in favor of species originally introduced by caring parents. In addition, caring parents and their larvae may be exposed to different bacterial numbers and composition as a function of carcass age, a factor that is known to have a dramatic influence on larval fitness (26, 42). Although much remains to be determined about these processes, our results clarify the importance of more completely understanding how parents influence the bacteria on the carcass and how this, in turn, affects larval microbiota establishment.

After larvae complete feeding, they migrate into the soil to pupate (25, 41). Bacterial numbers during this stage decline precipitously (Fig. 1), in part, due to the absence of feeding and also to the evacuation of the larval gut. In addition, larvae in some metamorphosing insects undergo a prepupal molt that would further reduce bacterial numbers (43, 44). Regardless of the mechanisms, our data are consistent with *Nicrophorus* larvae becoming effectively sterile during pupation, an outcome previously seen in several flies and mosquitoes (45–47). It is possible that host immunity facilitates pupal symbiont suppression during metamorphosis (44, 48, 49), as a decline of phagocytic hemocytes and increasing phenoloxidase activity were both detected in *Nicrophorus* pupa (50). Following this aposymbiotic state, bacterial densities are quickly recovered at eclosion with bacterial communities that significantly overlap those present prior to pupation (Fig. 1 and 2). To determine the source of recolonization, we sampled bacteria from the pupal molt as well as the wall of the pupal chambers, and in both cases, we observed striking similarity to the microbial communities of earlier developmental stages. Interestingly, this was true for all treatment groups, suggesting that there is no intrinsic bias to recolonization but rather that eclosing beetles are colonized by a subset of the bacterial species present in the pupal chamber.

The larval gut of *N. vespilloides* thus appears to be colonized via a combination of mechanisms that are dependent on the degree of parental care and the stage of development. With complete parental care, parents transmit bacteria to larvae through a combination of direct feeding and through an indirect effect mediated by the carcass. At present, it remains unclear if the latter component is because *Nicrophorus* symbionts outcompete the mouse carrion microbiota within the larval gut or if this occurs primarily on the carcass surface. However, the former seems more likely given the vast differences in larval exposure to these two groups of bacteria and the fact that larvae in the prehatch group remained colonized by beetle symbionts despite lacking any direct exposure to parents (Fig. 2). It is tempting, given the reliable mode of transmission from parents to larvae, to speculate about the function of these symbionts for *Nicrophorus* growth and development, particularly the role of these bacteria in limiting infection from carrion-borne bacteria (29, 33). It will also be important to supplement our studies using laboratory populations of *Nicrophorus* with work focusing on field-derived beetle adults and larvae, as exposure to the broader diversity of natural bacteria in the soil or carcass may potentially influence bacterial acquisition and transmission through distinct developmental stages (33). However, this remains an active area of research that we hope to address in future publications. In addition, it will be important to supplement the present work with more detailed analyses based on

sequencing (29, 33). Although culture-based methods play an essential role in unraveling the relationships between invertebrate host sociality and their symbiont strain-level diversity (51), they are clearly complementary to sequence-based methods that can recover bacterial groups that may be difficult or impossible to culture in the laboratory. Our work clarifies the key links between *Nicrophorus* social behavior and symbiont transmission. This is likely to have parallels in other animal systems where parents invest in the care of offspring.

MATERIALS AND METHODS

General procedures. Experimental beetles were taken from an outbred laboratory population derived from wild-caught *N. vespilloides* individuals trapped in Warmond near Leiden in The Netherlands between May and June 2014. Beetles were maintained in the laboratory at 20°C with a 15-h/9-h light-dark cycle. All adults were fed fresh chicken liver twice weekly. To generate outcrossed broods, nonsibling pairs of beetles were allowed to mate for 24 h in small plastic containers with soil. Next, the mated pairs were provided with a freshly thawed mouse carcass weighing 24 to 26 g in a 15-cm by 10-cm plastic box filled with approximately 1 to 2 cm of moist soil. Although fresh carcasses may differ in bacterial composition from aged carcasses (26, 29), our use of fresh carcasses in this study ensured higher brood success and is consistent with recent data showing that most mouse carcasses are discovered by burying beetles shortly after they are placed in experimental forests (52). Broods were reared in sterile soil until the point of larval dispersal from the carcass, after which larvae were transferred to new boxes for pupation with unsterilized peat soil to complete development. Soil was sterilized using two autoclave cycles at 121°C for 30 min in a volume of 160 liters (Tuttnauer 5075 ELV), with a cooling interval between cycles.

Maternal care manipulation. To examine the role of parental care on the acquisition and composition of beetle gut bacteria, we reared larvae under the following three treatment conditions that modified the degree of parental care they received (26, 30): (i) full care (FC) broods that experienced complete parental care, including prehatch and posthatch care; (ii) prehatch parental care (PPC) broods that were reared on a carcass that had been prepared by the female, after which she was removed prior to the hatch/arrival of larvae; and (iii) no care (NC) broods that experienced neither prehatch nor posthatch care. Broods in all treatments were initiated similarly. Mated females were provided with a fresh carcass and induced to lay eggs. Eggs were collected and surface sterilized within 12 to 24 h, and these were then used to generate replicate broods of 15 to 20 larvae each. Females remained with their prepared carcasses in FC broods, while females were removed prior to reintroducing larvae in the PPC broods. NC larvae were provided with a freshly thawed carcass with a sterile incision in the abdomen to permit larval entry.

Bacterial density and composition throughout development. We examined the dynamics of *N. vespilloides* intestinal microbiota through time by destructively sampling beetles throughout development. To quantify gut bacterial CFU, the whole intestinal tract from each beetle ($n = 3$ at each time point) from independent broods was carefully removed with fine forceps and suspended in 0.7 ml of sterile sodium phosphate buffer (phosphate-buffered saline [PBS]; 100 mM, pH 7.2); beetles were surface sterilized by 75% ethanol and PBS solution (100 mM, pH 7.2) prior to gut dissection. The inner contents of pupa were examined in their entirety owing to the absence of a clear gut at this stage. Because 0.1 ml was plated from 0.7-ml dilutions, our limit of detection is ~10 cells/larval gut. Newly eclosed adults were unfed prior to sampling. Individual gut/pupal contents were serially diluted in PBS and plated on one-third strength tryptic soy broth agar and incubated at 30°C. To directly compare bacterial densities determined from total microscopic counts versus those determined via CFU from plating, bacterial cells per beetle gut ($n = 5$ from each time point) from independent broods with full care were quantified on one-third tryptic soy agar (TSA) and by estimating total cell numbers via fluorescence-based microscopy. Microscopic counts were further partitioned into live and dead cells with LIVE/DEAD staining using SYTO9 and propidium iodide (BacLight bacterial viability kit; Invitrogen). Samples were observed under a Zeiss Axioplan 2 imaging microscope. The filters used were 470/40 (green) and 572/25 nm (red) for excitation and 525/50 (green) or 629/62 nm (red) for emission. Three images were counted for each sample at each dilution. Although plating for CFU consistently underestimates bacterial densities, this approach recovered up to 60% of total counts and the dynamics of bacterial densities perfectly mirror those based on total counts. The composition of the maternal microbiota was characterized from $n = 3$ mated females.

At each time point from each treatment, we isolated random colonies ($n \geq 100$) on one-third TSA from individual beetles to analyze for species identification using MALDI-TOF mass spectrometry with the Biotyper platform (Bruker Daltonik GmbH). By generating unique whole-cell protein-based fingerprints for each colony, the Biotyper permits highly reproducible identification of bacterial colonies to the genus or species level. Because of its reproducibility, ease of use, and cost effectiveness, the Biotyper is used extensively in clinical and public health microbiological laboratories (53) and is finding increased use in ecological studies (54–56). To standardize growth prior to analysis, individual colonies were tooth-picked onto a one-third TSA plate and grown overnight. Colonies were then transferred directly to a 96-well steel MALDI-TOF target plate and coated with 1 μl of an alpha-cyano-4-hydroxycinamic acid (HCCA) matrix comprised of acetonitrile (50%), trifluoroacetic acid (2.5%), and water (47.5%) and dried at room temperature. The target plate was subsequently inserted into the Biotyper system for analysis. Next, mass spectrometry was carried out using the MALDI Biotyper RTC (real-time classification) and analyzed using

Biotyper 3.0 (Bruker Daltonik GmbH). Spectra were collected under the linear positive mode in the mass range of 3 to 20 kDa and a sample rate of 0.5 gigasamples [GS]/s (laser frequency, 60 Hz; ion source 1 voltage, 20.08 kV; ion source 2 voltage, 18.6 kV; lens voltage, 7.83 kV). The Bruker bacterial test standard (BTS 8255343) was measured for standardization of MALDI calibration before the specimens were processed. Spectra were compared to the reference library provided by Bruker, which identified 62.3% of the colonies to species level overall using a stringent cutoff of 1.699, below which indicated no reliable identification (in the Bruker library) (57, 58). To confirm these assignments and to establish the identity of colonies whose spectra were not included in the Bruker database, all unique MS spectra (including both of those with positive hits and those not present in the Biotyper database) were subsequently analyzed using 16S rRNA sequencing. Colony PCR using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) was used for bacterial 16S rRNA gene amplification (59). The PCR cycling conditions were as follows: 95°C for 5 min and then 34 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 1 min. PCR products were directly sequenced via the DNA Markerpoint in Leiden, and 16S rRNA gene sequences were classified for bacterial taxonomy using a nucleotide BLAST against the NCBI database. The Bruker database was manually updated to include new samples thus obtained.

A second experiment was conducted to determine the source of bacterial recolonization following beetle pupation. Pupae were removed from their soil chambers, and the inside wall of the soil chamber and the cuticles of the pupae were swabbed with a sterile, moist cotton swab. The bacteria on the swab were resuspended in sterile water and serially diluted onto one-third TSA. Finally, soil from outside the pupal chamber was collected and diluted into PBS and plated. Colonies were isolated and identified as above using a combination of MALDI-TOF biotyping and 16S rRNA sequencing. To exclude rare or transient bacterial species, we established a minimum threshold frequency of 1%, averaged over all sampling periods for each treatment set, prior to analysis of community composition.

Statistical analysis. Bacterial CFU through time were analyzed using general linear models (GLMs) with time and treatment as factors. Community composition was analyzed using the Vegan package in R (60). Beta diversity among the different treatments was analyzed using ANOSIM, which is based on a Bray-Curtis dissimilarity matrix (43, 61). The R function *betadisper* was used along with ANOSIM to test for equal dispersion between groups. Analyses of dispersion between parental care treatments and larvae detected no significant differences between groups ($F = 1.796$; $P = 0.12$; number of permutations = 199) nor did we detect any differences in dispersion between any of the comparisons of microbial communities examined following microbial recolonization of pupae (all tests, >0.05). Dendrograms to examine community similarity were generated based on the matrix of mean within-group and between-group distances, and the R function *hclust* was used for hierarchical clustering.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03250-16.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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