Alteration of gut microbiota with rifampicin does not impair maternal care in the European earwig

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ABSTRACT

The microbes residing within the gut of an animal host often maximise their own fitness by modifying their host's physiological, reproductive, and behavioural functions. Whereas recent studies suggest that they may also shape host sociality and therefore have critical effects on animal social evolution, the impact of the gut microbiota on maternal care remains unexplored. This is surprising, as this social behaviour is widespread among animals, often determines the fitness of both juveniles and parents, and is essential in the evolution of complex animal societies. Here, we address this gap in knowledge by testing whether life-long alterations of the gut microbiota with rifampicin - a broad-spectrum antibiotic - impair the expression of pre- and post-hatching maternal care in the European earwig, an insect exhibiting extensive forms of maternal care towards eggs and juveniles. Our results first confirm that rifampicin altered the mothers' gut microbial communities and revealed that the composition of the gut microbiota differs before and after egg care. Contrary to our predictions, however, the rifampicin-induced alterations of the gut microbiota did not modify the expression of pre- or post-hatching care. Independent of maternal care, rifampicin increased the females' feces production and resulted in lighter eggs and juveniles. By contrast, rifampicin altered none of the other 23 physiological, reproductive and longevity traits measured over the females' lifetime. Overall, these findings reveal that altering the gut microbiota does not necessarily affect host sociality. More generally, our results emphasize that not all animals have evolved a co-dependence with their microbiota.

Keywords: Antibiotic, Dermaptera, Insect, Microbiome, Parental care

1-INTRODUCTION

Almost all animals harbour a gut microbiota, i.e. a community of microorganisms residing within the gut of the host [1]. Some of these gut microbes have long been known for their pathogenic effects in the hosts [2] and others for the benefits they provide to the hosts in terms of nutritional mutualism [3]. Over the last decades, however, a growing number of works has been revealing that the effects of gut microbes are much more diverse than previously thought and shape numerous physiological, reproductive, and behavioural functions of the host [4]. In the fruit fly *Drosophila melanogaster*, for instance, the gut microbiota is associated with hormone signalling, metabolism and ageing [5]. Gut microbes can also shape the hosts' immunocompetence and resistance against pesticides and toxic plant metabolites [6], such as in the mosquito *Anopheles stephensi* [7], the bean bug *Riptortus pedestri* [8] and the wasp *Nasonia vitripennis* [9]. Similarly, the gut microbiota is a key parameter in host reproduction and mating incompatibilities, as found in the fruit fly *Bactrocera minax* [10], in the terrestrial isopod *Armadillidium vulgare* [11], and the parasitic wasp *Asobara tabida* [12]. Finally, gut microbes shape the expression of numerous host behaviours, such as nutritional preference and kin recognition in *D. melanogaster* [13,14], offspring activity in the stinkbug *Megacopta punctissima* [15], and different behavioural tasks in honeybees [16].

In addition to these multiple effects, recent studies suggest that gut microbiota also play a critical role in the sociality of their hosts by shaping the expression and nature of social interactions among group members. For instance, individuals with an altered gut microbiota exhibited deficient sociability and increased social avoidance in family-living rats [17,18], as well as showed higher levels of aggressiveness toward conspecifics in colonies of the leaf-cutting ant

Acromyrmex echinator [19]. Experimental alterations of gut microbial communities also rendered hosts less attractive to conspecifics both in the gregarious German cockroach *Blattela germanica* [20] and in the swarm-living desert locust *Schistocerca gregaria* [21]. Most of these social alterations were reverted when individuals received transplants of their original gut microbiota [17–20], suggesting that certain microbes and/or the gut community as a whole were responsible for the sociality of their host and thus, more generally, supporting the hypothesis that gut microbes could have a key role in the evolution of their hosts' social life.

Despite these apparent links between the hosts' gut microbial communities and their social behaviours, the role of gut microbes on the expression of parental care remains experimentally unexplored. This is surprising, because this form of social behaviour is present in a large and taxonomically diverse number of animal species [22], has considerable effects on the fitness of both juveniles and parents [23] and because shedding light on this link may provide crucial information on the role of gut microbes in the early evolution of complex animal societies [24]. On one hand, gut microbes may indirectly drive parental care, because parents are expected to adjust their level of care to their own condition [25] and altered gut microbial communities can lower these conditions in multiple ways (see above). On the other hand, gut microbes could serve as a direct promoter of parental care because, by enforcing the expression of care in adult hosts, parental gut microbes could maximize their chances to reach novel hosts [26–31](but see [32]). The transfer of gut microbes through parental care has been reported in several insect species, such as the stinkbug *Parastrachia japonensis* [33], the Japanese common plataspid stinkbug *Megacopta punctatissima* [34] and the wood cockroach

71 *Cryptocercus punctulatus* [35,36]. However, whether the gut microbiota drives the expression
72 of parental care remains untested.

73 In this study, we address this gap in knowledge by investigating whether gut microbiota
74 alteration with rifampicin - a broad-spectrum antibiotic - impaired the expression of pre- and
75 post-hatching maternal care in the European earwig *Forficula auricularia* L. In this omnivorous
76 insect, mothers tend their clutch of eggs over winter. During this period, mothers stop their
77 foraging activity to provide extensive maternal egg care in the forms of protection against
78 desiccation, predators and pathogens [37,38]. Upon egg hatching, mothers continue tending
79 their brood of newly emerged juveniles (called nymphs) for two weeks, during which they
80 provide post-hatching care in the forms of fierce protections against predators, grooming
81 behaviours, and food provisioning through regurgitation [39,40]. Interestingly, pre-hatching
82 care allows mothers to transfer microbes exhibiting antifungal properties to their eggshell in
83 the maritime earwig *Anisolabis maritima* [41], a behaviour that could also occur in the
84 European earwig [42]. In *F. auricularia*, pre-hatching maternal care is necessary to ensure egg
85 development and hatching [42], whereas post-hatching maternal care is facultative for the
86 development and survival of nymphs [43]. Here, we altered the gut microbiota of *F. auricularia*
87 females by feeding them with rifampicin during their entire adult lifetime (about 14 months)
88 and measured whether and how this treatment affected gut microbial communities, maternal
89 care, and other life-history traits. Specifically, we first determined how the antibiotherapy alters
90 the diversity and the structure of the gut bacterial community of females at two periods of their
91 life-cycle (before oviposition and at egg hatching) by sequencing 16S rRNA gene (V3-V4 region)
92 amplicons. We then tested the effects of rifampicin on the expression of four pre- and two

post-hatching forms of maternal care. Finally, to disentangle whether the potential link between gut microbiota alteration and the level of maternal care is direct and/or indirect, we investigated the effects of rifampicin on 32 other traits measured throughout the females' lifetime and reflecting their general physiological state, reproductive success and longevity, as well as their juveniles' development, sex-ratio and survival.

2-MATERIALS AND METHODS

2.1 Insect rearing and rifampicin treatment

The experiment involved a total of 296 *Forficula auricularia* L. (clade B [44]) males and females. These were the first laboratory-born descendants of 74 females field-sampled in Pont-de-Ruan, France, in 2017 and then maintained under standard laboratory conditions [45]. For each of these 74 families, 2 males and 2 females were isolated at adult emergence and immediately fed with a standard food mixed with either 10 µL of rifampicin (1 male and 1 female per family; Sigma-Aldrich, PHR1806; 0.2 mg/ml) or 10 µL of water (1 male and 1 female per family). The standard food contained agar, carrots, pollen, and cat and bird dry food [45]. Two weeks later, 148 mating pairs were set up (1 female and 1 male from the same family and same treatment). The use of sibling pairs allowed us limiting the risk of cytoplasmic incompatibility due to inter-familial microbiome variability, and there are only limited signs of inbreeding depression in this species [46]. They continued to receive the same rifampicin- and water-treatment for about two months. At that time, females were isolated to mimic natural dispersal for oviposition [45]. From oviposition to egg hatching, four forms of egg care were measured (details below). During that time, females were not provided with food and thus not treated with rifampicin, as

mothers typically stop foraging during the period of egg care [43]. One day after egg hatching, each family (a mother with its newly hatched juveniles) was provided with either rifampicin or water to follow up on the pre-oviposition treatment. Three forms of maternal care towards juveniles were measured during the following 14 days (details below), which corresponds to the natural duration of family life [45]. At the end of these 14 days, families were split to allow newly isolated mothers to produce a 2nd clutch and permit groups of nymphs to continue their development. The mothers and groups of nymphs continued to receive the same treatment (rifampicin or water) until the end of the experiment, i.e. until the mother died and nymphs reached adulthood. Throughout the experiment, the treatments were renewed twice a week (except during egg care). All isolated adults, groups of nymphs, and families were maintained in Petri dishes (diameter 9 cm) lined with moistened sand. More details on the experimental setup in the supplementary material.

Rifampicin is considered one of the most potent and broad-spectrum antibiotics due to its high-affinity binding to the RNAP β subunit, which causes the inhibition of the bacterial DNA-dependent RNA polymerase RNAP by directly blocking the RNA elongation path [47]. It is also commonly used to experimentally alter gut microbial communities in insects (e.g. [48–50]). The high dose of rifampicin used in this study (about 10 times higher than the dose generally used in smaller insect species [49,50]) was chosen to ensure gut microbial alteration and because it did not trigger an excess of mortality in the German cockroach [48], an insect that is about the size of the European earwig.

2.2 Effects of rifampicin on the gut microbiota

To determine whether and how rifampicin treatment altered the earwigs' gut microbial communities, we extracted the gut of 10 females per treatment (n total = 20) on the day we observed the first oviposition (i.e. about 2 months after being fed with or without rifampicin), and 10 rifampicin- and 8 water-treated females one day after egg hatching (i.e. about 1 month later). For gut extraction, we first anaesthetized each female for 2 min at -20°C and then dissected them in a watch glass with sterilized double Q water. All dissections and manipulations were conducted on a sterilized bench, under a Bunsen burner's sterility area and using sterile material. Whole individual guts were extracted, placed in 100 µl of T1 extraction buffer (Nucleo-Spin Tissue, Macherey-Nagel), and stored at −80°C until DNA extraction. Protocol for DNA extractions is detailed in the supplementary material. Two PCR amplifications were performed for each sample in a final volume of 35 µl to amplify a 450-bp portion of the V3–V4 region of the 16S rRNA gene (forward primer: 5′-CTT TCC CTA CAC GAC GCT CTT CCG ATC T**AC GGR AGG CAG CAG**-3′; reverse primer: 5′-GGA GTT CAG ACG TGT GCT CTT CCG ATC T**TA CCA GGG TAT CTA ATC**-3′; the Illumina adapters and primers *per se* appeared in non-bold and bold, respectively). Fifty microliters of PCR product were then sent to the GeT-PlaGe genomic platform (GenoToul, Toulouse, France), which performed library preparation and 2 × 250 paired-end Illumina Miseq sequencing according to the manufacturer's instructions. Protocols of the sequencing process and bioinformatic pipelines are detailed in the supplementary material.

2.3 Measurements of pre- and post-hatching maternal care

We measured the effects of rifampicin on four classical forms of earwig maternal egg care: egg grooming, egg defence, delay of maternal return and egg searching exploration rate [38,51]. Egg grooming, which is used by earwig females to deposit chemical substances on the eggs and to clean eggshell from dirt and fungi [42], was measured 15 days after egg production. We first isolated each mother for 30 min, then returned them to their Petri dish and gently deposited them at a distance of 5 cm from their egg clutch, and finally recorded their behaviours for the subsequent 15 minutes on camera (SONY© Handycam HDR-CX700 camera). The resulting movies were analysed using the software BORIS v4.0.3 [52] and the total duration of egg grooming was defined as the total amount of time each female spent on cleaning eggs with their mandibles [42]. Clutch defence, which reflects the females' willingness to protect their eggs from a predator attack [53], was measured 16 days after egg production. We standardly poked each female on the pronotum with a glass capillary (one poke per second) and then recorded the number of pokes required until the female moved more than one body length away from the clutch. The delay of maternal return after clutch abandonment [38], which represents the delay after which females return to their clutch after being chased away by a simulated predator attack [53], was measured by recording the time the female took to return to its clutch just after the end of the clutch defence measurement. Finally, the egg searching exploration rate, which represents the level of exploration of a novel area by a mother looking for her eggs, was measured 21 days after egg production. We removed each mother from its clutch of eggs, subsequently deposited it at the centre of a square arena (W: 9 cm; L: 9 cm; H: 0.5 cm) covered by a glass sheet, and then video-tracked its activity during the following 35

min. The video was done under infra-red light, while the individual video tracking and calculation of exploration rate were conducted using the software ToxTrac v2.83 [54].

We then measured the effects of rifampicin on two classical forms of post-hatching maternal care: the defence of and search for juveniles [51,53]. These two forms of care were measured 10 days and 12 days after egg hatching, respectively, following the above-detailed protocols for egg defence and egg searching activity. All the measurements of pre- and post-hatching maternal care were conducted in the afternoon and under a red light as earwigs are nocturnal. These measurements were conducted blindly regarding the treatments (rifampicin versus control). The number of replicates for each of our measurements ranged from 21 to 56 (details in Tables 1 and S1).

2.4 Measurements of the 24 other life-history traits in mothers and offspring

We tested the effects of rifampicin on 7 proxies of female physiology, 16 proxies of female reproduction and on female longevity using standard protocols [45,55]. We measured the females' physiology through their feces production (reflecting their digestive/foraging activity) and weight gains between two life stages. Feces production was measured two months after the beginning of the treatments. Females were isolated in a new Petri Dish for 24 hours, after which we counted the number of feces pellets present on the ground. The weight gains of each female were measured between the days of adult emergence and oviposition, and between the days of oviposition and egg hatching. Reproductive success was measured in the 1st and 2nd clutches (if any), by counting the number of eggs produced, the number of days between oviposition and egg hatching (egg development time), and by measuring the mean egg weight

at oviposition, the egg hatching rate, and the mean offspring weight at egg hatching. We also counted the number of days between adult emergence and oviposition (delay until oviposition), between the females' isolation after family life and 2nd clutch oviposition (delay until 2nd clutch production), and between adult emergence and death (female longevity). We finally assessed the females' likelihood to produce a 2nd clutch (1 or 0) and the females' reproductive allocation between the two clutches (i.e. the females' reproductive strategy [45]). This allocation was defined as the number of 2nd clutch eggs divided by the total number of eggs produced by a female.

Because mothers and groups of nymphs continued to receive their treatment after the end of family life, we also tested the effects of rifampicin on juvenile developmental time between each developmental instar, on the survival rates from egg hatching until both the end of family life and adulthood, and on the sex ratio of the resulting adults. Juvenile developmental time was defined as the differences between the moulting date in a given instar (or hatching date) and the moulting date of its subsequent instar at the family-level, i.e. focusing on the first individual moulting in the clutch. Every weighing was done to the nearest 0.01 mg using a microbalance (OHAUS© Discovery DV215CD). Sample sizes for each measurement are detailed in Tables 1 and S1. More details on the methods are provided in the supplementary material.

2.6 Statistical analyses

Analyses of the α and β-diversity indices. The structure, composition and diversity of the microbial communities were analysed using PHYLOSEQ R package [56] implemented in the

222 FROGSSTAT Phyloseq tools [57]. Diversity within the gut microbial communities (alpha-
223 diversity) was assessed using two richness indices which estimate the number of species in the
224 microbiome with correction for subsampling (Chao1; ACE), and three metrics that aim to
225 measure diversity by accounting for evenness or homogeneity (Shannon, Simpson, Inverse
226 Simpson, and Fisher) [58]. Diversity between the gut microbial communities (beta-diversity)
227 was assessed using two non-phylogenetically informed (Bray Curtis dissimilarity; Jaccard indice)
228 and two phylogenetically informed (uniFrac; Weighed uniFrac) measures of community
229 similarity. The metrics were analysed individually using either a General Linear Model for α-
230 diversity, or a Permutational Multivariate Analysis of Variance Using Distance Matrices
231 (PERMANOVA) for β-diversity. In these models, the values (or distance matrix for β-diversity) of
232 each index were entered as a response variable, while the treatment (rifampicin or water), the
233 sampling stage of the female (before oviposition or at egg hatching) and the interaction
234 between them were used as fixed factors. When required, a post-hoc analysis was conducted
235 by splitting the data set according to the sampling stage and then conducting PERMANOVA on
236 each of the resulting subsets.
237
238 *Analyses of the life-history traits.* Although the presented experimental design was originally
239 paired, i.e. 2 females per family distributed among the two treatments, the 38 life-history traits
240 were often measured in only one of the paired individuals (detailed sample sizes in Tables 1 and
241 S1). This was mostly due to time constraints, and because some females died during the 18-
242 months course of this experiment. These overall led to critical reductions in the number of
243 replicates that could be involved in a paired statistical approach (details in Table S2). We,

244 therefore, analysed the effects of rifampicin on the 32 measurements using a series of 31 exact
245 Mann Whitney U tests and 1 Pearson's Chi-squared test (for the likelihood to produce a 2nd
246 clutch), in which we compared the values of all the available replicates fed with rifampicin to
247 the values of all the available replicates fed with water. Note that the results do not
248 qualitatively change when we use paired analyses with the associated smallest sample sizes
249 (results presented in Table S2). To correct for the inflated risk of Type I errors due to multiple
250 testing, all p-values were adjusted using the False Discovery Rate (FDR) method [59]. All these
251 analyses were conducted using the software R v4.0.2 (www.r-project.org).

3-RESULTS

252 *3.1 Description of the earwig gut microbiota*

253 A total of 1636435 sequenced reads of the 16S rRNA V3-V4 region were obtained from the 38
254 female earwig gut samples. After sequence processing, this number went down to 1130241,
255 with 21069 to 35385 sequences per sample (median = 30595.5). The sequences were
256 aggregated and filtered in a total of 161 unique OTUs, which were resolved down to the family
257 or genus level to increase the confidence in the taxonomic assignation. All detailed information
258 on OTUs is given in Table S3. More than 99.90% of the sequences were assigned to four
259 bacterial phyla: Proteobacteria (65.94%), Firmicutes (21.12%), Bacteroidota (9.89%) and
260 Actinobacteriota (2.96%) (Figure 1). The remaining OTUs were assigned to Bdellovibrionota
261 (0.04%) and Patescibacteria (0.04%).

262 *3.2 Comparative analyses of the α and β diversity of the gut microbiota*

263 The gut microbial α-diversity (i.e. species richness) decreased between oviposition and egg
264 hatching when this diversity was measured using Chao1 ($F_{1,34}$ = 21.63, P < 0.0001), ACE ($F_{1,34}$ =
265 24.46, P < 0.0001) and Fisher ($F_{1,34}$ = 20.85, P < 0.0001; Figure 2) indices. This decrease was,
266 however, not significant when α-diversity was measured using Shannon ($F_{1,34}$ = 3.18, P = 0.084;
267 Figure 2) and Simpson ($F_{1,34}$ = 1.60, P = 0.214) indices. The α-diversity was overall independent
268 of the rifampicin treatment (Chao1: $F_{1,34}$ = 0.72, P = 0.401; ACE: $F_{1,34}$ = 0.62, P =0.435; Fisher:
269 $F_{1,34}$ = 0.59, P = 0.447; Shannon: $F_{1,34}$ = 1.67, P = 0.205; Simpson: $F_{1,34}$ = 0.55, P = 0.465; Figure
270 2), and of an interaction between female sampling stage and rifampicin treatment (all P >
271 0.525).

272 The gut microbiota β-diversity (i.e. species composition) overall changed with female
273 sampling stage and rifampicin treatment. This was the case with the four measured indices of
274 β-diversity: Bray-Curtis (Stage: $F_{1,34}$ = 5.77, P < 0.0001; Rifampicin: $F_{1,34}$ = 4.23, P < 0.0001),
275 JACCARD (Stage: $F_{1,34}$ = 7.76, P < 0.0001; Rifampicin: $F_{1,34}$ = 2.37, P = 0.0036), unweighted
276 UniFrac (Stage: $F_{1,34}$ = 6.51, P < 0.0001; Rifampicin: $F_{1,34}$ = 3.39, P = 0.0006) and weighted
277 UniFrac (Stage: $F_{1,34}$ = 14.10, P < 0.0001; Rifampicin: $F_{1,34}$ = 6.42, P = 0.0006). In particular,
278 females before oviposition harboured less Actinobacteriota and Proteobacteria compared to
279 females at egg hatching, while rifampicin females overall harboured less Bacteroidota and more
280 Firmicutes compared to untreated females (Figure 1). Interestingly, the interaction between
281 female sampling stage and rifampicin had no effect on the β-diversity measured using all (all P >
282 0.117) but the weighted UniFrac indices ($F_{1,34}$ = 2.94, P = 0.026). This interaction reflected an
283 effect of rifampicin on the β-diversity before oviposition ($F_{1,34}$ = 0.17, P = 0.018) but not at egg
284 hatching ($F_{1,34}$ = 0.97, P = 0.356).

3.3 Rifampicin and maternal care

We did not detect any effect of rifampicin on the six measured forms of egg and nymph care (Table 1). In particular, rifampicin- and control-fed mothers spent the same amount of time in egg grooming (Figure 3A), showed the same levels of both egg and juvenile defences against a simulated predator attack (Figures 3B and 3E), exhibited the same delay to return to their eggs after egg defence (Figure 3C) and showed comparable exploration rates when looking for their eggs or their juveniles (Figures 3D and 3F).

3.4 Rifampicin and female's physiology, reproduction, and longevity

The consumption of rifampicin altered 3 of the 23 measured proxies of female physiology, reproduction, and longevity. In particular, females fed with rifampicin produced on average twice as many feces pellets per 24h (Figure 4; Table 1), had newly hatched nymphs that were 15% lighter (W = 1244, P = 0.002, adjusted-P = 0.025; Table S1) and laid 2nd clutch eggs that were 7% lighter (W = 628, P = 0.002, adjusted-P = 0.025; Table S1) compared to control females. By contrast, we did not detect any effect of rifampicin on the 20 other traits (Tables 1 and S1).

3.5 Rifampicin and nymphs' development, sex-ratio and survival

We did not detect any effect of rifampicin on the juveniles' developmental speed from hatching to adult and at every step of their development, as well as the sex-ratio of the 1st clutches (Tables 1 and S1). Similarly, the survival rate of nymphs throughout family life (i.e. from egg

303 hatching until day fourteen) and from the end of family life until they reached adulthood were
304 not impacted by the consumption of rifampicin (Tables 1 and S1).

4-DISCUSSION

305 Whereas gut microbial communities shape the physiology, reproduction and behaviours of a
306 great diversity of hosts, their importance on parental care – a key behaviour in social evolution
307 [22–24] - remains poorly explored [31]. In this study, we addressed this gap in knowledge by
308 treating females of the European earwig with rifampicin and measuring the effects on gut
309 microbial communities, maternal care and female physiology, reproduction, and longevity. Our
310 results first reveal that rifampicin altered the composition of the gut microbial community of
311 earwig females and show that this modification diminishes during the period of egg care.
312 Contrary to our predictions, however, the rifampicin-induced alterations of gut microbial
313 communities did not impair the expression of pre- or post-hatching care: rifampicin-treated and
314 control mothers showed similar levels of egg grooming, clutch defences against a predator,
315 maternal return and clutch searching. Independent of maternal care, our results reveal that the
316 consumption of rifampicin increased the females' production of feces pellets, as well as lead to
317 the production of lighter nymphs and lighter 2nd clutch eggs. By contrast, rifampicin affected
318 none of the other 23 physiological, reproductive and longevity traits measured over the
319 females' lifetime.

320 Our experiment first demonstrates that the ingestion of rifampicin by earwig females
321 induced stage-specific modifications in the species composition (β-diversity) of the gut
322 microbiota but did not shape its species richness (α-diversity). These findings confirm that the

earwig gut microbiota harbours both bacterial taxa (and/or genetic variants of certain taxa) that are sensitive and taxa that are resistant to rifampicin, and thus that our treatment successfully altered gut microbial communities (just like in other animal species [48–50]). Independent of rifampicin, our data also reveal that both α- and β-diversity changed from pre-oviposition to egg hatching. This stage-specific pattern may result from the absence of food intake for about four weeks before gut sampling in females at egg hatching compared to before oviposition [60], and/or from the different rearing temperatures [61] and differences in female age [62] between the two life stages. Notwithstanding the drivers of this stage-specific effect, it is important to note that all the tested females were re-treated with rifampicin (or water) after egg hatching so that the resulting alteration of their gut microbiota reported at oviposition likely operated during their entire lifetime and could thus have affected all the traits measured after egg-hatching.

Although gut microbial communities shape the expression of hosts sociality in numerous vertebrate and arthropod species [17–21], our findings reveal that rifampicin-induced alterations of this community did not affect the expression of pre- and post-hatching maternal care in earwigs. Gut microbes were expected to directly drive the expression of parental care, because enforcing the expression of this social behaviour may allow symbionts to reach new hosts (i.e. offspring) that are typically young (thus offering long-lived habitats), display poor immune defences (thus facilitating bacterial establishment and development [63]) and harbour only a few resident microbes (thus limiting the risk of competition within the microbiome [29]). This absence of a link between rifampicin and maternal care may first suggest that earwig parental care is shaped by microbes that are non-sensitive to rifampicin. In insects, gut

345 microbial communities do not only encompass a broad diversity of bacteria (among which some
346 are resistant to rifampicin) but also fungi, protists and other microorganisms that could have
347 key roles in hosts biology [41,64,65]. Even if the previous experimental works linking gut
348 microbiota and host sociality focused on bacteria [17–21], future studies will be required to
349 confirm that no other members of the gut microbiota shape parental care in our study species.
350 A second hypothesis is that microbial symbionts never developed any specific capabilities to
351 manipulate host sociality, either because adapted strain never occurred within the microbial
352 populations associated with these earwig species (or populations), or because certain
353 antagonistic interactions (e.g. competition) among the members of the microbial community
354 have prevented the emergence of host social manipulation. Any symbiont species (or strain)
355 investing its resources to manipulate host behaviour is indeed expected to be outcompeted
356 within the microbiome by other species or variants that, instead, direct their resources into
357 growth, survival or directly transmission [32](but see for the evolution of paternal care [31]).
358 Finally, a third hypothesis is that the symbionts' capability to manipulate host sociality may
359 have changed and/or disappeared during host social evolution and consequently vanished in
360 the European earwig. The evolutionary drivers of family life indeed change over time [24] and,
361 while gut microbes may have (at least partly) driven the ancestral evolutionary shift from
362 solitary to family living for the reasons detailed above, the resulting benefits of parental care
363 for the hosts could have consolidated the expression of care and thus reduced the capability of
364 symbionts to control host social behaviours and/or reduced the sensitivity of the hosts to this
365 manipulation. Based on this hypothesis, alterations in gut microbial communities should not
366 shape the expression of parental care once this behaviour is established. In earwigs, pre- and

post-hatching maternal care are well established (even if their levels of expression differ between females and the associated benefits appear to be limited for juveniles [43,53]), which may thus have limited the maintenance of symbiont control over parental care. Overall, our findings provide the first experimental evidence that alteration of the gut microbiota (with rifampicin) does not directly or indirectly impair the expression of maternal care and thus emphasize the potentially limited role of the gut microbiota in this important social behaviour.

Despite its apparent lack of effects on maternal care, rifampicin altered three female life-history traits related to physiology and reproduction. The first trait is the production of feces pellets, which was twice as high in rifampicin-treated compared to control females. This result was not surprising, as the gut microbiota often plays a key role in nutrient extraction and digestion [66] and its alteration by antibiotics typically disturbs the host's digestive efficiency and triggers an overproduction of fecal material. The two other traits were the weights of the 2nd clutch juveniles and 2nd clutch eggs, which were lighter in rifampicin compared to control females. Light eggs and newly hatched juveniles are often thought to reflect low offspring quality in insects [67]. In the present study, however, heavier eggs and newly hatched juveniles did not translate into higher offspring survival and improved development compared to lighter counterparts. On a proximate level, these findings suggest that rifampicin breaks the association between offspring weight and quality, either due to alteration in gut microbial communities and/or antibiotic toxicity. More generally, these findings stress that rifampicin only has a limited impact on offspring fitness, as least under laboratory conditions.

Rifampicin altered none of the 23 others developmental, physiological, reproductive and longevity traits measured in earwig mothers and offspring. Whereas these findings contrast

with a large body of literature showing the broad impact of altered gut microbial communities on host biology [4], they are in line with a few recent studies showing that antibiotic-induced alteration of gut microbial communities does not affect development and survival of the three Lepidopteran *Danaus chrysippus*, *Ariadne merione* and *Choristoneura fumiferana* [68–70]. Together with these findings, our results thus provide support to the idea that essential microbial symbioses are not universal across insect species [68,71]. In these three Lepidoptera species, the lack of microbial symbioses has been explained by the fact that they do not depend on specific gut bacteria to derive critical nutrition from their dietary resources [70,72,73]. This might also be the case in the European earwig because it is omnivorous [45] and thus a partnership with bacteria facilitating the digestion of specific food sources might not have been required during species evolution. Future works are nevertheless required to test whether (or which part of) its gut microbiota is transient.

To conclude, our study reveals that rifampicin consumption alters female gut microbial communities in earwigs, but provides no evidence for a link between this alteration and the expression of parental care, and no evidence for a strong impact of this alteration on earwig physiology, reproduction and survival. Our study also shows that earwig females harbour different gut microbial communities before and after the period of egg care, a result in line with temporal variation in the microbial communities present on eggshells in the maritime earwig [41]. Overall, these findings provide support to the recent proposal that microbial enforcement of host social interactions is unlikely to evolve [32] and to the emerging idea that not all animals have evolved a co-dependence with their microbiome [68,71]. Nevertheless, shedding light on whether and how a symbiotic community shape hosts biology is a difficult task, mostly due to

the number of players possibly involved and the complexity of their potential interactions [69]. Hence, our findings pave the way for follow-up studies testing whether other (non-rifampicin sensitive) members of the gut microbial community could shape the expression of parental care in family-living animals and/or drive important fitness parameters of earwig biology.

5-ACKNOWLEDGEMENTS

We thank Jos Kramer and Maximilian Körner for their comments on this manuscript. This research has been supported by a research grant from the French Ministry of Research (to S.V.M.) and a research grant from the ANR (project *MicroSoc* to J.M.).

6-DATA ACCESSIBILITY

The raw metabarcoding sequence data have been deposited in the NCBI Sequence Read Archive under the BioProject PRJNA646389 with BioSample accession numbers SAMN15547835 to SAMN15547872. The Dataset and R script used for analysis of life history traits and behaviour as well as the detailed bioinformatics pipelines reported in this manuscript are available in the public repository server Zenodo [74].

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Table 1. Effects of rifampicin on a representative selection of 15 of the 36 measured traits reflecting maternal care, female physiology, reproduction and longevity, as well as nymph development and survival. The effects of rifampicin on all 36 traits are presented in table S1. P-values significant after correction for multiple comparisons (Adj-P) are in bold. Med = Median; 1Q and 3Q = first and third quartile, respectively. N = sample size.

	Water				**Rifampicin**				**Statistics**		
	Med	1Q	3Q	N	Med	1Q	3Q	N	W	P	Adj-P
Maternal care											
Egg grooming (sec)	379.34	259.20	492.28	26	359.54	264.49	450.18	22	288.5	0.963	0.963
Egg defense	12.00	7.00	27.00	55	14.00	8.00	26.75	56	1397.0	0.398	0.582
Delay maternal return (sec)	32.00	17.00	54.00	55	27.00	10.75	60.50	56	1677.5	0.417	0.582
Egg searching (%)	68.37	59.72	76.28	27	69.55	50.78	81.88	24	339.5	0.775	0.807
Juveniles defense	6.00	3.00	13.50	35	5.00	3.00	8.75	30	592.5	0.377	0.582
Nymph searching (%)	80.61	77.55	85.20	21	84.16	77.17	89.55	22	208.0	0.584	0.716
Female physiology											
Feces production	6.50	4.00	11.00	36	13.00	10.00	14.00	36	303.0	**<0.001**	**<0.001**
Abs. weight gain during egg care (mg)	1.28	-1.28	4.82	52	2.32	0.19	4.48	59	1308.0	0.182	0.407
Female reproduction & longevity											
No. eggs produced in the 1st clutch	55.00	48.50	60.00	59	53.00	43.00	58.75	62	2037.0	0.280	0.560
No. eggs produced in the 2nd clutch	28.00	20.00	33.00	33	23.00	14.00	28.50	27	561.0	0.087	0.383
Total No. nymphs produced	32.50	22.00	52.00	32	21.50	7.50	32.50	28	596.5	0.027	0.203
Female longevity (days)	323.00	293.50	361.00	39	306.00	284.50	343.25	42	994.5	0.098	0.310
Nymph development and survival											
Dvptal time from hatching to adults	81.00	75.50	85.50	35	82.00	77.25	85.75	34	565.5	0.727	0.807
Survival rate from hatching to day 14	84.38	77.50	90.00	35	85.16	75.37	91.37	34	572.0	0.786	0.807
Survival rate from day 14 to adults	42.86	29.86	59.91	35	39.88	27.27	66.67	34	620.5	0.763	0.807

6-FIGURES

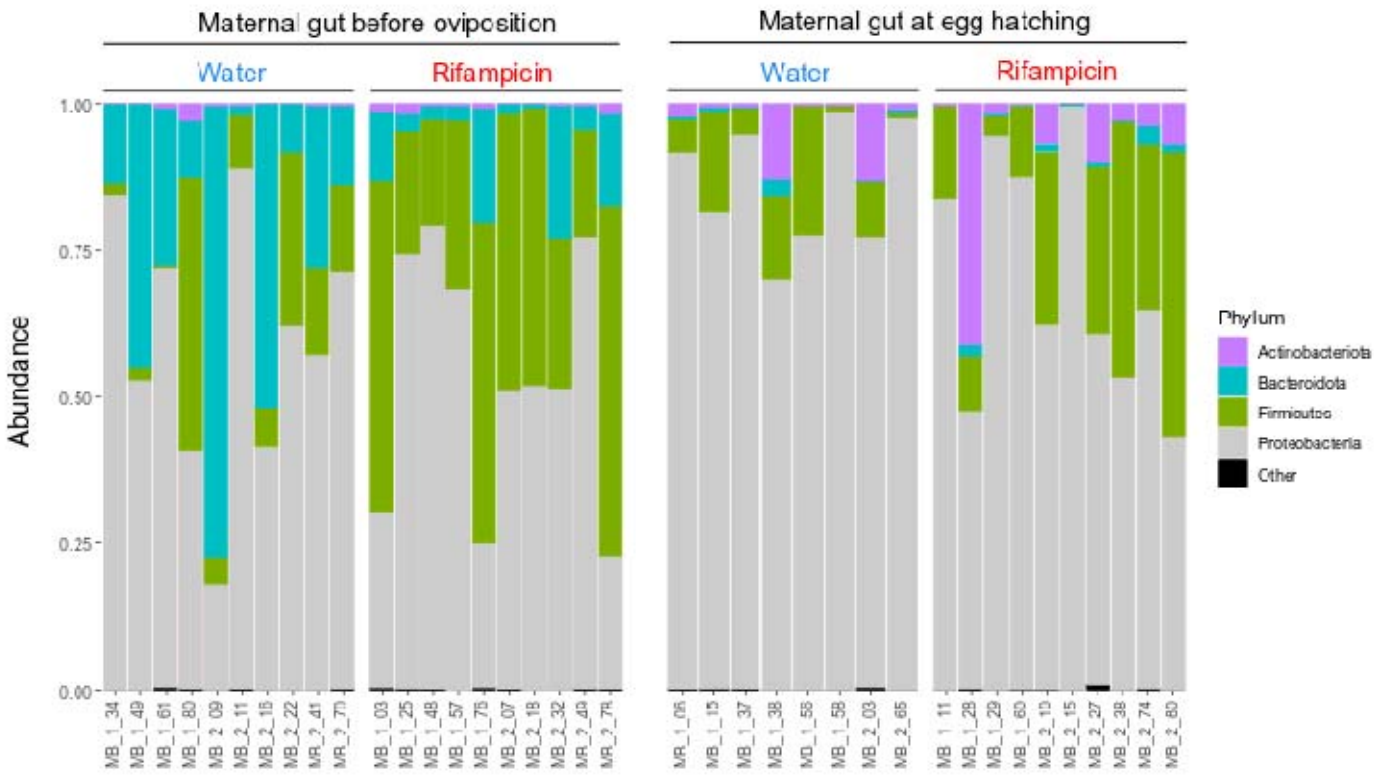

Figure 1 – Gut microbial composition in females. Guts were sampled either before oviposition or at egg hatching in females treated either with water or rifampicin. The ID of each female is provided on the x-axis. More details in table S3.

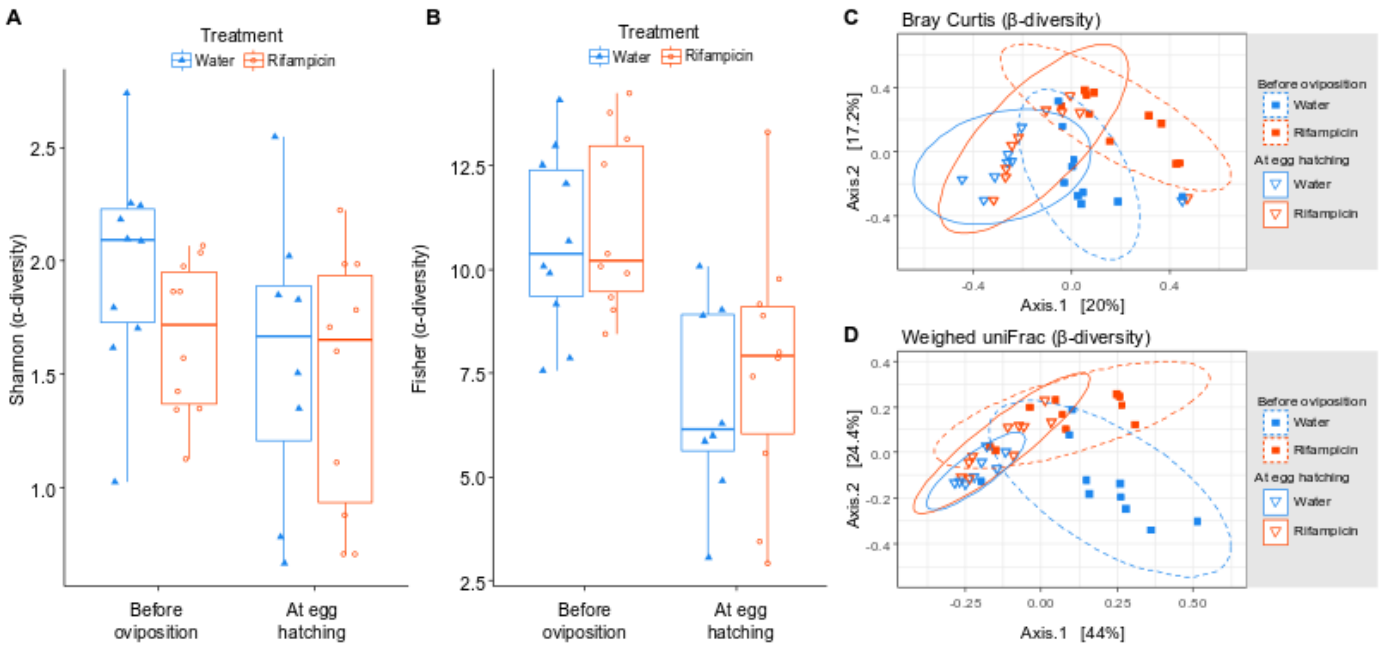

Figure 2 – Effects of rifampicin and female sampling stage on gut microbial *α*- and *β*-diversities. Guts were sampled either before oviposition or at egg hatching in females treated either with water or rifampicin. (A, B) *Alpha*-diversity based on Shannon and Fisher indices (representative of all the tested metrics), respectively. Box plots depict median (middle bar) and interquartile range (box), with whiskers extending to 1.5 times the interquartile range and dots/triangles representing values of each sample. (C, D) *Beta*-diversity based on Bray-Curtis and weighed- uniFrac indices (representative of all the tested metrics). Illustrations report multidimensional scaling (MDS) results, where dots show values and ellipses represent 95% confidence intervals.

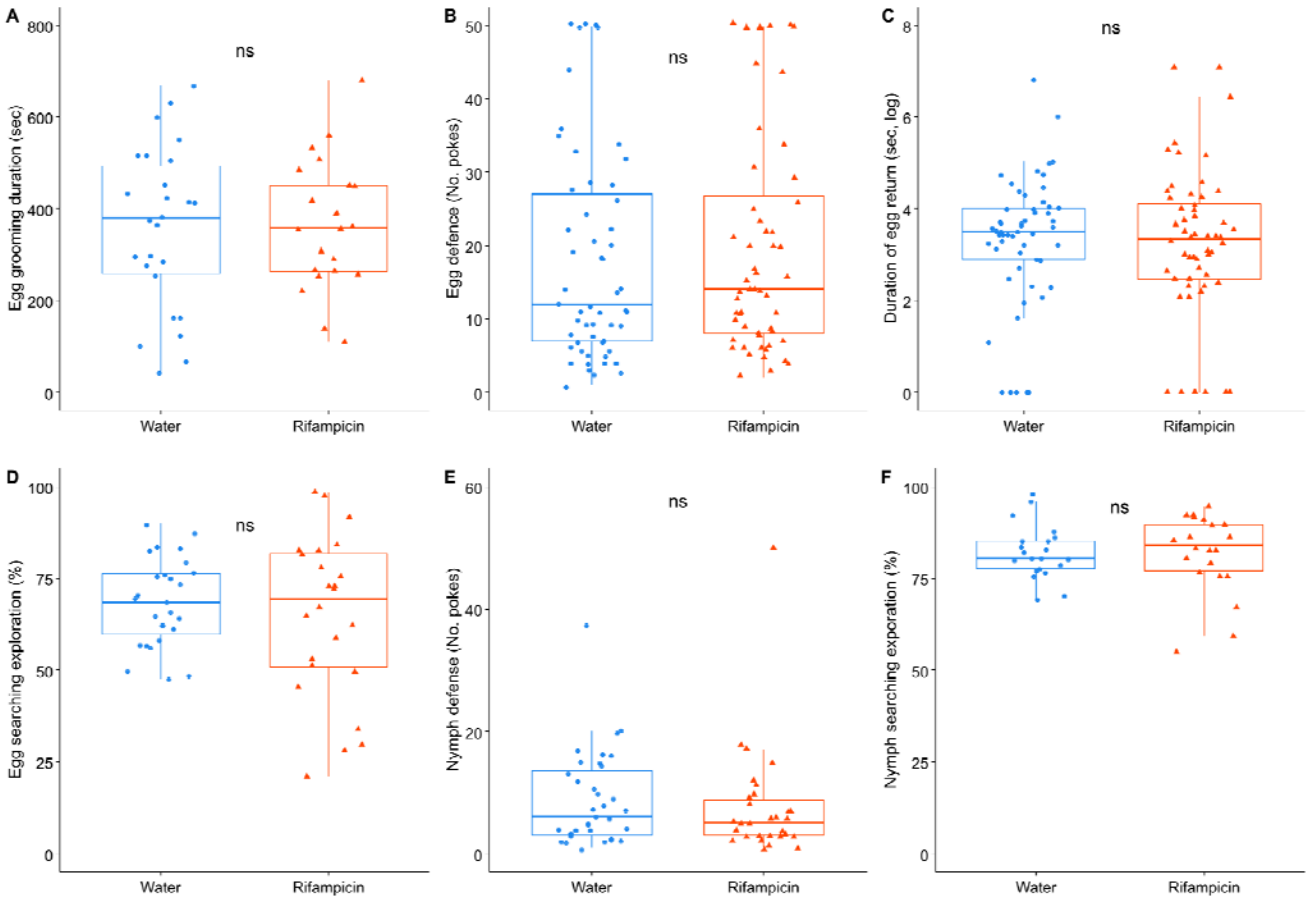

Figure 3 – Effect of rifampicin on maternal care. (A) duration of egg grooming, (B) egg defence against a simulated predator, (C) delay of maternal return after egg defence, (D) egg searching, (E) nymph defence against a simulated predator and (F) nymph searching. Box plots depict median (middle bar) and interquartile range (light bar), with whiskers extending to 1.5 times the interquartile range and dots representing experimental values. *ns* stands for $P < 0.05$.

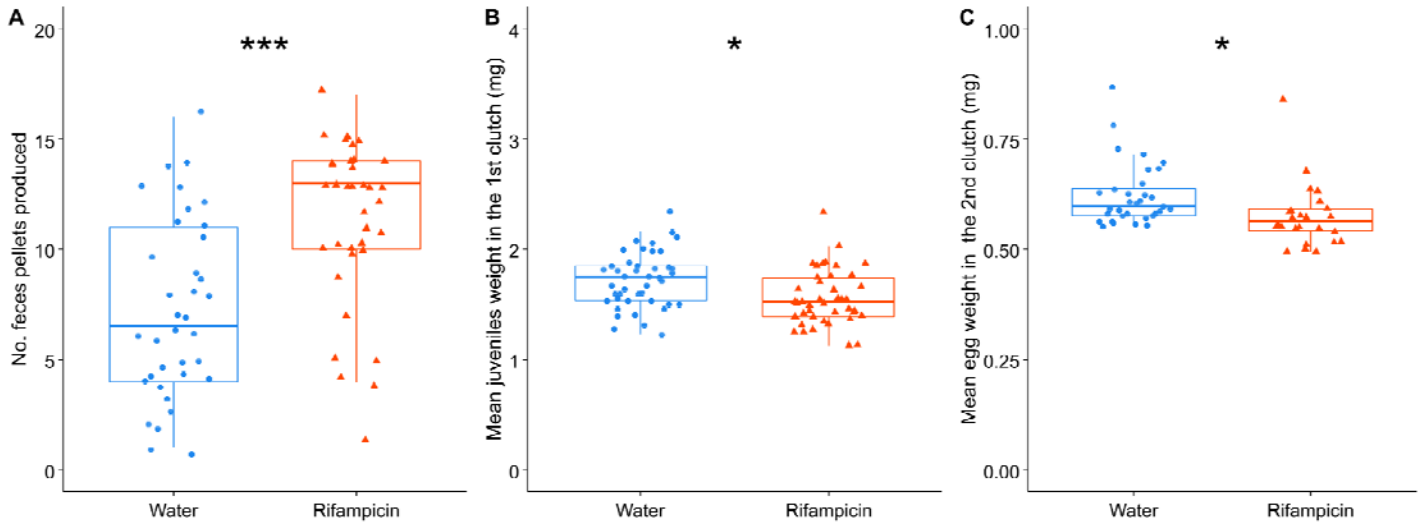

Figure 4 – Effects of rifampicin on (A) females' feces production, (B) mean juveniles weight in the first clutch and (C) mean egg weight in the 2nd clutch. Box plots depict median (middle bar) and interquartile range (light bar), with whiskers extending to 1.5 times the interquartile range and dots representing experimental values. ***$P < 0.001$ and *$P < 0.05$.