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TITLE

Interactive effects of warming, antibiotics and nanoplastics on the gut microbiome of the collembolan *Folsomia candida*

Running title: Interaction of warming, antibiotics and nanoplastics on the gut microbiome of collembolan

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ABSTRACT

Nanoplastics and antibiotics are among the most abundant chemical pollutants of soils, but their interplay with global warming remains poorly understood. The springtail *Folsomia candida* (Class Collembola) is a standard model for ecotoxicological assays with potential as a bioindicator of xenobiotics. Little is known, however, about their gut microbiome and how it might respond to warming and these pollutants. We exposed populations of *F. candida* to nanoplastics and antibiotic under two temperatures. The antibiotic treatment consisted of colistin addition, and the nanoplastic treatment consisted of polystyrene particles (50 $mg \cdot kg^{-1}$ and 0.1 $g \cdot kg^{-1}$ of dry soil, respectively). Both treatments were incubated at 20 and 22 °C for two months, and the bacterial gut microbiomes of springtails were then sequenced. Exposure to nanoplastics at 20 °C decreased the abundance of the dominant bacterial phyla and families, and decreased the evenness of the gut microbiome. At 22 °C, however, the abundances and evenness of the dominant families increased. Surprisingly, Gram-negative bacteria targeted by colistin were not globally affected. And at genus-level, the endosymbiont *Wolbachia* controlled the compositional shifts under nanoplastic addition, potentially driving the gut microbiome. Our results also indicated that warming was a major driver modulating the impacts of the antibiotic and nanoplastics. We illustrate how the gut microbiomes of springtails are sensitive communities responsive to xenobiotics and provide evidence of the need to combine multiple factors of global change operating simultaneously if we are to understand the responses of communities of soil arthropods and their microbiomes.

Key words: xenobiotics, bacteria, colistin, microplastics, *Folsomia candida*, global change

1. Introduction

Anthropogenic global change involves multiple drivers that interact simultaneously in complex ways, leading to increased uncertainty in the responses of organisms and ecological communities (Rillig et al., 2019). Advocating for the study of such responses of biodiversity that integrate the effects of climate change and environmental pollution by novel chemical entities is consequently a growing concern (Sigmund et al., 2023). The term "xenobiotics" represents molecules of anthropogenic origin that have a high potential for unwanted geophysical and biological effects (Steffen et al., 2015). Xenobiotics are recognized as a major obstacle to the delivery of ecosystem services and the conservation of biodiversity and interact complexly with other environmental stressors such as global warming (Moe et al., 2013; Rillig, de Souza Machado, et al., 2019; Rockström et al., 2009). These emergent pollutants have long been influencing ecosystems by worldwide littering, and their production has never been so large (European Environment Agency, 2018). We have only recently, however, begun to properly recognize their impact, with the unsettling discovery that we are already beyond the planetary boundary within which human activity and environmental stability are safe (Persson et al., 2022; Zortéa et al., 2017). Our concern for the well-being of life exposed to xenobiotics and their interplay with global warming thus constitutes a crucial gap in our knowledge of the ecology of global change that we must urgently address.

Plastics are one of the most abundant contaminants in the natural environment, but their fate in soil ecosystems is still mostly a mystery (Horton et al., 2017; Rillig, 2012). Of special interest are micro- and nanoplastics. Microplastics have been defined as plastic particles <5 mm (Thompson et al., 2004), and nanoplastics have been described as a subset of microplastics ranging from 1 to 100 nm (Jambeck et al., 2015). The smallness

of these particles confer them high mobility and surface-to-volume ratio, which allows them to directly interact with the biosphere but also adsorb and transport other pollutants (Law & Thompson, 2014; Qi et al., 2020; Wiesner et al., 2011). The bioavailability of microplastics on land reduces the growth and survival of earthworms, interferes with nematode reproduction and modifies bee and springtail behavior (Ji et al., 2021; Liebezeit & Liebezeit, 2013). Similarly, antibiotics are widely distributed and highly bioavailable xenobiotics (Jensen et al., 2003). The effects of antibiotic waste and abuse are notably recognizable by antibiotic resistance in target bacterial communities (Z. Li et al., 2022; MacFadden et al., 2018; McGough et al., 2020). Animals, however, are also susceptible to antibiotic contamination, causing bacterial dysbiosis, a disruption to the microbiome leading to an imbalance in the microbiota and to changes in their functional composition and metabolic activity (Wypych & Marsland, 2018). Such changes in gut microbiota due to antibiotic contamination affect host fitness in bees and springtails (Agamennone et al., 2015; Zortéa et al., 2017).

Experiments assessing single factors may allow a more thorough mechanistic understanding of their individual impact, but current global-change research stresses that the environment is a multifactorial scenario where the impact of xenobiotics is concomitant with other variables such as warming (Rillig, Ryo, et al., 2019). Indeed, interactions are key to the discovery of emergent and non-intuitive properties capable of increasing the loss of diversity and endangering the resilience of ecosystems (Darling & Côté, 2008; Paine et al., 1998; Rillig, Ryo, et al., 2019). Examining high-order interactions among two or more components, however, needs extensive prior knowledge of the factors being examined, which unfortunately is rarely available (Altenburger et al., 2013). Identifying pairwise interactions is a reasonable approach to study such complexity, because it may lead to a more realistic understanding of the

interplay of simultaneous drivers of global change but balancing the high uncertainty. For example, the coupled impact of warming and microplastics addition increase xenobiotic toxicity over aquatic arthropods reducing fecundity and growth rate (Chang et al., 2022; Lyu et al., 2021). Yet, it is still unclear how temperature might modulate the response of terrestrial species to nanoplastics. Similarly, the interactions between antibiotics and temperature on terrestrial arthropods warrants further examination.

Assessing the impact of xenobiotics in soils is thus mandatory to protect the delivery of the ecosystem services that soils provide around the world, and we need to find new methods that overcome previous limitations. The gut microbiomes of soil fauna link soil environmental conditions with animal physiology and ultimately with fitness (S. Li et al., 2021; Xiang et al., 2019; Q. Zhang et al., 2019). The term "holobiont" refers to the ecological unit comprising a host, its microbiome and their interplay (Margulis & Fester, 1991). Holobionts are useful in soil ecology because hosts and microbes influence each other under warming and soil contamination (Iltis et al., 2022; Zhu, An, et al., 2018; Zhu, Chen, et al., 2018). Assessing the impact of xenobiotics in the gut microbiomes of soil fauna is thus of great interest, because these entities are potential bioindicators of the ecotoxicological response of fauna to environmental pollutants under a scenario of rapid shifts in temperature. Early attempts focused on the bacterial diversity of springtail guts, which decreases under antibiotic treatment but increases when exposed to microplastics (Zhu, An, et al., 2018; Zhu, Chen, et al., 2018). Previous studies, however, have also suggested that the responses of gut microbiomes to xenobiotics in soil arthropods is highly variable within and between species (Thimm et al., 1998; C. Zhang et al., 2019), with some bacteria having differential responses between experiments (Agamennone et al., 2015; Pike & Kingcombe, 2009).

We characterized the response of the gut microbiome of a model soil organism experimentally exposed to nanoplastics and an antibiotic under two rearing temperatures (20 and 22 °C). We used *Folsomia candida* Willem (Class Collembola) as a host because of its cosmopolitan distribution and widespread use as a soil ecotoxicological model. We hypothesized that (1) antibiotic addition would reduce the bacterial diversity of the *F. candida* gut and that Gram-negative bacteria would be disproportionately affected because the antibiotic, colistin, specifically targets this polyphyletic group. (2) Nanoplastic addition would alter bacterial community structure by either increasing or decreasing evenness, depending on its preferential impact on dominant or rare taxa, respectively. And (3) the exposure to xenobiotics under moderate warming could exacerbate the potentially noxious effects of the nanoplastics and antibiotic, leading to larger compositional differences of the treated gut microbiomes compared to the control.

2. Materials and methods

2.1 Experimental conditions

The experiment was conducted between May and July 2019 in the laboratories of the Center for Ecological Research and Forestry Applications (CREAF, Barcelona, Spain). New colonies of the soil collembolan *F. candida* originated from the permanent culture at CREAF maintained at 20 °C. The antibiotic was colistin (also known as polymyxin E) in the form of colistin sulfate (Katz & Demain, 1977). Its industrial production has been associated with a therapy of last resort against Gram-negative bacteria. It has been widely used in animal production at the global level (Barlaam et al., 2019). Since 2015, stable plasmid-mediated mobile colistin resistance genes have been detected leading to regulations and policies to preserve the efficacy of colistin and side effects (Rhouma et al., 2023). The nanoplastic pollutants we used (Bangs Laboratories, Inc; Indiana, USA)

were polystyrene particles 0.044 µm in diameter. We tested six treatments: the addition of the antibiotic at 20 °C and 22 °C, the addition of nanoplastics at 20 °C and 22 °C and a control at 20 °C and 22 °C. Six replicates of each treatment were incubated at the original 20 °C of the permanent culture and at 22 °C in different climatic chambers, for a total of 36 microcosms (Fountain & Hopkin, 2005). The experiment was not fully factorial since we did not include a treatment with the three-order interaction between antibiotics, nanoplastics and temperature.

The soil in this study was collected from a Mediterranean forest dominated by holm-oak (*Quercus ilex*) at the Autonomous University of Barcelona campus (41° 30' 4.29489"N, 2° 6' 32.87871"E). After air-drying it for two weeks, it was defaunated following a standard procedure of five consecutive cycles of freezing at -30 °C for 24 h followed by thawing and heating to 40 °C for 24 h.

Each microcosm consisted of a glass pot containing 30 g of wet soil, with moisture adjusted to 50% of the water holding capacity. The relative humidity and soil moisture of each microcosm were monitored daily and kept constant along the experiment. At the start of the experiment, 10 *F. candida* individuals, aged 10 to 12 days, were added to each microcosm. Soil samples in the antibiotic treatment received colistin at a concentration of 50 $mg \bullet kg^{-1}$, while manured soils have been reported to reach antibiotic concentrations of up to 10 $mg \bullet kg^{-1}$ (Jensen et al., 2003). Soil samples in the nanoplastic treatment received nanoplastics at a concentration of 0.1 $g \bullet kg^{-1}$. In EU farm soils, microplastics have been estimated to range from ten to ten thousand tons per year, with a projected global accumulation of 12,000 Mt if no countermeasures are taken (Borrelle et al., 2020; Nizzetto et al., 2016). The control treatment only contained 30 g of untreated soil. The collembolans were not fed in any of the treatments to increase exposure to the xenobiotics. All individuals were extracted after two months of

incubation using small Berlese funnels and were immediately preserved in 80% ethanol. Gut microbiome sampling, DNA extraction, and sequencing were performed one month after the soil fauna extraction

2.2 Molecular analyses

The gut microbiome of three individuals (random selection) from each microcosm was then analyzed. Each collembolan was washed with 0.5% sodium hypochlorite for 10 s to eliminate surface bacterial contamination and then rinsed five times in sterile phosphate buffered saline (PBS). The guts were dissected using sterile forceps and individually placed into 1.5 mL centrifuge tubes. DNA was extracted following the protocol of the DNeasy Blood & Tissue Kit (QIAGEN; Hilden, Germany). We defined the gut microbiome by the total DNA extracted from the dissections quantified using high-throughput sequencing. Bacterial communities were characterized using primers 515F and 806R targeting the V4 region of the bacterial 16S rRNA gene (515F: 5′-GTGCCAGCMGCCGCGG-3′; 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2010). The reverse primer was designed with 24 unique barcodes to distinguish between the samples. Thermal cycling consisted of 95 °C for 5 min and 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. We used a Qubit 3.0 fluorimeter to determine the concentration of purified amplification products, and 24 products of equal concentration were pooled as a library. The amplification products were purified and then sequenced on an Illumina MiSeq platform (Meiji; Shanghai, China). The high-throughput sequencing data were then analyzed using Quantitative Insights Into Microbial Ecology (QIIME v1.9.1). Operational taxonomic units (OTUs) were classified at 97% similarity using the GreenGenes 13.8 bacterial database and UCLUST (Edgar, 2010; Glöckner et al., 2017; Kõljalg et al., 2013). OTUs with only one sequence (i.e. singletons) were discarded to obtain the final OTU table.

2.3 Data analyses

The microbiome communities were assessed using general linear models (LMs) to detect differences in OTU standardized abundance (Hellinger transformation), richness and evenness (Pielou's index). The LMs were always built separating the nanoplastic and antibiotic treatments, with the control at 20 °C as a fixed-effect term and the xenobiotics and the +2 °C warming treatment as predictors. Normal distribution was selected over other alternatives in all models based on Shapiro–Wilk tests of normalityand data linearity was confirmed with residual-fitted plots. Gram-negative species were identified using the FAPROTAX database and OTU phylogeny (Louca et al., 2016) for determining the impact of the antibiotic on the target taxa, conducting a LM with treatment as a fixed-effect term and relative abundances as response variable. Compositional dissimilarities in the gut bacterial communities were assessed using a permutational multivariate analysis of variance (PERMANOVA, with 999 permutations). Percentage analyses (SIMPER, using Bray-Curtis dissimilarities with 999 permutations) were used to identify which taxa are primarily responsible for an observed difference between treatments, pointing out *Wolbachia Sp.* and *Geobacillus Sp.* SIMPER can easily confound taxa driving community differences among two groups, with taxa that have high within-group variances (Warton et al., 2012). To circumvent this issue we did nonmetric multidimensional scaling (NMDS) and a *post hoc* test using a LM focusing only on the variations in the genus *Wolbachia* due to its known impact over the host fitness (Xi et al., 2008). All data handling, visualization and statistical analyses were carried out using R v4.0.6 (R Core Team, 2020).

3. Results

3.1 Characterization of the gut microbiota of *Folsomia candida*

The total number of sequences across all treatments was 14951. We identified 1022 OTUs after removing unassigned sequences and combining redundant assignments. The most dominant phyla (>5% total abundances) were *Firmicutes* (46.2%), *Proteobacteria* (43.7%) and *Actinobacteria* (8.0%) (Figure 1); while the most dominant families were *Bacillaceae* (46.0%), *Rickettsiaceae* (13.3%), *Sphingomonadaceae* (8.4%), *Caulobacteraceae* (7.3%) and *Nocardiaceae* (6.1%). Moreover, genus *Geobacillus* accounted for 50.7% of all identified genus abundances, followed by *Wolbachia* with 15%.

3.2 Response of the gut bacterial community to addition of colistin and nanoplastics at 20 °C

The exposure to nanoplastics decreased the relative number of sequence reads and hence the abundance of bacteria in the gut of *F. candida* (Table 1, and Figure 2a). This smaller gut microbiome did not affect OTU richness but was coupled with a lower evenness of the gut bacterial communities, indicating that the most dominant taxa (i.e. *Sphingomonadaceae*) became proportionally even more abundant after exposure to the antibiotic and nanoplastics (Table 1, and Figure 2b and c). Nonetheless, the relative abundances of *Proteobacteria*, *Actinobacteria* and *Firmicutes* (excluding *Bacillaceae*) were lower. Moreover, *Wolbachia* sp. drove the compositional dissimilarities between the nanoplastic addition treatments and the control (Table 2 and A4).

On the other hand, colistin addition did not modify the abundance or the richness of the overall gut bacterial communities, showing a slight decrease in evenness (Table 1, and Figure 2c). Nonetheless, the relative abundances of the family *Sphingomonadaceae*

241 decreased with the exposure to the antibiotic, while that of *Proteobacteria* (including
242 *Rickettsiaceae*), *Firmicutes* and *Actinobacteria* were higher (Figure 1 and Tables A1).

243 **3.3 Response to +2 °C warming and its interaction with xenobiotic particles**

244 An increase of 2 °C during the incubation significantly altered how the *F. candida* gut
245 microbiome reacted to the presence of the two chemical pollutants. The increased
246 warming led to a loss of abundances visible in most of the dominant phyla and families
247 (Table 1 and A1), which was coupled with a decrease in the evenness of the gut
248 microbiome due to increases in *Sphingomonadaceae*.

249 The effect of the nanoplastics was temperature-dependent (see significant interactions
250 between temperature and treatment for abundance and evenness in Table 1). The
251 exposure to nanoplastics at 22 °C increased the abundances across bacterial taxa and the
252 evenness of the gut microbiome, suggesting that the most dominant taxa were
253 disproportionately affected by the simultaneous effect of temperature and nanoplastics.
254 However, nanoplastic addition decreased the abundance of all main phyla and the
255 family *Rickettsiaceae*, and increased the abundances of *Sphingomonadaceae* (Table
256 A1).

257 On the other hand, antibiotic exposure combined with +2 °C warming did not modify
258 the abundance and richness of bacterial taxa in the gut microbiome (Table 1). But
259 dominant taxa (excluding *Bacillaceae*) decreased abundances under antibiotic addition
260 at 22 °C (Table A1). Warming also led to shifts in the composition of the gut
261 microbiome of *F. candida*. The combination of antibiotic and warming did not cause
262 overall changes in composition (Figure 3 and Table 2), but the abundance of
263 *Actinobacteria*, *Firmicutes* and *Protobacteria* (including *Rickettsiaceae*) decreased
264 while that of *Sphingomonadaceae* increased (Figure 1 and Table A1). Additional
265 analysis, however, indicated that the abundance of Gram-negative bacteria was not

affected by colistin addition (P = 0.26; Table A3). In contrast to the control and antibiotic treatments, a significant interaction between warming and nanoplastic exposure (Table 2) indicated that the compositional effect of the nanoplastics on the gut bacterial communities depended on the temperature of the incubation with increases in abundances, evenness and shifts in composition (Table 1 and 2). Under combined warming and nanoplastic addition, we found increases in *Actinobacteria, Firmicutes* and *Proteobacteria* (including *Rickettsiaceae*), but decreases *Sphingomonadaceae*, which made the gut microbiomes structurally more similar to the microbiomes in the control incubated at 20 °C (Figures 1 and 3).

4. Discussion

The main finding from our experiment is the pivotal role of temperature modulating the response of the gut microbiome of *F. candida* to the exposure of antibiotics and nanoplastics. At 20 °C, antibiotic addition led to a loss of evenness while nanoplastic exposure reduced relative abundances, evenness and caused minor compositional shifts. In contrast, under a 2 °C warming, antibiotic exposure increased evenness, while nanoplastics increased relative abundances, evenness and produced larger compositional shifts. Interestingly, at a lower taxonomic level the response became taxon-dependent, and target Gram-Negative bacteria were not affected by colistin. This finding raises important concerns about the uncertainty surrounding the responses of soil fauna to the interactive effects of simultaneous drivers of global change. We also provide additional evidence of the sensitivity of the gut microbiomes of soil fauna to antibiotics and we are the first to report the impact of nanoplastic exposure, which was an interesting contrast to previous observations involving larger microplastics (Zhu, Chen, et al., 2018). By highlighting the sensitivity of the gut microbiome in response to changes in temperature and to pollutants, this research emphasizes the need for a comprehensive understanding

of how various environmental stressors can interact and potentially buffer their effects in soil ecosystems (see Figure 3b; Rillig, Ryo, et al., 2019; Sigmund et al., 2023).

4.1 Impact of warming on the gut microbiome

The response to warming suggested a decrease in the fitness of the most dominant taxa in the control treatment. The decrease in abundance, linked with the lack of differences in richness and the decrease in evenness indicated that the most dominant taxa were favored by warming (Table 1, A1 and Figure 1). Our results did not support previous observations of increases in the relative abundance of *Proteobacteria* in arthropods under experimental warming, although we corroborated the loss of abundance in members of the phylum *Actinobacteria* (Table A1) (Moghadam et al., 2018; Horváthová et al., 2019). Such shifts in community composition can lead to the loss or increase of specific guilds capable of modifying the fitness of the host. For instance, previous studies suggest how the loss of Rickettsiaceae and Actinobacteria observed in Table A1 can lead to worse heat acclimation and lower protection against pathogens, respectively (Brumin et al., 2011; Feder & Hofmann, 1999; Horváthová et al., 2019). Nonetheless, dissimilarities in the composition of the *F. candida* gut microbiome between studies are common (Thimm et al., 1998). **4.2 Interactions between warming and xenobiotics**

Warming was a strong driver of shifts in the impact of the novel chemical entities on the *F. candida* gut microbiome. We expected that an increase in temperature of 2 °C during the incubation would amplify the effects of the antibiotic and nanoplastics, but this extra warming radically changed the response of the gut microbiome to these chemical pollutants. Dissimilarities between warming treatments under xenobiotic addition are surprising and responses varied between phyla and families, but altogether point out to a

strong temperature modulation of the effects of soil contaminants over *Folsomia*'s gut microbiome.

Antibiotic addition decreased evenness, suggesting an unequal response in taxa abundances that favored *Firmicutes* (albeit not *Bacillaceae*) and decreased the overall abundances of phyla such as Gram-positive *Actinobacteria* (Table 1 and A1). In contrast, the response to antibiotic combined with a temperature increase of 2 °C only favored Gram-negative *Sphingomonadaceae* (Table A1). These findings were surprising because colistin targets Gram-negative bacteria, suggesting the up-regulation of colistin resistance genes (i.e. *mcr*) (Arcilla et al., 2016; Wang et al., 2020). We also expected antibiotic exposure under warming to increase the antibacterial activity of colistin, reducing the abundance of vulnerable bacteria (Beveridge & Martin, 1967). The lack of response from targeted bacteria nonetheless led to a similar response between Gram-negative and -positive bacteria, suggesting a more transversal impact on the entire microbiome. Moreover, it is important to note that our antibiotic concentration was five times higher than what is typically observed in farmland soils after manure application (Jensen et al., 2003). This suggests a potentially lower impact of colistin in managed soils, as well as a reduced effect on its target bacteria. Despite the significant variation in OTUs observed at 20 °C with colistin addition, the assembly composition was similar to that under antibiotic exposure at 22 °C, suggesting no external contamination. However, the possibility of processing errors cannot be entirely ruled out (Figure 2b).

Similarly, the effects of nanoplastic addition varied depending on the temperature of incubation. At 20 °C, the nanoplastics decreased the relative abundances of the main phyla; but at 22 °C, the response was reversed with a buffer-like response over community composition (Table 1 and A1, and Figure 3b). We expected that the effects of the nanoplastics would be more general than those of the antibiotic colistin, affecting

all bacterial taxa in the gut microbiome. The results, however, were similar in magnitude albeit taxa were affected differently (Table 1). Interestingly, the nanoplastic treatments differed from the control through *Wolbachia* sp. (Table A2 and A4). A loss of *Wolbachia* under nanoplastic addition at 20 °C is especially important, because they are endosymbionts that control the reproduction of *F. candida* and down-regulate immune responses and responses to heat stress (Table S5) (Xi et al., 2008). Nanoplastic addition may thus have modified the gut microbiome by altering the abundance of *Wolbachia*, thereby altering evenness (Figure 2). Furthermore, the feeding habits *of F. candida* may have been influenced by both temperature and nanoplastics. The selective grazing of *F. candida* could allow it to avoid the unpalatable plastics. However, nanoplastics interact with soil microorganisms covering them with eco-coronas, which could be appetizing for bacterial-feeding soil fauna (Fountain & Hopkin, 2005; Ng et al., 2018). Thus, changes in dietary habits should affect the holobiont through shifts in gut environmental conditions and springtail's fitness (Xiang et al., 2019). As a result, we could hypothesize that nanoplastic exposure under higher temperatures might have facilitated the growth of eco-coronas around the nanoplastic particles, altering *F. candida* feeding habits and its gut environmental conditions. At the same time, temperature and nanoplastics also have a direct effect over microbial communities both inside and outside of Folsomia. This may result in complex population dynamics of microorganisms and shifts in their competitive interactions. These direct and indirect effects could explain the surprising responses observed in Folsomia's gut microbiome. However, further experiments are needed to elucidate the mechanisms driving these complex interactions between xenobiotics and temperature.**5. Final remarks**To our knowledge, no previous study has investigated the interactions between soil-emergent pollutants and soil warming and their combined effects on the gut microbiome of soil

fauna. Our research extends the current understanding by highlighting the role of temperature in modulating the effects of antibiotics and nanoplastics. The variable responses of *F. candida*'s gut microbiome to antibiotic and nanoplastic exposure under different temperature regimes underscore the high uncertainty and complexity of the mechanisms driving these interactions between environmental stressors. However, we acknowledge that our experimental design was not fully factorial, and therefore, potential interactions between different pollutants and temperature have yet to be tested. Given the high idiosyncrasy of our results and previous studies combining a high number of environmental stressors (Rillig et al. 2019), we anticipate that significant three-order interactions may arise. Additionally, we suggest that focusing more on the size of plastic particles could yield interesting insights into the gut microbiome's response to micro- and nanoplastics. The Gram-negative bacteria targeted by the antibiotic were only partially affected, indicating the need for further research on mcr genes to assess their expression in intestinal microbiomes. Lastly, we did not investigate whether changes in the gut microbiome led to detrimental dysbiosis in *F. candida*, potentially impairing its functions in soil ecosystems, nor did we explore the possible impact of fungi. Consequently, future research on colistin and nanoplastics should connect bacterial and fungal responses to fitness variables such as the abundance, growth rate, and reproduction of *F. candida*. These additional studies will enhance our understanding of how interactions between xenobiotics and warming impact the biodiversity and functioning of soil ecosystems.

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642

643 Table 1
644
645

Table 1. Linear model outputs of *Folsomia candida*'s gut microbiome community abundance, richness and evenness after exposure to warming, antibiotics and nanoplastics at 20º and 22º.

Variable	Factor	Warming		Antibiotics		Nanoplastics	
		Estimate ± SE	R^2	Estimate ± SE	R^2	Estimate ± SE	R^2
Abundance	Intercept	23.0 ± 3.55**	0.68	17.0 ± 3.99**	0.20	23.0 ± 3.21**	0.79
	22 °C	-0.80 ± 0.16**		-0.51 ± 0.19**		-0.80 ± 0.15**	
	Xenobiotic	NA		-0.10 ± 0.38		-39.0 ± 4.56**	
	Interaction	NA		-		1.88 ± 0.21**	
Richness	Intercept	273 ± 21.8	0.00	486 ± 304	0.00	169 ± 208	0.00
	22 °C	5.60 ± 32.3		-8.41 ± 14.6		5.08 ± 9.95	
	Xenobiotic	NA		-34.4 ± 28.9		9.16 ± 19.9	
	Interaction	NA		-		-	
Evenness	Intercept	0.85 ± 0.00**	0.85	0.85 ± 0.00**	0.56	0.85 ± 0.00**	0.88
	22 °C	-0.05 ± 0.00**		-0.05 ± 0.01**		-0.04 ± 0.00**	
	Xenobiotic	NA		-0.02 ± 0.01*		-0.05 ± 0.00**	
	Interaction	NA		0.05 ± 0.01**		0.11 ± 0.00 **	

Linear models were done with gaussian distribution of response variables. Models were optimized deleting all non-significant interactions, leaving "-" instead. Intercept group are controls incubated at 20 °C. Adjusted-R^2 was calculated for all linear models. SE, standard error of the estimate; NA, non-applicable to the xenobiotic-free warming experiment models; *, $P < 0.05$; **, $P < 0.01$. Degrees of freedom are 9, 18 and 17 for warming, the nanoplastic and antibiotic models, respectively.

646 Table 2
647

Table 2. Composition of *Folsomia candida* gut microbiome after exposure to antibiotics and nanoplastics at 20º and 22º.

Response variable	Antibiotic		Nanoplastics	
	Sum of squares	R^2	Sum of squares	R^2
22 °C	0.39	0.06	0.29	0.04
Xenobiotic	0.29	0.05	0.29	0.04
Interaction	-	-	0.70*	0.11

The change in the composition of the gut microbiome was tested by means of permutational analyses of variance (PERMANOVA), with 18 and 17 degrees of freedom for the nanoplastic and antibiotic models, respectively. *, $P < 0.05$. Number of permutations is 999 per model.

Figure captions:

Figure 1. Taxonomic composition of the gut microbiome of *Folsomia candida* at the phylum (a) and family (b) levels for each combination of treatment and rearing temperature. Taxa with relative abundances <5% have been grouped as "Others".

Figure 2. Violin plots of mean bacterial abundance (a), richness (b) and Pielou evenness (c) of the gut microbiome of *Folsomia candida* across experimental treatments. Horizontal lines inside each violin from lowest to highest denote the first, second and third quartiles. Violins were trimmed to adjust to the data.

Figure 3. Ordination of the gut microbiome of *Folsomia candida* by nonmetric multidimensional scaling showing ellipses and points to separate the two temperature levels of control and antibiotic addition (a) and for the two temperature levels of control and nanoplastic addition (b).

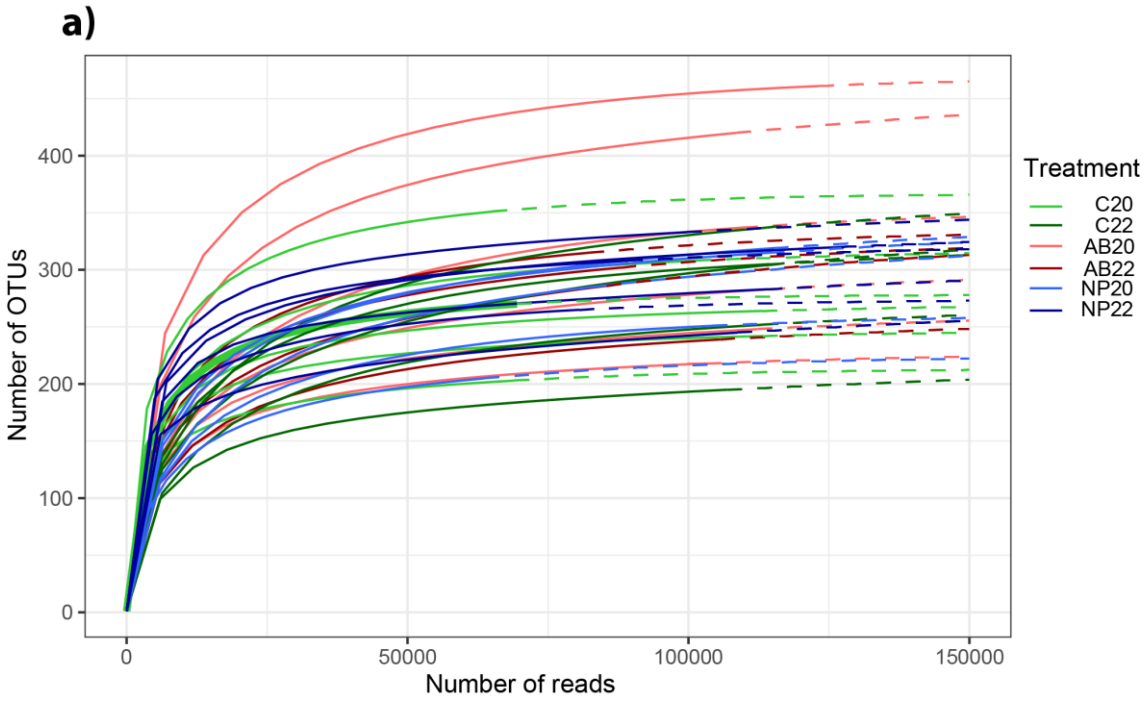
APPENDIX

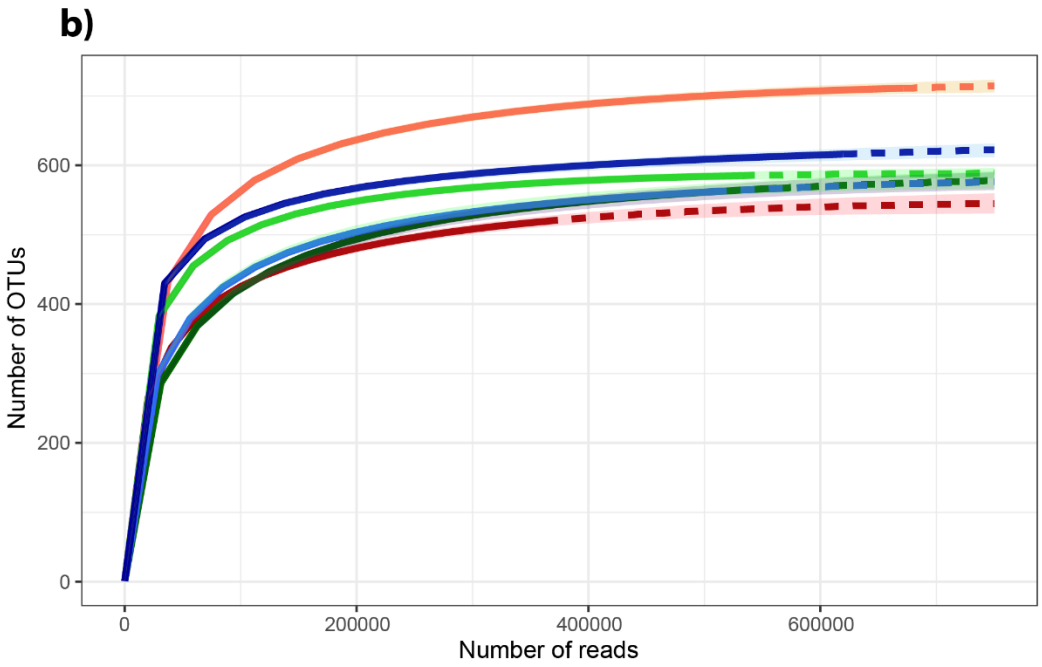
Interactive effects of warming, antibiotics and nanoplastics on the gut microbiome of the collembolan *Folsomia candida*

Miquel Ferrín, Laura Márquez, Xavier Domene, Dong Zhu, Yong-Guan Zhu, Josep Peñuelas, Guille Peguero

Figure A1

Rarefaction curves for each treatment using replicates (a) and mean reads per replicate (b). Dashed lines correspond to the extrapolated portions of the curves, which were mean-rarefied.




b)
600
400
200
0
Number of OTUs
0
200000
400000
600000
Number of reads

672

673 **Table A1**

Table A1. Linear models of species abundances for the main phyla and families against each treatment.

		Phyla			
	Response variable	Antibiotic		Nanoplastics	
		Estimate ± SE	R^2	Estimate ± SE	R^2
Actinobacteria	Intercept	2.22 ± 0.88*	0.55	5.03 ± 0.72**	0.86
	22 °C	-0.06 ± 0.04		-0.20 ± 0.03**	
	Xenobiotic	2.80 ± 1.21*		-10.8 ± 1.02**	
	Interaction	-0.13 ± 0.05*		0.52 ± 0.04**	
Firmicutes	Intercept	0.03 ± 0.71	0.37	2.29 ± 1.07*	0.36
	22 °C	0.06 ± 0.03		-0.04 ± 0.05	
	Xenobiotic	2.26 ± 0.97*		-4.67 ± 1.52**	
	Interaction	-0.11 ± 0.04*		0.22 ± 0.07**	
Proteobacteria	Intercept	5.01 ± 2.09*	0.46	11.6 ± 1.25**	0.76
	22 °C	-0.08 ± 0.10		-0.39 ± 0.06**	
	Xenobiotic	6.64 ± 2.87*		-14.4 ± 1.78**	
	Interaction	-0.30 ± 0.13*		0.69 ± 0.08**	
		Families			
	Response variable	Antibiotic		Nanoplastics	
		Estimate ± SE	R^2	Estimate ± SE	R^2
Bacillaceae	Intercept	0.55 ± 0.22*	0.00	0.84 ± 0.29*	0.00
	22 °C	0.01 ± 0.01		0.00 ± 0.01	
	Xenobiotic	0.29 ± 0.31		-0.60 ± 0.41	
	Interaction	-0.01 ± 0.01		0.02 ± 0.01	
Rickettsiaceae	Intercept	1.29 ± 0.35**	0.76	2.47 ± 0.45**	0.72
	22 °C	-0.05 ± 0.01**		-0.10 ± 0.02**	
	Xenobiotic	1.17 ± 0.48*		-4.87 ± 0.64**	
	Interaction	-0.05 ± 0.02*		0.23 ± 0.03**	
Sphingomonadaceae	Intercept	1.14 ± 0.17**	0.58	-0.22 ± 0.22	0.60
	22 °C	-0.03 ± 0.00**		0.03 ± 0.01**	
	Xenobiotic	-1.37 ± 0.24**		1.83 ± 0.31**	
	Interaction	0.06 ± 0.01**		-0.08 ± 0.01**	

LMs where intercept group are controls incubated at 20 °C. Linear models for abundances were done with Gaussian family. SE, standard error; **, $P < 0.01$. Degrees of freedom = 18 and 17 for the nanoplastics and antibiotic models, respectively.

674

675 **Table A2**

676

Table A2. Similarity percentage analyses under paired treatments.			
Paired variables	Taxon	Contribution ± SD	Contribution to all taxa contribution
C20-NP20	*Wolbachia* sp.	2.14e-02 ± 5.56e-03 **	5.7e-02
Discriminating taxon between pairs of treatments using Bray-Curtis dissimilarities with 999 permutations. Taxa are those with $P < 0.05$ and contributions >5%, if none is shown means that none met the criteria. "Contribution" corresponds to taxa contribution to average between-group dissimilarity, and "Contribution to all taxa contribution" corresponds to the contribution of a given taxon to the overall taxa contribution totaling 1. SD, standard deviation; *****, $P < 0.05$; ******, $P < 0.01$.			

677 **Table A3**

678

Table A3. Linear model for the abundance of Gram-negative species		
Response variable	Estimate ± SE	Adjusted-R^2
Intercept	747 ± 265 **	0.00
22 °C	506 ± 371	
Colistin	674 ± 397 ·	
Interaction	-1.09e+3 ± 570 ·	
Linear model with Gaussian family for Gram-negative taxa at the temperature treatments and colistin. Intercepts are at control 20 °°C. SE, standard error; ·, $P < 0.10$; **, $P < 0.01$. Degrees of freedom = 415.		

679 **Table A4**

Table A4. Linear model for *Wolbachia* sp. abundance with nanoplastic addition and warming.

Response variable	Estimate ± SE	Adjusted-R^2
Intercept	0.35 ± 0.01**	0.89
Nanoplastics	-0.27 ± 0.02 **	

Linear model with Gaussian family for *Wolbachia* sp. in the temperature and nanoplastic treatments. The intercept is at the control. We found no interaction and no impact from warming, limiting the model to the impact of nanoplastics. **, $P < 0.01$. Degrees of freedom = 9.