How to thrive on carrion

The role of burying beetle's microbiota in carcass preservation and digestion

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ABSTRACT

Associations between insects and symbiotic microorganisms has shaped the ecology and evolution of insects. Through these associations, insects gain access to a wider range of resources and are able to colonize complex ecological niches such as carrion. Necrophagous insects like burying beetles (*Nicrophorus vespilloides*) have to cope with strong competition from microbial decomposers. To do so, beetles coat carrion with anal exudates which have antimicrobial properties and a rich and complex microbial community that is vertically transmitted to its offspring. Here we provide evidence of how burying beetles microbiota facilitates carcass exploitation by preventing microbial decay and maximizing carcass digestion. Our data also support the hypothesis that the ascomycetous yeast *Yarrowia* and the burying beetles have a mutualistic symbiosis. We provide evidence supporting how *Yarrowia* could facilitate the access of larvae to proteins and lipids -with positive effects on burying beetle's fitness- by producing extracellular enzymes on the carcass surface. In this study we provide important insights about the host-symbiont interactions and how diverse microbial metabolites can assist insects into colonizing a challenging an ephemeral resource such as carrion.

INTRODUCTION

Insects are one of the most successful groups of organism on Earth comprehending approximately 58% of animal's global diversity (Foottit & Adler 2009; Stork et al. 2015) They have successfully colonized a wide range of ecological niches by exploiting novel resources and adapting to extreme environments (Engel & Moran 2013). Understanding how they have been able to adapt and radiate in such environments is an ongoing question of insect's evolution. The innate adaptive capacity of single species may be restricted by its mutational rate, pre-existing genetic variation, phenotypic plasticity and dispersal capacity as well as molecular and environmental factors. Nevertheless, associations with other species can lead to symbiotic interactions that ultimately impact the species evolutionary trajectory (Engel & Moran 2013). Insect evolutionary success has been particularly driven by its symbiotic interactions with microorganisms. Both obligate and transient symbionts are known to provide important contributions to their insect hosts: microorganisms can play a role in development, immune response, nutrition, and metabolism among other processes (Engel & Moran 2013; Shropshire & Bordenstein 2016). In termites, for example, the nutritional services provided by its microbiota marked the major evolutionary transitions of this group. In the first transition, the common ancestor of termites and wood-feeding cockroaches acquired cellulolytic flagellates which provided a higher capacity of lignocellulose digestion; this association gave rise to the wood-feeding clade of termites and their subsequent radiation. Later, the loss of these flagellated symbionts, along with a shift of the core microbiota towards a prokaryotic community, lead to a dietary diversification of the Termitidae family and its successful

colonization of novel ecosystems (Abe et al. 2000; Brune & Dietrich 2015). Other insect groups also harbor complex microbial communities that aid their host to exploit nutritionally poor, unbalanced or toxic food sources. For instance, in stink bugs bacterial endosymbionts provide the essential amino acids missing from its phytophagous diet (Nikoh et al. 2011); in the coffee-berry-borer, its core microbiota mediates caffeine detoxification thus making this beetle a very effective coffee pest (Ceja-Navarro et al. 2015); and the bean bug symbionts confer pesticide resistance to its host (Werren 2012). The ubiquitous association between insects and microbes, and their deep implications on the ecology and evolution of their host raised the need of studying the host and its microbiota as an ecological unit (Bordenstein et al. 2015; Guerrero et al. 2013). Integrated studies of these associations, instead of the study of its isolated units promise to expand our understanding of the adaptive capacity of insects.

The burying beetle *Nicrophorus vespilloides* (Herbst, 1783) and their associated microbiota represents a promising model to study the evolution of these interspecific interactions. These beetles exploit vertebrate carcass as its main resource, and this phenomenon has been the focus of multiple studies for more than two decades. But only until very recently, with the study of its associated microbiota (Kaltenpoth & Steiger 2014; Shukla et al. 2017; Vogel et al. 2017) we are starting to grasp the complexity of this system and the ecological dynamics underlying *N. vespilloides* necrophagous lifestyle.

Carrion is a highly nutritious but ephemeral resource, and its random distribution and rapid physicochemical and biotic degradation puts carrion at the center of intense intra- and inter-specific competition from scavengers and microbial decomposers. Coping with microbial pathogens and decomposing bacteria is one of the major challenges of necrophagous insects. Yet, burying beetles poses behavioral and chemical strategies for exploiting this challenging resource. Beetles prepare the carrion by burying and shaping it into a carcass ball, around which eggs are laid. During this process, anal and oral exudates are constantly applied to the carcass. Once larvae hatch, they migrate onto the carcass where both females and males provide care by directly feeding the developing larvae and maintaining the carcass through constant application of oral and anal exudates (Scott 1998). The extensive biparental care of *Nicrophorus spp*. is known to increase its fitness (Arce et al. 2012; Cotter & Kilner 2010a). From this set of parental behaviors, the application of oral and anal secretions has been of particular interest. Already from the seminal works of Scott (1998) and Pukowski (1933) these secretions have been hypothesized to preserve the carcass, as little evidence of microbial decay is observed during *Nicrophorus* breeding. Experimental evidence has proved that these exudates possess antimicrobial properties and are effective against gram positive bacteria (Arce et al. 2012; Cotter & Kilner 2010a; Hoback et al. 2004), which in turn has positive effects on beetles fitness (Arce et al. 2012). Together, these findings have framed the relationship of burying beetles and its microbiota as one of intense competition for carrion. Although this can be the case for specific species or groups of microbes (i.e. *Staphylococcus*, *Clostridia*), there is no evidence that it remains true for most of the microbial groups associated with *Nicrophorus*.

The benefits that *Nicrophorus* can derive from its association with microbes were overlooked until recently. Novel findings suggest that burying beetle's secretions are not only rich in antimicrobial compounds but also enclose a core microbial community with potential benefits for carcass utilization, detoxification, and defense (Kaltenpoth & Steiger 2014; Shukla et al. 2017; Vogel et al. 2017). Culture-based (Wang & Rozen 2017) and next-generation sequencing data (Shukla et al. 2017) revealed that *Nicrophorus* microbiota is vertically transmitted from adult beetles to larvae directly through regurgitation and indirectly with the anal secretions applied onto the carcass surface. During breeding, beetles seed their microbiota onto the carcass inducing a microbial succession from carcass and soil associated taxa towards a community monopolized by the beetle's microbiota (Shukla et al. 2017). *Nicrophorus* core-microbiota is dominated by bacteria from the genus *Morganella, Myroides, Providencia, Vagococcus, Wohlfahrtiimonas* and *Clostridiales* and several strains of the ascomycetous yeast *Yarrowia sp.* (Shukla et al. 2017; Vogel et al. 2017). Most of these taxa are unique to the beetles, but some (e.g. *Myroides, Clostridiales*) can be environmentally acquired from the soil or carcass and correspond to transient associations (Duarte et al. 2016; Kaltenpoth & Steiger 2014; Shukla et al. 2017). Several members of *Nicrophorus* microbiota possess desirable traits that could potentially benefit their host. For example, the Firmicutes (*Vagococcus, Enterococcus*) and Enterobacteriaceae (*Morganella, Providencia, and Proteus*) are known produce antimicrobial compounds and bacteriolytic enzymes that make them good antagonists of meat-spoilage and pathogenic microorganism (Ammor et al. 2006; Thompson et al. 2013), while some Clostridiales could facilitate digestion (Harms et al. 1998). Distinctively, the two strains of *Yarrowia* isolated from *Nicrophorus* (Vogel et al. 2017) are closely related with

Yarrowia lipolytica, an aerobic yeast capable of using multiple carbon sources and with high enzyme production yield (Coelho et al. 2011). Transcriptomics data of the beetle's gut and carcass shows a high abundance of *Yarrowia* transcripts, indicating that this yeast has a high metabolic activity on the breeding cavity including functions such as carbohydrate metabolism, lipid metabolism (extracellular lipase production), protein metabolism (extracellular protease production), amino acid metabolism, vitamin and cofactor metabolism and, glycerolipid and terpenoid biosynthesis (Vogel et al. 2017).

This study was motivated by the recently gained knowledge about the metabolic potential of burying beetles' microbiota. Here we focused on understanding how *Nicrophorus* and its microbiota as an ecological unit cooperate to exploit vertebrate carcass. The aims of this study were to evaluate the capacity of *Nicrophorus* microbiota to preserve (1) and digest (2) vertebrate carcass; and to elucidate the ecological nature of the association between *Nicrophorus* and its symbiotic yeast *Yarrowia* (3).

Carcass preservation (1) was evaluated by measuring the concentration of the biogenic amines (produced during microbial decay) in beetle-attended and unattended carcasses. If carcass are preserved by *Nicrophorus* and its microbiota we expected to find a lower amount biogenic amines in the beetle attended carcass. We also tested the antimicrobial activity of carcass-derived solutions as the mechanism for carcass preservation. Here we present for the first time quantitative data supporting that burying beetles do preserve carcass by reducing microbial decay.

Given that *Yarrowia* genes encoding for the extracellular enzymes protease and lipase have been found to be highly expressed on the breeding cavity of beetle-attended carcasses (Vogel et al. 2017); to evaluate carcass digestion (2) we measure the enzymatic activity of the protease and lipase and the protein concentration between beetle-attended and unattended carcasses; we also performed a metatranscriptomic analysis to unravel the metabolic activity of *Nicrophorus* microbiota on the carcass.

Finally, to elucidate the ecological nature of the association between *Nicrophorus* and its symbiotic yeast –*Yarrowia* (3), we tested the hypothesis of a mutualistic interaction between these species. To do so, we manipulated *Nicrophorus*' microbiota to obtain *Yarrowia*-free beetles (aposymbiotic), and tested the fitness of aposymbiotic vs. symbiotic beetles.

Our results show that *Nicrophorus* and its microbiota act together to slow down the microbial decay of the carcass, and thus they effectively preserve its breeding resource while enhancing digestion. Our data also support the role of *Yarrowia* as a mutualistic symbiont of *Nicrophorus*.

MATERIALS AND METHODS

Insect rearing

A *Nicrophorus vespilliodes* colony was established at the Max Plank Institute of Chemical Ecology (Jena, Germany) in 2016 from adults collected near Ulm, Germany by Dr. Sandra Steiger, University of Ulm. The beetles were maintained in the laboratory at 20°C with a 16:8 hour light: dark cycle and 80% relative humidity and fed (if not stated differently) with freshly

decapitated larvae of *Tenebrio molitor* twice a week until sexual maturity (approx. 20 days after eclosion). Virgin non-sibling male and females were paired in transparent plastic containers filled two-thirds with garden soil and provided with a freshly thawed mouse (*Mus musculus*) carcass of approximately 22g (b.t.b.e. Insektenzucht GmbH, Germany).

1. Carcass preservation and digestion

General procedures

To understand how *Nicrophorus* and its associate microbiota regulates carcass degradation and digestion, we performed a comparative study of beetle attended-carcass and unattended-carcass (naturally decomposing). Freshly thawed mice (*Mus musculus*) carcasses of approximately 22g (b.t.b.e. Insektenzucht GmbH, Germany) were set in clear plastic boxes filled 2/3 with soil. In the first treatment (beetles attended-carcass), *N. vespilloides* mating pairs were allowed to prepare and breed on the carcass for approximately 9 days; at this stage 3rd instar larvae are actively feeding inside the breeding cavity. In the second treatment, unattended-carcasses were left to naturally decompose for approximately 9 days; before the start of the experiment unattended-carcasses received a small abdominal incision to simulate the breeding cavity usually formed by the beetles. On the second day the unattended-carcasses were buried with sterile, DNA-free cotton swabs (Sarstedt, Germany).

At the end of the experiment, the breeding cavity of the carcass of both treatments was swabbed with sterile, DNA-free cotton swabs (Sarstedt, Nümbrecht, Germany) by gently rotating the swabs inside the cavity for approximately 30 seconds. Three consecutive swab samples were taken from each cavity. 1) The first swab was preserved in power-bead tubes of the PowerSoil DNA isolation kit (MoBio); the samples were kept for further experiments and were not analyzed for the present report. 2) The second sample was used for quantification of proteins, enzymes, and biogenic amines; this swab was submerged in 1.5 ml of Milli-Q water on ice and vortexed for 10 seconds before removing the swap. The carcass-derived solution was then centrifuged for 30 seconds at 10,000g to remove debris and subsequently filter-sterilized (PVDF 0.22 µm sterile syringe filter, ROTH, Germany); the filtered fraction was stored at 4C and the unfiltered fraction at -20C. The sample weight was taken to normalize for any difference in the amount of biomass that adhered to the swabs. 3) The last swab was preserved in TRIsure (Bioline, Berlin-Brandenburg, Germany), after sampling it was immediately frozen in liquid nitrogen and stored at −80 °C. for subsequent RNA extraction.

An independent set of beetle attended-carcasses was used for testing the antimicrobial activity of the breeding cavity. The cavity was washed with 2 ml of ice cold phosphate-buffered saline (1X PBS) for 30 seconds by constant pipetting. The recovered solution was filter-sterilized (PVDF 0.22 um sterile syringe filter, ROTH, Germany) and immediately used for the assay.

Carcass digestion

Yarrowia genes encoding for the extracellular enzymes protease and lipase are expressed on the breeding cavity of beetle-attended carcass thus, this symbiont has been hypothesized to play an important role in carcass digestion (Vogel et al. 2017). To test the digestive role of *Nicrophorus* microbiota, protein, protease and lipase concentration was measured in beetle-attended and unattended carcasses.

Protein concentration assay

The total protein content of the unfiltered carcass-derived solution was quantified with the Bradford method (1976) using IX Coomassie brilliant blue G-dye reagent (BioRad, 500-0006) and bovine serum albumin (BSA) as standard. In this colorimetric assay, the dye reagent binds to the amino acid residues producing a blue color that can be measured at a wavelength of 595 nm [Bradford1976]. A dilution series of the BSA standard was included for quantification purpuses. The carcass samples (dilution factor 1:2 in Milli-Q water) and standards were assayed in a FluoroNunc flat-bottom clear polystyrene 96-well microplate. In each well 10 µl sample or standard were mixed with 200 µl of dye reagent and incubated for 10 minutes at room temperature. Absorbance (595 nm) was measured in a spectrophotometer (Tecan Infinite 200). The protein concentration of the samples was calculated by comparing the absorbance with the BSA (0-0.5 mg/ml) calibration curve.

Protease assay

Protease concentration was measured using the Pierce Fluorescent Protease Assay Kit (#23266, Thermo Fischer, Germany). This protocol is based on the fluorescence produced by the proteolytic digestion of fluorescein-labeled casein (FTC-Caseine). A dilution series of Trypsin, a standard protease, was included for quantification proposes. Samples and standards were assayed in a FluoroNunc flat-bottom black polystyrene 96-well microplate. Hundred microlitres of samples (dilution factor 1:20 in TBS assay buffer) and standards were mixed with 100 µl of FTC-Casein substrate (1:500 in TBS assay buffer) and incubated for 18 minutes at room temperature. Fluorescence was measured in a spectrophotometer (Tecan Infinite 200) with 485/538 nm excitation/emission filter. The enzyme concentration of the samples was calculated by comparing the fluorescence with the Trypsin (0-0.5 µg/ml) calibration curve.

Lipase assay

Lipase enzymatic activity was calculated using the Lipase Activity Assay Kit II (#MAK047, Sigma-Aldrich, Germany). In this method, lipase substrate conversion is colorimetrically measure as the amount of product (TNB) released during the reaction time. A dilution series of TNB standard was included for quantification proposes. Samples and standards were assayed in a FluoroNunc flat-bottom clear polystyrene 96-well microplate. Fifty microliters of samples (dilution factor 1:10 in Lipase Assay Buffer) and standards were mixed with 5 µl of lipase substrate, 10 µl of DTNB Probe, and 85 µl Lipase Assay Buffer. After an incubation period of 3 minutes (T-initial) at 37 °C the initial absorbance (412nm) was measured in a

spectrophotometer (Tecan Infinite 200). The incubation continued, with absorbance measures every 5 minutes, until the value most active sample exceeded the 50 nmole TNB standard; the penultimate absorbance reading was considered as T-final and used for further calculations. The change of TNB produce by the test samples (between T-initial and T-final) was calculated by comparing with the TNB (0-50 nmole/well) calibration curve. Lipase activity was estimated according to the manufacturer's instructions (Eq. 1):

$$Lipase\ Activity = \frac{\Delta TNB \times Sample\ dilution\ factor}{(t_{final} - t_{initial}) \times V_{sample}} \qquad Equation\ 1.$$

Antimicrobial assay

An agar diffusion test was performed to evaluate the antimicrobial activity from the beetle-attended-carcass against four microorganisms: *Bacillus subtilis* (G+), *Escherichia coli* (G), *Mucor sp*.(previously isolated form an unattended-carcass) and *Saccharomyces cerevisiae*. The test microorganisms were incubated (30°C for *B. subtilis* and *E. coli*; 26°C for *S. cerevisiae*) overnight in yeast-malt broth (YM), then 1.5 ml of each culture was centrifuged for 1:30 minutes at 8,000g, the supernatant was removed and the pellet was re-suspended in NaCl. A fresh spore suspension of *Mucor sp*. was collected, from a sporulating culture, in Milli-Q water. The concentration of the microbial suspensions was measured as the optical-density at 600 nm (OD600) in a spectrophotometer (BioPhotometer, Eppendorf, USA). Finally, all the suspensions were diluted down to 0.08 OD600 and then use for inoculating the test Mueller-Hinton agar

(MHA) plates. A total of 50 µl of each carcass-derived solution, and one pooled sample (of all fitered carcass-derived solutions) was filled in agar holes of 9 mm diameter previously inoculated with one of the test microorganisms. After 12h and 36h of incubation (30°C for *B. subtilis and E. coli*; 26°C for *S. cerevisiae* and *Mucor sp*.), the diameter of the inhibition zone was measured with the software ImageJ v1.51p (https://imagej.nih.gov/ij/).

Biogenic Amines

Biogenic amines are produced during microbial decomposition of organic matter and can be toxic in large doses. In particular, cadaverine and putrescine are good indicators of the decomposition state of food, being responsible for the characteristic foul smell of putrefying food (Shalaby 1996). To directly assess if together *Nicrophorus* and its microbiota are capable of preserving the carcass, the amount biogenic amines, cadaverine and putrescine, in the different carcass samples were measured with a high-performance liquid chromatograph (HP 1100, Agilent Technologies, Germany) coupled with a fluorescence detector. Because amines cannot be directly detected, the samples were first derivatized with o-phthalaldehyde (OPA); the resultant derivates are highly fluorescent and can be accurately quantified. Derivatization was also performed in a dilution series of cadaverine and putrescine standards (Sigma-Aldrich, Germany). This later allowed to construct a standard curve to infer the concentration of amines in the test samples. Fifty microliters of either samples or standards were injected on the HPLC, the separation of the polyamine derivates was performed on a reversed-phase column (RP-C18, Merck) with acetonitrile as the mobile phase and a gradient elution from 0 to 40% for 15

minutes at room temperature. The eluted derivatives were detected by fluorescence using 340 nm excitation filter and 425 nm emission filter.

RNA extraction

Total RNA was isolated from the swabbed carcass samples. RNA extractions were performed using TRIsure (Bioline, Berlin-Brandenburg, Germany) according to the manufacturer's instructions and DNA contamination was removed using a DNase Digest treatment (Turbo DNase, Thermo Fisher Scientific, USA). Finally, RNA was further purified using RNA Clean and Concentrator 5 (Zymo Research, USA) columns. RNA samples were quantified with a Nanodrop ND-1000 spectrophotometer (PeqLab) and sample quality was verified using an Agilent 2100 Bioanalyzer and an RNA 6000 Nano Kit (Agilent Technologies, USA).

RNA-Seq and de-novo assembly.

We performed RNA-Seq and *de novo* (meta-)transcriptome assemblies to evaluate the functional role of *Nicrophorus* microbiota on the carcass. In this approach, we target both eukaryotic and prokaryotic mRNAs. To do so, we performed rRNA depletion utilizing biotinylated rRNA capture probes (Illumina Ribo-Zero rRNA Removal Kit) for both prokaryotic and eukaryotic rRNAs, which, following hybridization to the target rRNA molecules, are captured by magnetic beads and removed from the total RNA samples. The rRNA depletion procedure was subsequently followed by a poly(A)+ mRNA enrichment using the Dynabeads™

mRNA Purification Kit (Thermo Fisher Scientific). During this last step the mRNA was separated into eukaryotic and prokaryotic fractions and sequenced independently. Library generation and sequencing was carried out by the Max Planck Genome Center Cologne on an Illumina HiSeq2500 Genome Analyzer platform, using paired-end (2x150 bp) reads. Approximately 25 million paired-end reads were obtained for each of the eukaryotic and the prokaryotic libraries. Verification, quality control and transcriptome assembly was performed on the software CLC Genomics Workbench v10.1 (http://www.clcbio.com). The eukaryotic (libraries based on poly(A)+ mRNAs) fractions of both beetle-attended and unattended carcasses were first individually assembled and then jointly assembled to test for the optimal approach; the same was done for the prokaryotic (libraries based on poly(A)- mRNAs) fractions. The presumed optimum consensus *de novo* (meta-)transcriptome assemblies were tested by generating several assemblies with different assembly parameter settings. The selected assemblies were based on the highest N50 contig size, lowest total number of contigs and lowest number of chimeric sequences with the following parameters: word size = automatic, bubble size = 200; minimum contig length = 350 bp; nucleotide mismatch cost = 2; insertion = deletion costs = 2; length fraction = 0.7; similarity = 0.9. The beetle-attended eukaryotic metatranscriptome contained 70,130 contigs with an N50 contig size of 988 bp and a maximum contig length of 17,065 bp, while the unattended eukaryotic metatranscriptome contained 84,671 contigs with an N50 contig size of 1,143 bp and a maximum contig length of 18,175 bp. The beetle-attended prokaryotic metatranscriptome contained 116,634 contigs with an N50 contig size of 1,771 bp and a maximum contig length of 299,065 bp, while the unattended prokaryotic metatranscriptome contained 136,023 contigs with an N50 contig size of 1,347 bp and a

maximum contig length of 86,121 bp. Homology searches (BLASTx and BLASTn) and functional annotation according to GO terms (http://www.geneontology.org), EC codes, and metabolic pathways (Kyoto Encyclopedia of Genes and Genomes (KEGG)) were carried out using BLAST2GO PRO v4.1.9 (http://www.blast2go.de). Homology searches were conducted remotely on the NCBI server by QBLAST using a sequential strategy.

2. Symbiont manipulation

To determine if *Yarrowia* has any fitness effect on the burying beetle, we generated aposymbiotic (symbiont free) lines. We tested two different protocols: (1) oral antifungal treatment of adult beetles and a (2) yeast-specific egg wash.

Oral antifungal treatment. Recently emerged beetles (2-3 days old) were separated into two treatment groups, antifungal fed (N=20) and control (N=20). Beetles were trained to feed on 10% meat extract solution (Sigma-Aldrich, Germany) for seven days by daily providing 100 µl of the solution in an Eppendorf cap. After the training period, the beetles were treated for six consecutive days with Voriconazole (Sigma-Aldrich, Germany), a broad spectrum antifungal, by adding 10 µl (1 ng/ µl) on the daily 100µl meat extract; control beetles were fed on meat extract only. Because the yeast is also found on the beetle's cuticle, the Voriconazole treatment was followed by a Nystatin (yeast-specific anti-fungal; Sigma-Aldrich, Germany) wash. The beetles were washed with 1 ml of Nystatin (24mg/L) inside a 1.5 ml Eppendorf microcentrifuge tube, for one minute, by constant inversion; control beetles were washed with Milli-Q water only. After a resting period of 5 days (in which they were fed with two meal worms), the beetles

were washed and fed with Nystatin for 3 days, as described before. 48 hours after the end of the treatment, 2 µl of anal secretions were collected from all beetles to test the effectiveness of the treatment. To evaluate the aposymbiotic state of the adult beetles the anal secretions were collected for DNA extraction. The secretions were obtained by disturbing the beetles with forceps and collected with a pipette tip; the samples were immediately frozen in liquid nitrogen and stored at −20 °C.

Yeast-specific egg wash. Freshly laid eggs were collected from 4 broods 18 hours after the mating pairs were allowed to breed. Approximately 16 eggs were recovered from each brood, these were assigned to two treatment groups: yeast-specific egg wash (N=11 per brood) and control (N=5 per brood). We allocated more eggs to the yeast-specific egg wash treatment to increase our chances of obtaining enough aposymbiotic beetles to establish a *Yarrowia*-free line. The treated eggs were washed in 1X PBS for 60 seconds, followed by 15 seconds in 0.1% TritonX, then rinsed for 60 seconds with Milli-Q water and finally submerged in Nystatin (10000 units/L) for 60 seconds. The control eggs were only washed in 1X PBS for 60 seconds. After washing the eggs were placed on autoclaved soil until hatching. First instar larvae of each replicate and treatment were placed in a freshly thawed mouse. To facilitate larval feeding in the absence of parental care, the ventral side of the mouse was shaved and two incisions were made in the joint of two legs (young larvae without parental have been observed to select the joints to access the carcass, personal observation). Once the larvae migrated out of the carcass they were transferred to individual boxes with new autoclaved soil. When adults reached

maturity 2 µl of anal secretions were sampled to test for the presence of *Yarrowia*, the secretions were obtained as mentioned in the previous section.

DNA extraction

DNA extraction from the anal secretions was performed using the MasterPure DNA Purification Kit (Epicentre, Germany) according to the manufacturer's instructions. Anal secretions can be contaminated with gut content and soil particles that interfere with downstream applications, thus the obtained DNA was purified using the OneStep PCR Inhibitor Removal Kit (Zymo-Research, Germany) according to the manufacturer's instructions. The quality and quantity of the Purified DNA were checked with a Nanodrop ND-1000 spectrophotometer (PeqLab).

Quantitative PCR

To confirm the aposymbiotic state of the treated beetles, the abundance of *Yarrowia* in the anal secretions was estimated using a quantitative real time PCR. Specific qPCR primers (fwd: 5'TCAACAACGGATCTCTTGGC -3' and rev: 5'- ATACCATACCGCGCAATGTG -3') were designed [Shukla2017] for the *Yarrowia* strains previously isolated from *N. vespilloides* anal secretions by [Vogel et al 2017], these primers target a 109bp region of the internal transcribed spacer (ITS). To estimate *Yarrowia's* ITS gene copy numbers of the test samples, a standard curve was constructed as follows: DNA extract from pure cultures of the *N. vespilloides*-associated *Yarrowia* were amplified using the ITS primers, then purified from an agarose gel (With

Zygmoclean gel DNA recovery Kit, Zymo Research Germany), and serially diluted from 10-1 to 10-8 ng/ µl. A 20 µl total reaction volume containing 8 µl of H2O, 0.5 µl of each primer, 10uL of ABsolute Blue qPCR SYBR Green Mix (Thermo Scientific, Germany), and 1 µl of template DNA of each sample or standard, was carried out in a Bio-Rad CFXConnect instrument. The qPCR cycler conditions were: 95 °C for 15 min, 40 cycles of 95 °C for 15 s, 66 °C for 30 s and 72 °C for 20 s. All runs were followed by a melt curve analysis that included raising the temperature of the reaction mixtures from Ta up to 95 °C (held for 10 s). qPCR results were analyzed with the CFX Manager v3.1 (Bio-Rad) software, the copy number of ITS genes was estimated from the standard curve of Log DNA concentrations of *Yarrowia* serial dilutions against its Ct values.

Fitness assessment

Oral antifungal treatment. Once the aposymbiotic state of the treated beetles was confirmed by qPCR, the beetles were allowed to breed. Non-sibling pairs were selected to set five aposymbiotic (antifungal treated beetles) and five symbiotic (control beetles) broods. Each female-male pair was provided with a freshly thawed mouse (*Mus musculus*) carcass of approximately 22g in transparent plastic containers filled two-thirds with garden soil. The carcass weight before and after breeding was recorded. The development of the brood was tracked until the 3rd instar larvae migrated out of the carcass. The larvae were separated and maintained in individual 37 mL plastic cups filled with soil. Five fitness measurements were assessed: (1) larval weight, taken immediately after migration; (2) time to pupation, time from larvae migration to pupae formation; individuals were considered pupae once adult features

were clearly observed (i.e. antennae, wing buds, legs) and they had completely shaded the larval skin; 3) pupation time, time from pupae formation until adult eclosion; and (4) adult pronotum size (maximum width in dorsal view). The larval weight fitness assessment was done for two consecutive broods of the same parents, with one week break between breeding events.

Sexually mature beetles of the F1 generation were mated to identify potential trans-generational effects, four broods of F1 aposymbiotic and symbiotic beetles (of the first brood) were set in the same conditions previously described and the brood development was tracked until the 3rd instar larvae migrated out of the carcass.

Yeast-specific egg wash. Because the control larvae did not survive the breeding period, only aposymbiotic broods (N=4) were set. The breeding conditions and fitness data were as described in the previous section.

RESULTS

1. Carcass preservation and digestion

The breeding cavity of *N. vespilloides* has antimicrobial activity against gram-positive bacteria.

The antimicrobial activity of the carcass samples was tested against two bacterial species and two fungi. We found that the samples from beetle-attended carcass display antimicrobial activity against *Bacillus subtilis*. After 12h a small inhibition zone (mean=80, sd=3.5 mm) was evident in 3 out of five replicates (Figure 1A); after 36h a larger zone with a *Bacillus subtilis* growth gradient (mean=260.5, sd=15.7 mm) was observed in all replicates (Figure 1B). In the pooled sample the observed inhibition zone (mean=125 mm) against *Bacillus subtilis* at 12h was larger than the one observed for individual samples; after 36h the well continued to show a full inhibition of equal size (Figure 2). None of the samples showed inhibition against *E. coli, S. cerevisiae, or Mucor sp.*

Microbial decay is reduced on beetle-attended carcasses.

The biogenic polyamines cadaverine and putrescine were quantified in beetle-attended and unattended carcasses to establish if beetles are capable of altering the normal decomposition process of carrion. Both biogenic amines were found in all samples of unattended-carcass, although its concentration was highly variable between replicates (Cadaverine mean=1.4 sd=2.1 μg/ml ; Putrescine mean=3.7 sd=2.3 μg/ml). In contrast, cadaverine was undetectable in the

beetle-attended carcass (Figure 3). We found that the unattended-carcasses had a significantly higher amount of cadaverine (Welch t_{13} = 2.5, $p<0.05$) and putrescine (Welch t_{15} = -5.3, $p<0.001$) than the beetle-attended carcasses (Figure 3).

Beetle-attended and unattended carcass differ in their protease and lipase activity

We performed a comparative analysis between the cavities of naturally decomposing mice and the breeding cavities of *N. vespilloies* attended carcasses. The total protein concentration, protease concentration, and lipase enzymatic activity were measured from swab samples. We found that naturally decomposing carcasses had ~ 4 times higher protease concentration than breeding cavities (Welch t_{15} =-1.7, $p<0.05$, Figure 4A), but similar amounts of total protein content (Welch t_{15} =-10.4 $p>0.05$, Figure 4B). In contrast, the lipase activity was undetectable for most of the samples of unattended carcasses while beetle-attended carcasses had a high lipase activity (Welch t_{13} t=-2.2 $p<0.05$, Figure 4C).

The transcriptome of the beetle-attended carcass is dominated by transcripts from *Yarrowia* and *Nicrophorus* gut core microbiota.

We identified the active eukaryotic (Figure 5). and prokaryotic (Figure 6). communities of beetles-attended and unattended carcasses. We observed overlapping between the two transcriptomes as a result of the initial rRNA depletion and the high abundance of some of these groups. However, the eukaryotic and prokaryotic transcriptome will be discussed

independently. The eukaryotic transcriptome of beetle-attended carcass was dominated by transcripts of several species of nematodes followed by *Yarrowia* and *Trichosporon* (Basidiomycota:Tremellales). While the unattended carcass was completely dominated by fungi transcripts, been the filamentous fungi *Mucor sp.* the most abundant*;* and in a minor degree *Rhizopus, Polysphondylium, Mortierella, Absidia, and Lichtheimia* (Figure 5B).

The prokaryotic transcriptome was evenly represented by several bacterial species with no major contribution of a single group like in the eukaryotic transcriptome (Figure 6). The beetle-attended transcriptome was mainly represented by a gram-negative bacterial community, being *Clostridium* the only gram-positive bacteria (Figure 6A). On the contrary, a mixed of gram-positive and gram-negative bacteria characterized the transcriptome of the unattended carcass (Figure 6B).

2. Symbiont manipulation

The two treatments used for obtaining aposymbiotic (symbiont free) beetles were effective. The load of *Yarrowia* (measured as *Yarrowia*-ITS gene copy numbers) of the antifungal feed beetles (Welch t_{14} = -8.9, $p<0.001$) and the yeast-specific egg wash beetles (Welch t*9* =-6.9, $p<0.001$) was significantly lower than in the control beetles (Figure 7). There were no significant differences between the two treatments (Welch t*12*=-2.0, $p<0.05$) nevertheless in 3 out of 10 the yeast-specific egg wash beetles *Yarrowia* was undetectable. Given these results, all the treated beetles were considered as aposymbiotic. Unfortunately, the control beetles of the egg

wash treatment did not survive, thus we focused on the results from the antifungal feed beetles.

Larvae from aposymbiotic beetle's parents have lower body mass

We followed the progress of aposymbiotic and symbiotic broods to determine potential fitness consequences due to the lack or presence of *Yarrowia.* The carcass preparation of both groups took place normally: the hair of the mice was removed and the carcass was buried and rolled into a ball between during the first two days of breeding and the females of all treatments laid eggs. Once all larvae migrated out of the carcass (~12 days) their weight was measured. We found that the weight of the larvae from aposymbiotic parents were significantly lower than the larvae from symbiotic beetles, for both the first (Welch t_{180}=-2.1, $p<0.05$, Figure 8) and the second brood (Welch t_{155}=-7.8, $p<0.01$). The brood size (number of larvae per brood) was equivalent (Welch t_8=0.5, p=0.6) for treated (mean=17, sd=4.7 larvae) and control beetles (mean=19, sd=4.9 larvae). The developmental times were also equivalent between treatments. The larval weight of the larvae was highly correlated (R-square= 0.65) to the adult size (pronotum width). Additionally, we observed a high incidence of deformed adult beetles from the aposymbiotic broods (24% vs.5% in control beetles), deformed beetles showed an incomplete development of both forward and hind wings which had a wrinkled appearance.

The weight of the carcass before and after breeding was also measured to determine the biomass conversion. There were no significant differences between the broods of aposymbiotic and symbiotic parents. (Welch t_7= 2.36, p=0.69)

To identify potential transgenerational effects, F1 aposymbiotic and symbiotic beetles were mated. While carcass attended by the symbiotic beetles were normally prepared, those from aposymbiotic pairs were loosely prepared, i.e. incomplete hair removal (in 2/4 carcasses), incomplete burial (in 1/4 carcasses), and not shaped into a carcass ball (in 2/4 carcasses). No larvae or eggs were observed in the aposymbiotic broods. At the end of the breeding period we observed that aposymbiotic adults had big masses of nematodes under the elytra, some symbiotic adult also had some nematodes but in lower numbers.

DISCUSSION

In this study, we explored how *Nicrophorus vespilloides* microbiota facilitates the necrophagous life style of its host. We provide for the first time quantitative evidence of carcass preservation due to the joint action of *N. vespilloides* and its microbiota (Figure 3). Furthermore, our study supports the hypothesis of a mutualistic interaction between *Nicrophorus* and its symbiotic yeast *Yarrowia*; the presence of digestive extracellular enzymes on the beetle attended carcass and the reduced fitness of aposymbiotic beetles suggest that *Yarrowia* enhances the fitness host by maximizing larval nutrition (Figures 4 & 7).

Carcass preservation

The antimicrobial peptides (AMPs) and lysozymes from the anal and oral exudates have been identified as agents responsible for at least part of the observed carcass preservation (Jacobs et

al. 2016; Palmer et al. 2016). However, these molecules can only inhibit gram-positive bacteria (Arce et al. 2012; Cotter & Kilner 2010b; Cotter et al. 2010; Steiger et al. 2011). Despite the fact that functional interactions between AMPs could lead to inhibition of gram-negative bacteria (Rahnamaeian et al. 2015), it seems likely that the host-derived innate immune effectors alone cannot regulate all the pathogens and microbial decomposers on the carcass. Alternatively, carcass preservation could be achieved by the join antimicrobial activity of the host and its microbiota. Several members of *Nicrophorus* microbiota, belonging to the Firmicutes and Enterobacteriaceae groups, have bacteriolytic and/or antimicrobial capacity (Ammor et al. 2006; Thompson et al. 2013) that could enhance the host immunity. Until now, the antimicrobial activity of *Nicrophorus* microbiota remained untested.

Several studies (Sheena C. Cotter & Kilner 2010; Cotter et al. 2010; Steiger et al. 201) have analyzed the antimicrobial activity of anal secretions, but none of these were designed to test the activity of *Nicrophorus* microbiota. In the first place, these studies collected anal secretions directly from the beetle's anus; this is insufficient to test the antimicrobial capacity of the microbiota given that it is most likely expressed in a context dependent manner (i.e. when the microbiota is in contact with carrion). Additionally, they only tested the lytic activity of anal secretions against a single gram-positive bacterium (Sheena C. Cotter & Kilner 2010; Cotter et al. 2010; Steiger et al. 2011 but see Arce et al. 2012). To overcome these constraints we sampled the carcass breeding cavity, were the beetles´ microbiota is more likely to express antimicrobial activity, and tested its effectiveness against gram-positive and gram-negative bacteria, and two fungi.

Our results are consistent with Arce et al. (2012), namely that the active compounds from the carcass breeding cavity were effective against the gram-positive bacteria *B. subtilis*, but not against the gram-negative *E. coli,* or fungi (*S. cerevisiae* or *Mucor sp.*). These results indicate that neither the beetles nor its microbiota produce antibiotics effective against gram-negative bacteria or fungi. Although the carcass-derived solution did not inhibit *Mucor sp*, from the transcriptomic data (Figure 5B) we can infer that either *Nicrophorus* or its microbiota are capable of outcompeting this fungus. It is possible that due to the constant carcass maintenance of adult beetles, *Mucor sp*. spores are constantly repressed.

The inhibition bias towards gram-positive bacteria is consistent with the observed community composition of the gut core microbiota, were the most abundant and diverse groups (i.e. *Wohlfahrtiimonas, Providencia, Morganella, and Myroides*) are gram-negative (Shukla et al. 2017; Vogel et al. 2017). The only gram-positive members of *Nicrophorus* core microbiota correspond to the genus *Vagococcus,* indicating that this group may be resistant to the antimicrobial activity of both the host and its microbiota. Some species of the *Vagococcus* genera are known to possess a cell membrane with a particular lipid and fatty acid pattern configuration (Fischer et al. 1988) that has been associated with antimicrobial peptide resistance (Ernst & Peschel 2011). As proposed by Shukla (2017) the host innate immunity is likely to favor those microbes involved in mutualistic interactions with the host, including those that can out-compete pathogens and microbial decomposer. Thus, the lack of inhibition against

gram-negative bacteria is not necessarily disadvantageous, as it may allow the coexistence with microbes with desirable traits.

As we only found antimicrobial activity against gram-positive bacteria, we cannot tell apart the contribution of the host and its microbiota. It remains to be tested how much *Nicrophorus* microbiota contributes to carcass preservation through the expression of antimicrobial properties. In comparison with previous studies we tested for the inhibition against a broader group of microbes; nevertheless, our approach was also limited to cultured strains that do not necessarily reflect the competitors that *Nicrophorus* encounters on the carcass. It is necessary to design specific assays to elucidate the interactions between *Nicrophorus* microbiota and the microbial decomposers of carrion, and how these interactions promote carcass preservation.

Even though multiple papers have deal with the potential mechanism underlying carcass preservation by the burying beetles (Arce et al. 2012; Cotter & Kilner 2010b; Rozen et al. 2008), preservation itself has not been measured. In this study, we present for the first time quantitative data of carcass preservation. During microbial decomposition organic matter is broken down by amino-acid decarboxylases producing biogenic amines. These amines are toxic in large doses and can lead to intoxication and disease in animals (Shalaby 1996). The production of biogenic amines by microbial decomposers, such as Enterobacteriaceae, *Clostridium*, and *Lactobacillus* among others, has been hypothesized to be a strategy of microbes to compete with animals by making food resources inaccessible (Shalaby 1996; Janzen

1977). We showed that beetle-attended carcasses have low levels of biogenic amines when compared with a naturally decomposing carcass (Figure 3); thus providing strong evidence of carcass preservation. The observed levels of biogenic amines of the two carcass treatments reflects the microbial succession, from a carcass community governed by decomposing microbes –with high production of biogenic amines-, to a community dominated by *Nicrophorus* core microbiota where producers of biogenic amines are outcompeted (Shukla et al. 2017). Interestingly, cadaverine was below the detection limit on the beetle attended carcass. Generally, cadaverine and putrescine co-occur in decomposing meat (Ruiz-Capillas & Jiménez-Colmenero 2004; Durlu-Özkaya et al. 2001) as observed on the unattended carcass. The concentration asymmetry between the two amines may imply that *Nicrophorus* and its microbiota not only possess antimicrobial activity against microbial decomposers (Arce et al. 2012; Cotter & Kilner 2010; Cotter et al. 2010; Steiger et al. 2011), but also a potential detoxification mechanism of biogenic amines.

Previous transcriptomic and proteomic data (Jacobs et al. 2016) on adult beetles and their anal secretions does not indicate any kind of host extracellular enzymes involved in detoxification, although this analysis has been focused mainly on antimicrobial peptides and lysozymes. If detoxification of biogenic amines occurs on the carcass, it is most likely driven by *Nicrophorus* microbiota. Some members of the gram-positive group Bacilli are capable of degrading biogenic amines through the action of amine oxidase enzymes (García-Ruiz et al. 2011; Gardini et al. 2002), members of this bacterial group have been found in both culture-based (Wang & Rozen 2017) and molecular (Shukla et al. 2017; Duarte et al. 2017) analysis of *Nicrophorus* microbiota. We found high gene expression *Nicrophorus* microbiota on the beetle-attended carcass (Figure

6A), a deeper homology search with *Nicrophorus* microbiota as specific target will reveal important aspects of the role of *Nicrophorus* microbiota in carcass preservation, making it possible to determine if the bacterial community is involved in the degradation of biogenic amines.

Carcass digestion

Besides carcass preservation, *Nicrophorus* microbiota has been also hypothesized to play an important role in carcass digestion (Vogel et al. 2017). In particular, *Yarrowia* genes encoding for the extracellular enzymes protease and lipase have been found to be highly expressed on the breeding cavity of beetle-attended carcasses (Vogel et al. 2017). To evaluate the digestive role of *Nicrophorus* microbiota, we measured total protein, protease and lipase concentration in beetle-attended and unattended carcasses. Our results showed that only beetle-attended carcasses are coated with lipases, thus supporting the previous findings by (Vogel et al. 2017) and reinforcing the hypothesis that *Yarrowia* metabolism facilitates beetle's access to fatty acids. In contrast, proteases were found in both attended an unattended carcass, and while the latter one had the highest concentration, the protein concentration was surprisingly similar in both treatments. This discrepancy between enzyme and substrate concentration may reflect a difference in the catalytic efficiency of the proteases due to the intrinsic capacity of the different microbial communities or due to environmental factors such as pH, oxygen, and temperature (Braaksma et al. 2009). As both yeast and bacteria are known to produce extracellular proteases (Glenn 1976), it remains to be identified if *Yarrowia* is the main producer of these enzymes on the beetle-attended carcass.

The transcriptome of the beetle-attended and unattended carcasses (Figure .4) reflects the distinctive microbial communities between the treatments. As discussed before by Shukla et al. (2017) burying beetles induce a microbial shift by seeding their core microbiota into the carcass, this shift is also reflected by the carcass transcriptome. The gut core-microbiota was highly represented in both the prokaryotic (i.e. *Wohlfahrtiimonas, Vagococcus, Providencia, Morganella,* and *Myroides;* Shukla et al. 2017) and eukaryotic (i.e. *Yarrowia, Trichosporon,* and *Candida*) transcriptomes; only *Candida* and *Vagococcus* were not significantly represented (Figures 5A & 6A). In general, our results are consistent with previous findings (Shukla et al. 2017; Vogel et al. 2017) and continue to support the ecological relevance of *Nicrophorus* microbiota and *Yarrowia* to utilize carrion as a breeding resource.

In the unattended carcass transcriptome we found an important contribution of microbial decomposers associated to the production biogenic amines in meat (e.g. *Clostridium, Pseudomonas, Enterobacter;* Shalaby 1996) (Figure 6B)

We observed an important contribution of several species of nematodes to the transcriptome of the beetle-attended carcass. Even though this high transcript abundance is likely biased due to the multicellular nature of nematodes, we suspect that roundworms have an important effect in burying beetle's fitness. Several of the nematode species here identified (Figure 5A) are known to be pathogenic, thus their presence could be detrimental for burying beetles. Given that we observed a particularly high amount of nematodes on the aposymbiotic beetles,

we will be interested in investigating about a potential interaction between *Yarrowia* and the nematodes. We propose to further analyze the roundworm transcripts to formulate a hypothesis regarding the presence of the nematodes and how it affects burying beetle's ecology.

Symbiont manipulation

Yarrowia was experimentally removed from adult beetles to establish an aposymbiotic (*Yarrowia*-free) line, and test for potential fitness costs due to the absence of the yeast. We found that larvae from broods of aposymbiotic parents had a lower weight than control larvae (Figure 8); however, the number of larvae per brood and the carcass consumption was equivalent between treatments.

The extracellular enzymes (lipases and proteases) produced by *Yarrowia* (Vogel et al, 2017, Figure 5) may facilitate the access of larvae to proteins and lipids. Maximization of larval nutrient intake is highly desirable given the ephemeral nature of carrion and the intense competition for this resource. Even though burying beetles possess complex behavioral (Scott 1998; Eggert et al. 1998) and antimicrobial adaptations (Arce et al. 2012; Cotter & Kilner 2010b; Cotter et al. 2010) to overcome those challenges, these strategies can be costly (Cotter & Kilner 2010b; Smith et al. 2015; Reavey et al. 2014). For example, upregulation of antimicrobial peptides during breeding (Jacobs et al. 2016) can lead to lifetime reproductive fitness costs

(Cotter et al. 2010). Thus, fast development times and an efficient nutrient intake are advantageous.

The positive effect of *Yarrowia* on *Nicrophorus* larval body weight can cascade through beetle's adult life with important fitness consequences. In general body size in insect has a positive fitness effect by increasing survival and reproductive success (Andersson 1994). At the larval stage, a larger size is advantageous for overwinter survival (Smith 2002) while as adults larger individuals have a competitive advantage during direct combat and can exploit a broader range of carcass sizes (Scott 1998). In the present experiment we found that mated F1 aposymbiotic adults were unable to breed successfully, this can be due to a combined effect of its aposymbiotic state and its lower body mass and poor nutrition. To discard any secondary effect due to the antifungal treatment and confirm the fitness consequences here reported, a *Yarrowia* recovery experiment should be performed. If the results here discuss hold true, by re-feeding *Yarrowia* to the aposymbiotic beetles we expect to recover the phenotype of control beetles.

Is probable that *Yarrowia* supports the burying beetle's development in further ways than the here described. During the experiment, we made several observations such as increased presence of nematodes (discussed above); and deformations in the elytra of aposymbiotic beetles which could be caused due to the lack of essential lipids for cuticle formation (Lockey 1988; Vogel et al. 2017). Formal experiments should follow to further explore this symbiotic association.

Associations between beetles and yeast are widespread but poorly studied, a meta-analysis of beetle guts (Suh et al. 2005) across 40 beetle species has shown that these beetles harbor at least 200 undescribed yeast species. Many of these associations are host-specific, suggesting that they are ecologically relevant. In bark beetles, yeasts have been hypothesized to provided multiple services such as semiochemical emission, chemical detoxification and nutrient provisioning (Davis 2015). Similarly, the symbiotic yeast of the wood-boring beetle is believed to provide nutrition through the fermentation of xylose (Nguyen et al. 2006), while cigarette beetle larvae benefit from the detoxification role of its symbiotic yeast. Together, the high abundance of *Yarrowia* transcripts on beetle attended carcasses (Vogel et al. 2017; Figure 5 A), the expression of lipases and proteases (Vogel et al. 2017; Figure 4), its vertical transmission through the anal exudates (Shukla et al. 2017) and it's positive fitness effect on the larvae indicate that *Yarrowia* is a mutualistic symbiont of the burying beetles.

Modern microbiology has challenged our view of eukaryotic ecology and evolution. We no longer see animals and plants as autonomous entities, we understand that symbiotic microbes can influence almost every trait of their host, from physiology to behavior up to driving speciation (Bordenstein et al. 2015; Shropshire & Bordenstein 2016). Together the host and its symbionts can exploit a wider range of resources and colonize complex ecological niches. Our results provide important insights about host-symbiont interactions and how diverse microbial

metabolites can assist insects into colonizing a challenging an ephemeral resource such as carrion.

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FIGURES

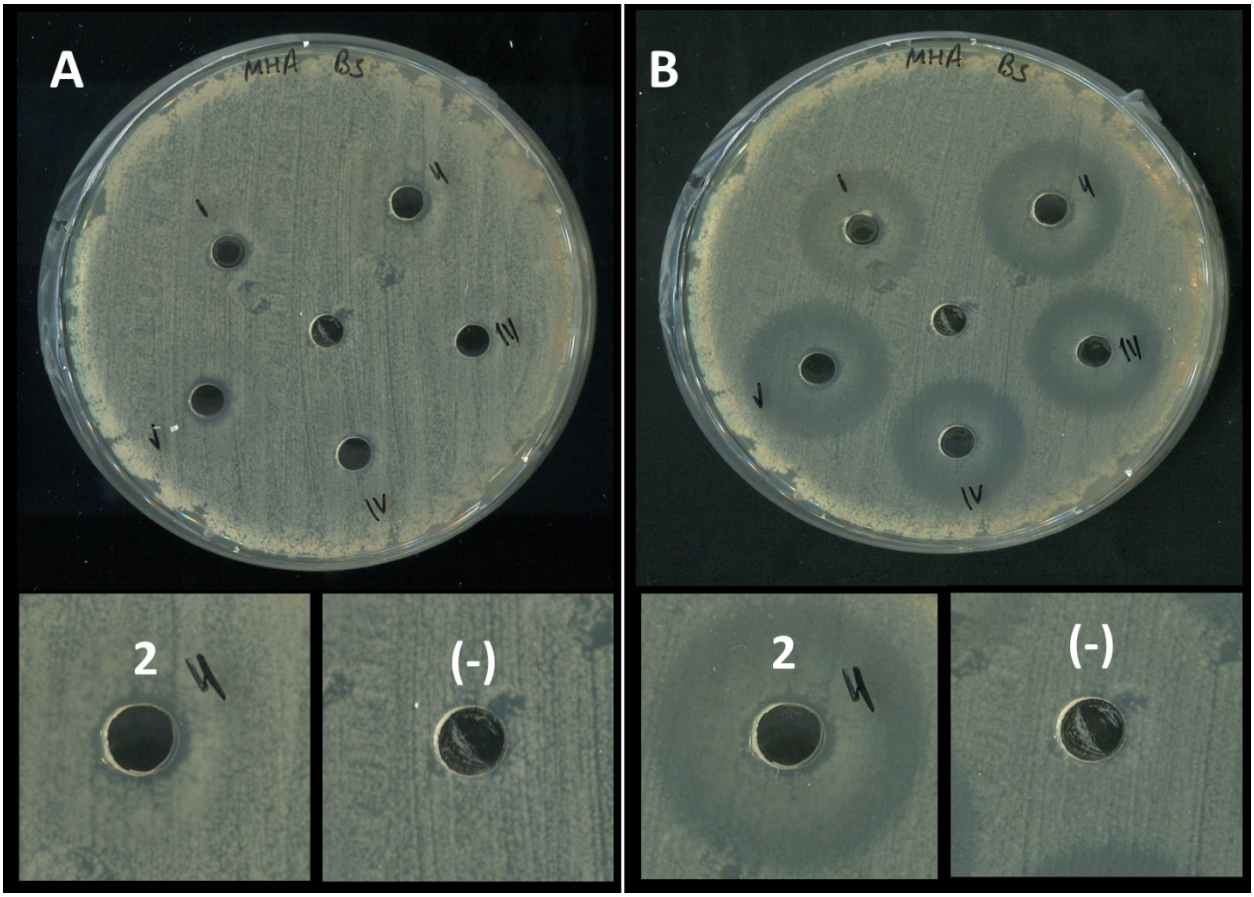

Figure 1. Agar-diffusion test against *Bacillus subtilis*. A) At 12h a small inhibition zone is observed in 3 out of 5 wells, see a close-up of sample two. B) At 36h all samples showed an inhibition zone with a growth gradient of *B. subtilis*. The negative control (-) in the middle of the plate is shown for reference.

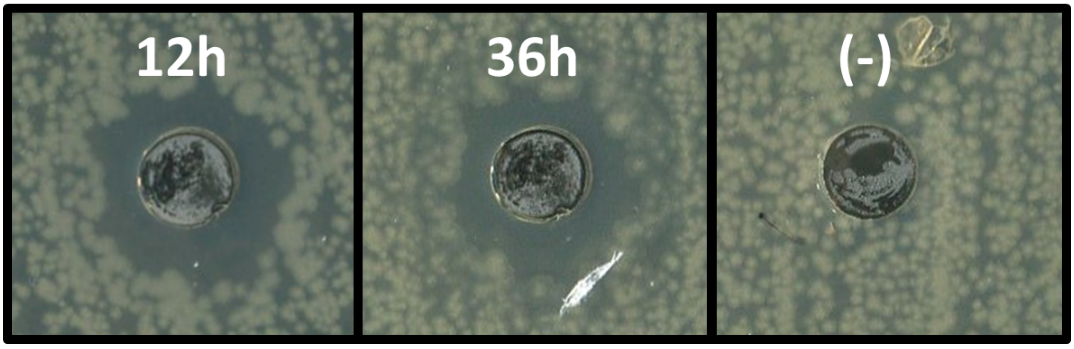

Figure 2. Agar-diffusion test against *Bacillus subtilis* for the pooled swab samples. Development of the assay at 12 and 36 hours. The negative control (-) is shown for reference.

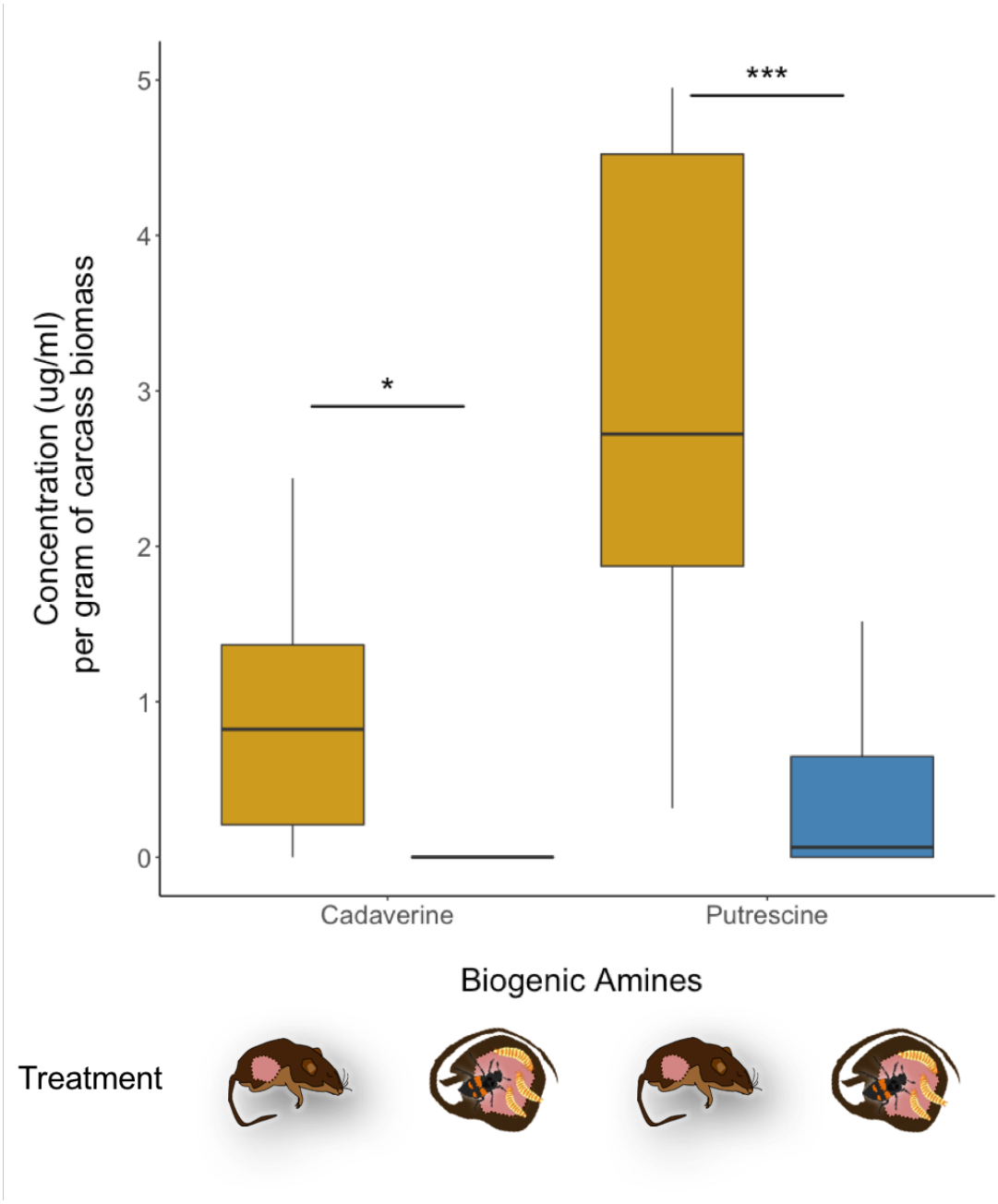

Figure 3. Concentration of the biogenic amines cadaverine and putrescine on unattended carcass (yellow) and beetle-attended carcass. Boxplots show median and interquartile range.

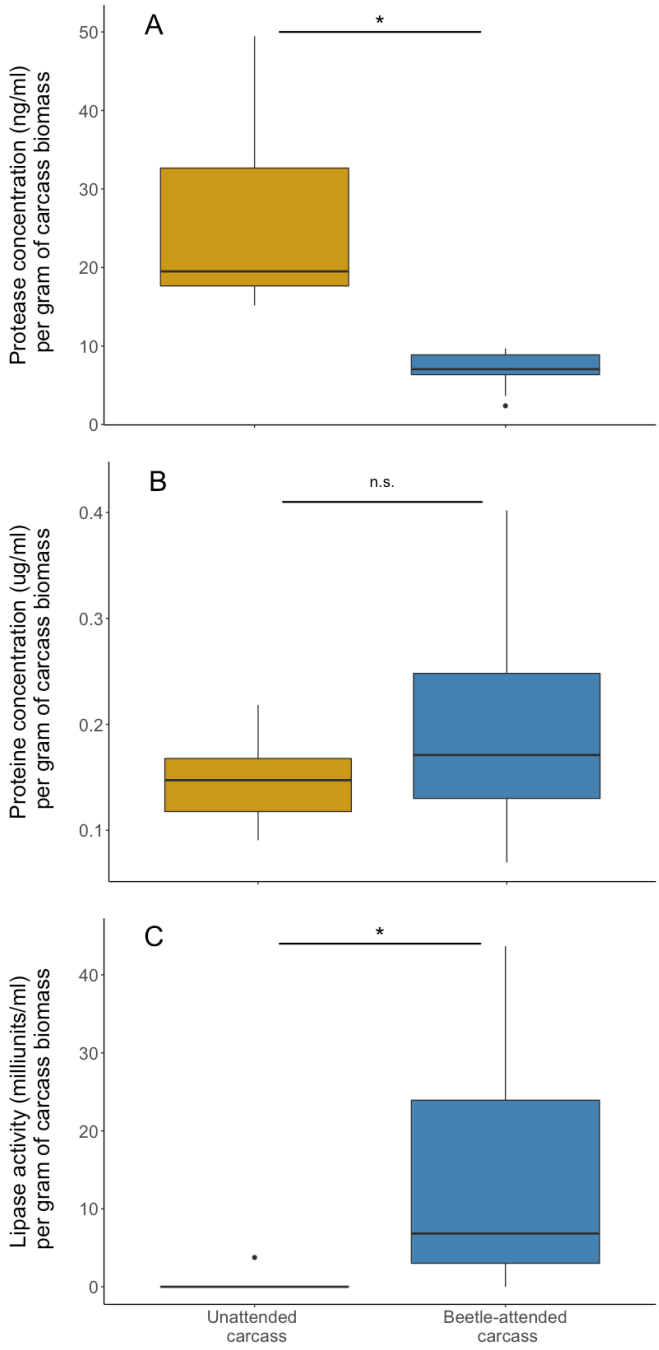

Figure 4. Enzymatic differences between unattended (yellow) and beetle-attended (blue) carcass. The A) protease concentration, B) protein concentration and C) lipase activity were estimated. Boxplots show median and interquartile range, samples outside the interquartile range (1.5> interquartile range) are shown as dots.

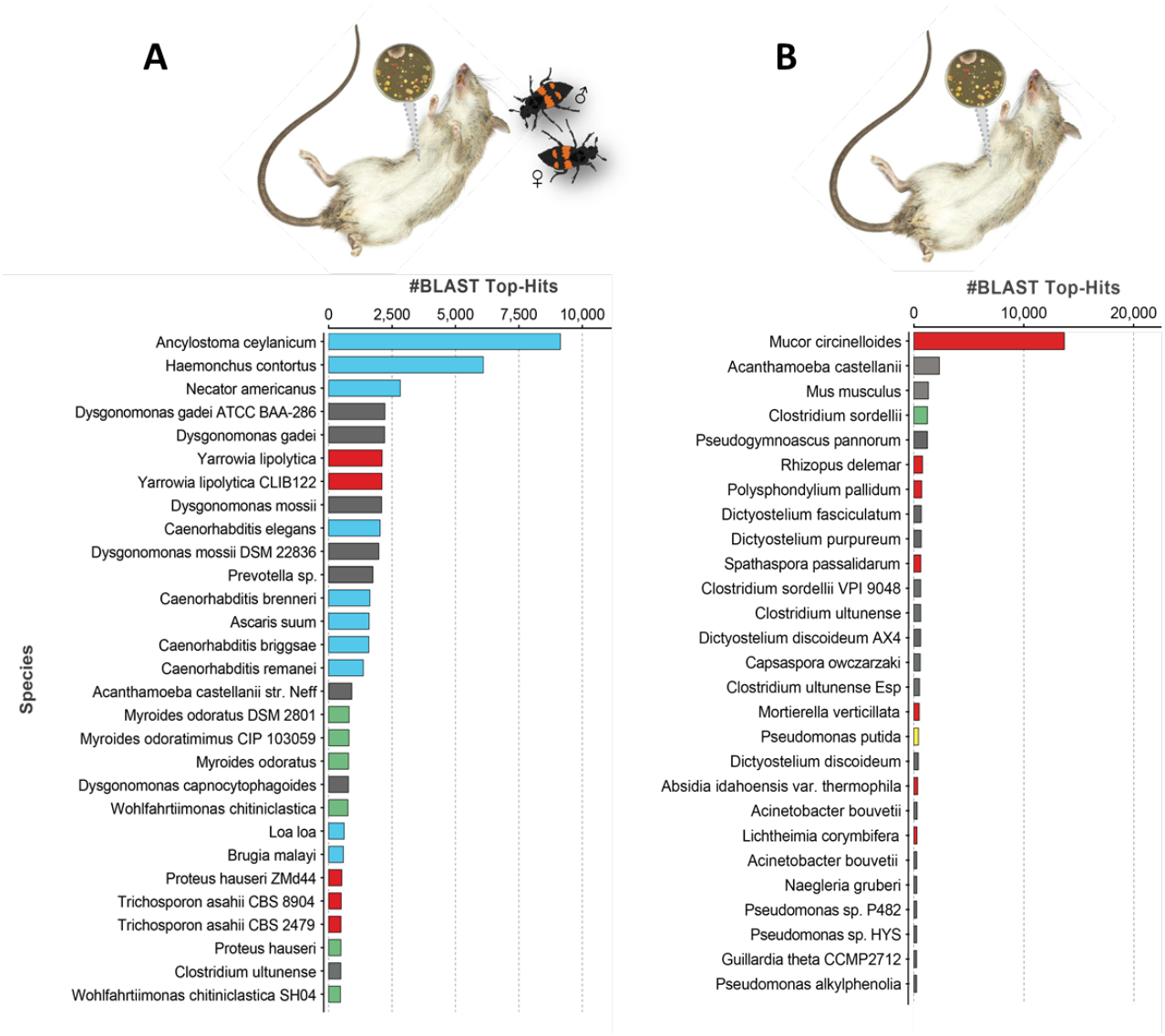

Figure 5. Top blast hit species distribution of the A) beetle-attended carcass and B) unattended eukaryotic carcass transcriptomes. Fungal taxa are shown in red, nematodes in blue, *Nicrophorus* gut core-microbiota in green, and others in grey bars.

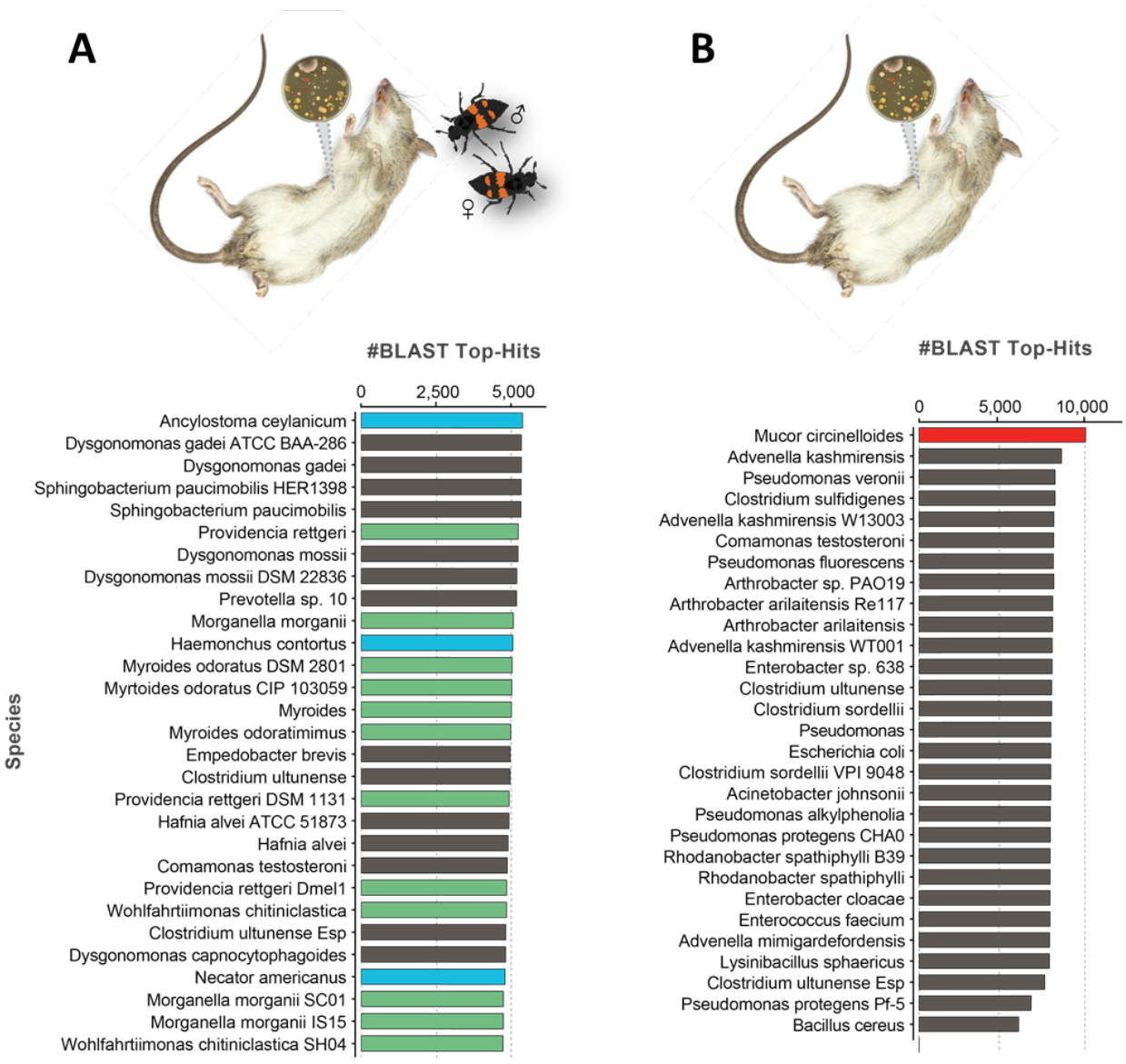

Figure 6. Top blast hit species distribution of the A) beetle-attended carcass and B) unattended prokaryotic carcass transcriptomes. Fungal taxa are shown in red, nematodes in blue, *Nicrophorus* gut core-microbiota in green, and others in grey bars.

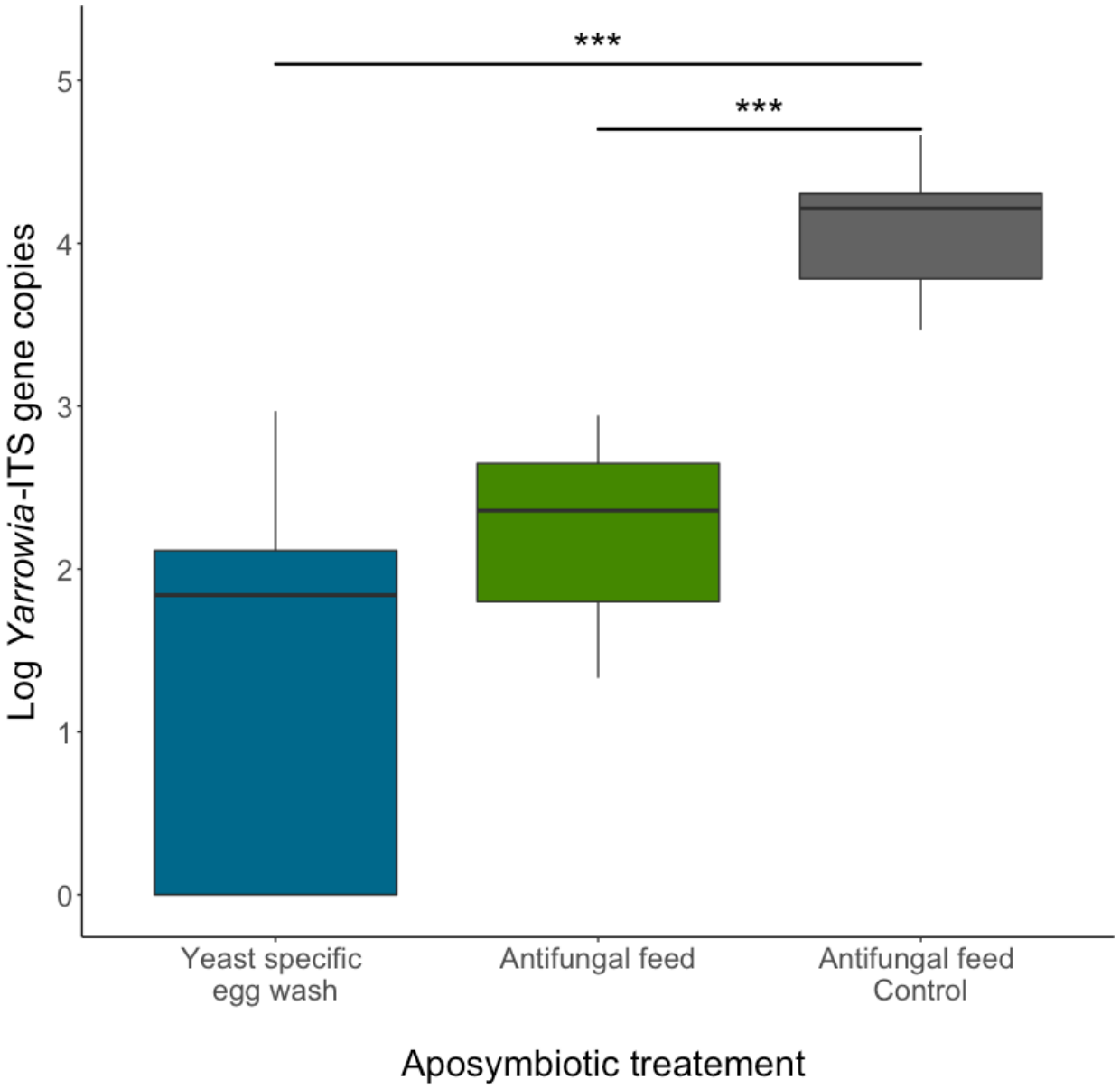

Figure 7. *Yarrowia* load on the anal secretion of adult beetles under two aposymbiotic treated and control beetles. Yeast specific egg-wash (N=10), antifungal feed (N=10) and, antifungal feed control (N=13). Boxplots show median and interquartile range.

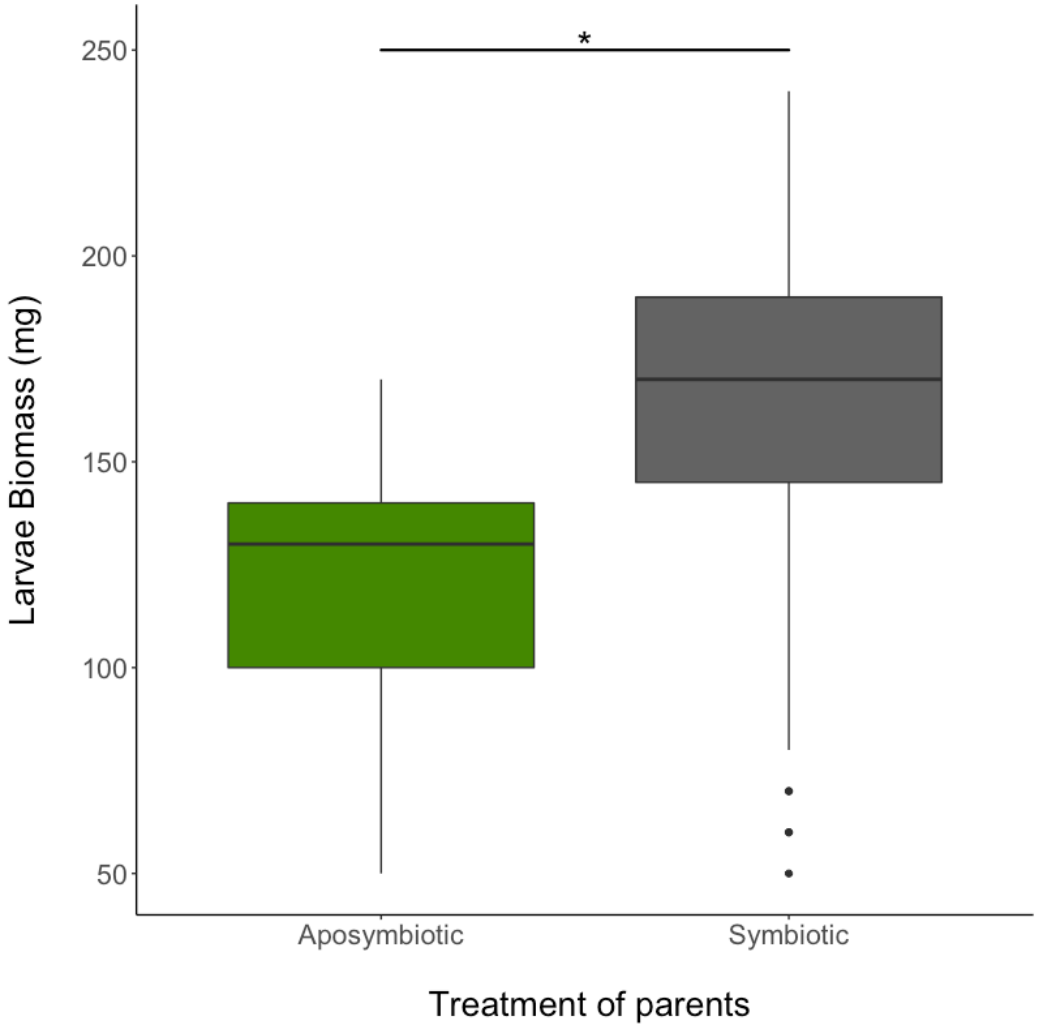

Figure 8. Larvae biomass from aposymbiotic (green, N=100) and symbiotic (grey, N=87) broods. Boxplots show median and interquartile range. Larvae outside the interquartile range (1.5> interquartile range) are shown as dots.