The intestinal microbiota of *Hermetia illucens* larvae is affected by diet and shows a diverse composition in the different midgut regions

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ABSTRACT The larva of the black soldier fly (*Hermetia illucens*) has emerged as an efficient system for the bioconversion of organic waste. Although many research efforts are devoted to the optimization of rearing conditions to increase the yield of the bioconversion process, microbiological aspects related to this insect are still neglected. Here we describe the microbiota of the midgut of *H. illucens* larvae showing the effect of different diets and midgut regions in shaping microbial load and diversity. The bacterial communities residing in the three parts of the midgut, characterized by remarkable changes in luminal pH values, differed in terms of bacterial numbers and microbiota composition. The microbiota of the anterior part of the midgut showed the highest diversity that gradually decreased along the midgut, whereas bacterial load had an opposite trend, being maximal in the posterior region. The results also showed that the influence of the microbial content of ingested food was limited to the anterior part of the midgut and that the feeding activity of *H. illucens* larvae did not affect significantly the microbiota of the substrate. Moreover, a high protein content compared to other macronutrients in the feeding substrate seems to favor midgut dysbiosis. The overall data indicate the importance of taking into account the presence of different midgut structural and functional domains, as well as substrate microbiota, in any further study that aims at clarifying microbiological aspects concerning *H. illucens* larval midgut.

IMPORTANCE The demand for food of animal origin is expected to increase by 2050. Since traditional protein sources for monogastric diets are failing to meet the increasing demand for additional feed production, there is an urgent need to find alternative protein sources. The larvae of *Hermetia illucens* emerge as efficient converters of low quality biomass into nutritionally valuable proteins. Many studies have been performed to optimize *H. illucens* mass rearing on a number of organic substrates and to maximize quantitatively and qualitatively the biomass yield. On the contrary, although insect microbiota can be fundamental for bioconversion processes and its characterization is mandatory also for safety aspects, this topic is largely overlooked. Here we provide an in depth study of the microbiota of *H. illucens* larval midgut taking into account pivotal

aspects such as the midgut spatial and functional regionalization as well as microbiota and nutrient composition of the feeding substrate.

The black soldier fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae), is a true fly that occurs worldwide in tropical and temperate regions. Adults of this insect have never attracted interest because they do not approach humans, do not bite and are not known to vector pathogens. On the contrary, BSF larvae have been object of intense research efforts because of their remarkable utility for humans that take advantage of their feeding regime of generalist detritivore (1). In particular, BSF larvae are largely used in forensic entomology to estimate human postmortem interval (2, 3) but the major promising potential of the voracious BSF larvae is their use as efficient bioconverters (4-6). Indeed, BSF larvae can be reared in mass cultures on a very wide variety of organic waste (e.g. crop and food processing residues, food waste, manure and feces) leading to the conversion of low quality material into valuable biomass. The latter is exploitable for the isolation of bioactive compounds (e.g. antimicrobial peptides, chitosan and degrading enzymes), biodiesel production, and as feed or feed ingredients (mainly for their content of high-quality proteins and lipids) for poultry, aquaculture, and livestock (1, 6). The production of BSF larvae is technically simple, cost-effective and environmentally sustainable (1, 6). However, the rate of waste recycling and the final value of the biomass obtained depend on the rearing strategy, in terms of feeding substrate composition, feed consumption rate, and environmental parameters (i.e. temperature, humidity and photoperiod) (1). For this reason, many research efforts now focus on the characterization of nutrient and micronutrient content of BSF larvae in response to different rearing conditions and substrates, in order to optimize biomass yield and quality (1, 6-10).

Safety aspects concerning the microbiological load of intermediate and final products of bioconversion processes are also crucial, especially when BSF is exploited for feed applications. In principle, this issue can be approached by classical food microbiology methods to establish whether a product meets the recommendations imposed by current hygiene criteria. On the other hand, an in-depth characterization of BSF larvae microbiota and the factors that influence its composition is particularly important. Microbiota composition is known to impact insect health and performance, and has to be considered in the effort to optimize biomass yield (11). In addition, the analysis of the

microbiota could allow the identification of bacterial species with peculiar and unique characteristics, such as the capacity to degrade complex substrates, as cellulose, hemicellulose and lignin (12), or xenobiotics. These microorganisms, or even the enzymes responsible for the degradation, could be isolated and exploited at industrial level for waste recycling and bioremediation. Populations of gut bacteria able to compete with pathogens or to act as probiotics could be boosted for the improvement of BSF larvae performances and bioconversion efficiency or may be used in other animal hosts with similar purposes. Moreover, the study of BSF microbiota has a strong potential in contributing to the global problem of the identification of new antimicrobials. Indeed, BSF larvae feeding activity is able to reduce the bacterial load of substrates and, importantly, this capacity is not accompanied by the accumulation of pathogens in their gut (13-16). Such evidence implies the presence of potent antimicrobial effectors produced by BSF larvae and their intestinal microbiota. It should be pointed out that the latter is implicated in turn in the maintenance of gut homeostasis and supports gut immune functions (11, 17, 18).

A few studies on microbiota of BSF larvae have already been performed (19, 20). Rearing substrate and insect development stage have a significant impact on the overall composition of the microbial community (19, 20). A very critical issue that these preliminary microbiological surveys have not taken into account is the high complexity of the gut of fly larvae. In fact, this organ, and in particular the midgut, shows peculiar regional structural and functional features associated with changes of luminal pH (21-23). The differences in gut morphology and epithelial architecture along different intestinal tracts of some insects are in fact accompanied by remarkable differences in physiological, metabolic and immune features that impact on microbiota composition (24-26). These complex relationships have been exhaustively described in the model insect, the fly *Drosophila melanogaster* (Diptera: Drosophilidae) (17, 27-30).

In all insect, the digestive tract is divided into three regions with different embryonic origin and peculiar morphological and functional features: a short initial tract, the foregut, a long midgut where digestion and absorption occur and a final hindgut where water, salts, and other molecules

99 are absorbed prior to elimination of the feces. Even though a detailed morphofunctional description
100 of *H. illucens* larval midgut is lacking, it is expected that, as in other non-hematophagous
101 brachycerous Diptera, discrete regions with peculiar pH values can be recognized along the midgut
102 and that each distinct midgut region possesses its own features, at both structural and functional
103 levels, and a peculiar resident microbiota (17, 24-30).

104 In the present work we analyzed the effects of different diets and their microbial community
105 on the midgut microbiota of BSF larvae, and the impact of the insect feeding activity on the diet
106 microbiota. Most importantly, we analyzed the different tracts of BSF larval midgut separately, and
107 highlighted the need of having future research on BSF larval midgut considering each midgut
108 domain independently.
109

RESULTS

Determination of the pH values of the midgut lumen content. Since luminal pH is a good marker for midgut regionalization in flies (21-23), we evaluated how the pH of the lumen content of BSF larvae changed along the midgut in order to have a clear identification of the regions in which this organ could be subdivided. For this purpose, last instar *H. illucens* larvae were fed with diet containing two pH indicators, bromophenol blue and phenol red. The color of the luminal content of larvae fed with diet containing bromophenol blue was clearly visible through the isolated epithelium (Fig. 1A). The anterior region of the midgut presented a blue color, indicating that its luminal content has a pH $\geq$ 4.6. Then, a marked change was observed, since the middle region turned yellow, revealing that its lumen has a pH $\leq$ 3. Moving towards the posterior midgut, the color gradually turned blue. Bromophenol blue turns at pH values between 3.0 and 4.6, thus differences in the pH values of the anterior and posterior midgut contents could not be evidenced. Figure 1B shows the gut isolated from a larva fed with diet containing phenol red, a dye that turns yellow at pH $\leq$ 6.8 and fuchsia at pH $\geq$ 8.2. Since the anterior and the middle regions of the midgut presented a golden yellow color, whereas the posterior midgut content appeared fuchsia, it is possible to state that the luminal content of the anterior and middle regions have an acidic pH and the posterior has an alkaline pH. The evidence obtained with phenol red supported and completed results obtained with bromophenol blue. In conclusion, the luminal content of the midgut of *H. illucens* larvae presents different pH values: the anterior region has an acid luminal content, the middle region presents a strongly acid pH (pH $\leq$ 3) and the posterior region has an alkaline luminal content. These three regions are separated by transition zones, in which the pH values gradually change (Fig. 1A and B). Taking into account this evidence, we could easily distinguish three main regions of the larval midgut of *H. illucens*, a fundamental aspect to isolate midgut samples for the analyses reported below (Fig. 1C).

Insect performances on different diets. The microbiota analyses were performed on larvae reared on three different feeding substrates: Standard diet, an optimal diet for fly larvae rearing

(31), Veg Mix diet, containing a mixture of fruits and vegetables, and Fish diet, based on fish meal (see Material and Methods for detailed composition). We thus evaluated the performances of the BSF larvae on these substrates. The maximum weight reached before pupation by BSF larvae reared on Standard diet was significantly higher compared to the other two diets (Table 1) (One Way ANOVA: $F_{(2,12)}$=15.50, P=0.0005, df=14). There was also a trend in the increase of larval period duration ($F_{(2,12)}$=12.00, P=0.0014, df=14). This was particularly evident for the larvae reared on Fish diet, that showed doubled developmental time and almost halved maximum weight compared to larvae grown on Standard diet (Table 1).

Evaluation of relative bacterial counts in the different regions of BSF larval midgut. The bacterial loads in different midgut regions of *H. illucens* larvae (Fig.1C) were determined by qRT-PCR on RNA samples in order to narrow in the analysis on live bacteria. The results demonstrate that the profile of the relative bacterial counts in the different midgut regions was similar for the three diets. In particular, while anterior and middle midgut had comparable bacterial loads, they were higher in the posterior portion (Fig. 2) (One Way ANOVA: Standard $F_{(2,12)}$= 8.869, n=5, P=0.0043, df=14; Veg Mix $F_{(2,12)}$= 295.51, n=5, P<0.0001, df=14; Fish $F_{(2,12)}$= 33.882, n=5, P<0.0001, df=14). We observed a statistically significant interaction between the effects of diet and midgut region on bacterial load ($F_{(4,36)}$=17.601, P<0.0001) which was significantly affected from both the considered independent variables (diet: $F_{(2,36)}$=23.339, P<0.0001; midgut region: $F_{(2,36)}$=137.170, P<0.0001).

Microbiota composition in the different regions of BSF larval midgut and diet substrates. We analyzed the microbiota by *16S rRNA* gene sequencing starting from cDNA obtained from RNA samples in order to consider communities of live bacteria. A total of 2,175,325 high quality reads were analyzed, with an average of 29,000 reads/sample. Our study included also the analysis of the microbiota of the feeding substrates prior to BSF larvae administration (fresh diet) and after BSF larvae feeding (conditioned diet). This is particularly important because BSF larvae feed and develop inside the food substrate, which is not renewed but periodically added with

fresh one. The anterior part of the midgut was always characterized by a high microbial diversity (P<0.05), that progressively decreased going from the anterior to the posterior part (Fig. 3), and this trend happened regardless of the diet. The microbiota of the feeding substrate showed a strong impact in shaping the midgut microbiota in larvae fed with Standard or Fish diet, at least in the first regions of the midgut (Fig. 4); by contrast, the microbiota of Veg Mix diet was not found in the midgut (Fig. 4). The posterior part always showed a significantly different microbiota when compared with middle and anterior part of the midgut, as determined by MANOVA based on Bray Curtis distance (Standard: $F_{(2,12)}$= 24.945, P<0.001; Veg Mix: $F_{(2,12)}$= 46.287, P<0.001; Fish: $F_{(2,12)}$= 16.968, P<0.001) and the composition of the microbiota in this region reflected a strong selection of the species that were present in the food substrate, an aspect of particular extent for Fish diet (Fig. 4 and 5). The composition of the microbiota determined a clear differentiation of the samples according to both midgut portion and diet (Fig. 5). Indeed, a significant effect of both diet type and midgut region was found by MANOVA, for both the independent variables (diet: $F_{(2,36)}$=57.047, P<0.001; midgut region: $F_{(2,36)}$=39.256, P<0.001) and for the interaction between them ($F_{(4,36)}$=19.540, P<0.001). Fish diet microbiota seemed to have the strongest effect on the gut microbiota, leading to a higher abundance of Proteobacteria taxa in the posterior tract of the midgut, while Firmicutes prevailed in the anterior and middle tract (Fig. 4A). On the contrary, the midgut of BSF larvae fed with Standard and Veg Mix diets were more similar and characterized by higher levels of Bacteroidetes (Fig. 4). Indeed, the midgut of larvae fed with Fish diet showed significantly higher weighted Unifrac distance from Standard and Veg Mix diets, compared to the distance of between Standard and Veg Mix, in all the three portions (Fig. S1). Although the larvae feed and develop into the diet, the data show that BSF larvae do not significantly alter microbiota composition of the substrate, except for an increase in *Lactobacillus* population in Veg Mix diet (Fig. 4B). A complete list of the taxa identified is reported in Supplementary Tables S1, S2, S3, S4.

DISCUSSION

Despite the great and exponentially increasing interest in BSF larvae for bioconversion (4-6) and bioremediation (32), several aspects concerning the biology of this insect are still neglected. Surprisingly, there is still paucity of information on its intestinal microbiota (11), an issue that should be instead considered a priority for an organism that can be used for such purposes. A recent review on the microbial community associated to BSF (11) highlights knowledge gaps and provides suggestions on criticisms to unravel, rather than presenting a summary of the available data.

Firstly, none of the few studies on BSF intestinal microbiota has taken into account the correlation between the different regions of the midgut of this insect and the microbiota. In this paper we provide evidence that discrete regions can be recognized along the midgut of BSF larvae as clearly demonstrated by the differences in the luminal pH (Fig. 1). Anterior region is characterized by an acid luminal content, followed by a strongly acidic middle region and an alkaline posterior tract. These data are partially in accordance with previous reports on non-hematophagous brachycerous Diptera. Indeed, in the larvae of *Musca domestica* (Diptera: Muscidae) three main segments can be identified: the anterior and the posterior midgut are characterized by a slightly acidic luminal pH, while the middle midgut presents a very low pH in the lumen (21) that is generated by the so called “copper cells”, a distinctive cell type present in the acidic segment of the midgut of flies (23, 33-35). The midgut of *D. melanogaster* larvae presents distinct regions as well (23, 35) with different pH of the luminal content: the anterior segment and the anterior part of the posterior segment is between neutral to mild alkalinity, while the middle segment is highly acidic and the posterior part of the posterior segment is highly alkaline (23). The differences of the pH in fly midgut regions are associated to peculiar physiological, immune and microbiological features (22, 26-28, 30).

Here we demonstrate that in BSF larvae the presence of different midgut regions associates to differences in microbial density and composition. We have observed that each tract is characterized by a different bacterial load, which is higher in the posterior compared to the anterior midgut. Interestingly, microbial diversity has an opposite trend, since it gradually decreases along

the midgut, suggesting that a selection of fewer taxa takes place. A simple explanation may be a reduced flow rate of luminal content to the posterior region due to the possible presence of sphincters or epithelium folding. In alternative or in addition, most bacteria are killed in the anterior and middle region and only a selection of the initial microbiota proliferate in the posterior midgut using the available nutrients, thus leading to higher numbers. This process of selection may result by the fine combination of extreme pH values in the middle region of the midgut and the activity of antimicrobial peptides, lysozyme and digestive enzymes produced and secreted by midgut cells into the lumen of anterior and middle midgut (17, 21, 27, 36, 37).

To understand whether and how food affects the microbial communities that colonize the digestive tract of BSF larvae, we have examined dietary substrates that strongly differ in terms of nutrient composition. In particular, the three diets were characterized by a different protein/carbohydrate ratio, a parameter that has been demonstrated to impact on the gut microbiota (38-40) and insect performances (41-43). Indeed, we detected differences in BSF larvae development on the different diets. A major novelty introduced by our study is the characterization of the microbiota of the dietary substrates, an aspect that was previously overlooked (11) and that could strongly affect the composition of the bacterial community of the midgut. In addition, we studied the influence of feeding activity of BSF larvae on dietary substrates. A comparative analysis of the results shows that diet composition plays a major role in shaping the diversity of the midgut microbiota. Similarly, the microbiota present in the diet influences the composition of the microbiota resident in the anterior/middle tracts of the midgut and less the one occurring in the posterior that presented a very narrow selection of the species in the food substrate. Interestingly, BSF larvae do not have detrimental effects on the microbiota of the substrates on which they feed and develop. They are not able to significantly change the bacterial community of the Standard and Fish diet substrates, and, although an increase of a specific population (i.e. *Lactobacillus*) occurs in Veg Mix substrate, these bacteria are known as non-pathogenic for their potential probiotic properties for humans (44-46) and some species are involved in detoxification of pesticides and

xenobiotics in humans and insects (47-50). This evidence is in contrast with previous claims about the capacity of BSF larvae to change the microbiota of substrates and, in particular, to reduce pathogenic bacteria of substrates (1, 11), but is a valuable trait for an organism that has to be mass-reared for bioconversion and bioremediation on a variety of substrates.

The differences found in the microbiota of larvae fed on different diets could reflect their physiological performances and bioconversion efficiency, and the posterior midgut, where the resident microbiota results from a selection of microbes present in previous midgut tracts, may have a relevant contribution in nutrient conversion and thus in energy harvest and overall fitness. Standard and Veg Mix diets were associated to an overall similar microbiota composition, both leading to increased levels of Bacteroidetes in the midgut, bacteria known as glycan degraders because of the presence of polysaccharide utilization loci in their genome (51). Genera of *Sphingobacterium* and *Dysgonomonas* were particularly abundant, likely reflecting a remarkable potential for complex polysaccharide degradation, and worthy to be isolated and explored for biotechnological purposes. Bacteroidetes have been identified as core members of the gut microbiome in many *Drosophila* species across the globe and also in other insects, including termites and honeybees (52), and several have xylanases directly involved in hemicellulose digestion (53, 54). On the other hand, Fish diet apparently induces a more putrefactive environment, with a microbiota severely dominated by Proteobacteria (Fig. 4A), mainly *Providencia* (Fig. 4B), which are highly transmitted vertically throughout insect life cycle (11) but can also be pathogens of many organisms including humans and insects (55). On the basis of the above consideration, Fish diet may induce a gut dysbiosis which may contribute to the reduced performance that we detected for BSF larvae reared on Fish diet compared to the other two feeding substrates. These data, along with a previous study performed on the same insect (7), suggest that unbalanced diets with a high protein/carbohydrate ratio content are not optimal for BSF larvae rearing.

Despite the great potential of *H. illucens* larvae (see Introduction for details), information on its microbiota is surprisingly very limited. Apart from a recent study on mycobiota (56), only two

266 studies have previously examined the microbiota of *H. illucens* larvae. In the first study (19, Table
267 2) the microbiota of the entire gut from larvae reared on three different feeding substrates were
268 investigated. In the second one (20, Table 2) the microbiota analysis was performed on whole
269 larvae. The differences in the experimental samples analyzed make it difficult to compare the
270 results from those studies and, for the same reason, results from previous studies and the present.
271 Moreover, both studies completely overlooked the bacteria communities present in the feeding
272 substrates, that we demonstrated can affect midgut microbiota composition. Nevertheless, as
273 summarized in Table 2, a few considerations can be done. In Zheng et al. (20), larvae were reared
274 on a diet with a composition very similar to Standard diet used in this study and the major Phyla
275 that characterize the microbiota quite match (both considering each midgut tract separately or the
276 average value of the different tracts). This evidence, along with the differences associated to the
277 microbiota of larvae reared on different substrates, suggests that diet composition had a role in
278 shaping bacterial communities. In particular, when diets were very unbalanced (i.e. cooked rice and
279 Fish diet) the diversity of microbial communities decreased compared to nutritionally more
280 balanced diets. In those unbalanced diets Proteobacteria were the major group identified, whereas in
281 all other cases Bacterioidetes were one of the dominant Phyla (Table 2). Interestingly, our data
282 (Table 2) demonstrate that the overall gut microbiota does not mirror the microbiota composition of
283 each tract, confirming the relevance of working with each tract separately.

284 Our study focused on the effect of midgut morphofunctional regionalization in shaping the
285 residing microbiota. Future work on microbiota in the hindgut of *H. illucens* larvae is also needed,
286 although the establishment of a stable bacterial community in the hindgut of insect larvae is
287 problematic (due to the molts during the larval period that involve the removal of the cuticle lining
288 the hindgut epithelium) and often requires the presence of special structures that provides a stable
289 environment for bacterial colonization (57), structures that have never been reported for *H. illucens*
290 larvae.

In conclusion, the presence of different midgut domains, diet composition and diet microbiota have a non-negligible effect on BSF larvae microbial ecology. These factors and their interdependence are going to play a major role for a proper exploitation of the biotechnological uses of insects.

MATERIALS AND METHODS

Insect rearing. BSF eggs were collected from a colony established in 2015 at the University of Insubria (Varese, Italy), and maintained in a humid chamber at 27°C until hatching. The eggs were laid on a Petri dish (9×1.5 cm) with the experimental diet. Three diets were used in the current study: standard diet for Diptera (Standard), a diet containing fruits and vegetables (Veg Mix), and a diet based on fish feed (Fish). Standard diet (31), was composed by wheat bran (50%), corn meal (30%) and alfalfa meal (20%) mixed in the ratio 1:1 dry matter/water (approximately 13% protein, protein/carbohydrate ratio 1:1). Veg Mix diet was composed by seven fruits and vegetables (apple, banana, pear, broccoli, zucchini, potato and carrot) in equal quantity and appropriately minced (approximately 1% protein, protein/carbohydrate ratio 1:9). Fish diet was composed by fish meal (FF type, Mazzoleni SpA, Bergamo, Italy), mixed in the ratio 1:1 dry matter/water (approximately 35% protein, no carbohydrates). Percentages are calculated on diet weight, including water. The values in parenthesis concerning protein and carbohydrate content were estimated on data available on the web for Standard and Veg Mix diet, whereas for Fish diet they were reported in the product data sheet. Nipagin (Methyl 4-hydroxybenzoate) was added to the diet administered to larvae the first 4 days after hatching to avoid mold growth (a 18% (w/v) stock solution in absolute ethanol was prepared; each gram of Veg Mix diet was added with 20 µl of this stock solution, whereas each gram of Standard and Fish diet was added with 1 ml of a 1.7% (v/v) dilution in water of the stock solution). Four days after hatching, 300 larvae were placed in a plastic container (16×16×9 cm), and fed *ad libitum* with the three experimental diets described above without nipagin. The larvae were maintained at 27.0 ± 0.5 °C, 70 ± 5% RU, in the dark. Fresh diet was added every two days, until

317 larvae reached the last larval instar. Five independent rearing groups were set up for each diet.
318 Random samples of 30 individuals were weighed every two days. For each experimental diet, the
319 sampling and the annotation of the larval weight were made in triplicate. Before weighing, the
320 larvae were washed in tap water to remove diet matter from their body and then wipe dried. The
321 weights were recorded until 25% of insects reached the pupal stage. Last instar, actively feeding
322 larvae were used for the measurement of midgut lumen pH and microbiota analyses.

323 **Determination of pH in the midgut lumen with colorimetric indicators.** The presence of
324 different pH in the midgut lumen of *H. illucens* larvae was assessed using phenol red and
325 bromophenol blue, two pH indicators that assume different coloration at different pH values.
326 Bromophenol blue is yellow at pH values lower than or equal to 3.0, blue at pH higher than or equal
327 to 4.6; phenol red is yellow at pH lower than or equal to 6.8, fuchsia at pH higher than or equal to
328 8.2, with a gradual color transition for intermediate values. *H. illucens* larvae were fed *ad libitum*
329 with Standard diet until they reached the last instar as described above. Larvae with a weight
330 ranging between 180 and 200 mg were selected and transferred to plastic containers on Standard
331 diet added with 0.2% (w/w) bromophenol blue or phenol red. After 24 h the larvae were removed
332 from the diet, placed in a plastic tube, and anesthetized on ice with CO_2. The guts were isolated and
333 the coloration of the midgut content was evaluated by means of a stereomicroscope.

334 **Collection of midgut and diet samples and RNA extraction.** Last instar larvae were
335 washed with 70% ethanol in autoclaved distilled water and then dissected with the help of a
336 stereomicroscope, under a horizontal-flow hood, by using sterile tweezers and scissors, to avoid
337 cross-contaminations of the samples. Each midgut was isolated in autoclaved 1× PBS (Phosphate
338 Buffered Saline: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4) in a
339 sterile Petri dish (5.5×1.3 cm). Once collected, the midgut was divided into three districts: anterior,
340 middle, and posterior region (see Results and Fig.1). For the dissection of each larva a new Petri
341 dish was used, and tweezers and scissors were washed with 70% ethanol in water. For each diet,
342 pools of five midgut regions samples for each of the five replicates of insect rearing were collected

in a cryovial, immediately put into TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and kept at -80°C until total RNA extraction that was performed according to the manufacturer's instructions. Briefly, after homogenization with eppendorf-fitting pestles to lyse samples in TRIzol reagent, total RNA was precipitated with isopropanol, washed with ethanol, and suspended in RNase-free water. Samples of fresh (before administration to larvae) and conditioned diets (on which larvae have fed) were also immediately put into TRIzol reagent and kept at -80°C until total RNA extraction. Ten samples of both fresh and conditioned diets were collected for each of the 5 experimental replicates on the 3 different feeding substrates.

RNA concentration was assessed by measuring the absorbance at 280 nm, with a Varioskan™ Flash Multimode Reader (Thermo Scientific, Waltham, MA, USA), and sample purity was evaluated by assessing 260/280 nm absorbance ratio. Total RNA preparations were then treated with TURBO DNase I (Life Technologies), according to the manufacturer's instructions and RNA quality was checked by electrophoresis on 1% agarose gel.

qRT-PCR for relative bacterial load determination. Total RNA was isolated as described above. The relative bacterial load in the three midgut regions (n=5 for each sampling point containing pools of 5 midgut portions each), was quantified by normalization of the relative expression of the *16S rRNA* gene (accession number SRP064613; *16S rRNA* forward primer: ACTCCTACGGGAGGCAGC, *16S rRNA* reverse primer: ATTACCGCGGCTGCTGGC) to that of the ribosomal protein L5 gene of *H. illucens* (*Hi RPL5*). The primers used for *Hi RPL5* (*Hi RPL5* forward primer: AGTCAGTCTTTCCCTCACGA, *Hi RPL5* reverse primer: GCGTCAACTCGGATGCTA) were designed on conserved regions of *RPL5* in other insect species and their sequence checked by sequencing the PCR product. Changes in relative bacterial loads were measured by one-step qRT-PCR (58-60), using the SYBR Green PCR Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's instructions, using the primers reported above. Relative gene expression data were analyzed using the $2^{\Delta\Delta CT}$ method (61-63). Expression data were normalized taking into account the differences in the area of the cross-section

of the different intestinal tracts (81,000 ± 7,300 µm^2, 250,000 ± 17,200 µm^2 and 46,000 ± 1,700 µm^2 for the anterior, middle and posterior midgut, respectively, n=10 for each tract) by dividing the Ct values (for both *16S rRNA* and *Hi RPL5* transcripts) by the area of the cross-section of the corresponding midgut tract. The areas were calculated using the diameter of the lumen of each midgut tract obtained by direct measurement on the micrographs of different cross-sections acquired from semithin cross- sections of BSF larval midguts stained with crystal violet and basic fuchsin, prepared for light microscopy analysis (64). For validation of the ΔΔCt method the difference between the Ct value of *16S rRNA* and the Ct value of *Hi RPL5* transcripts [ΔCt = Ct(*16S rRNA*)-Ct (*Hi RPL5*)] was plotted versus the log of two-fold serial dilutions (200, 100, 50, 25 and 12.5 ng) of the purified RNA samples. The plot of log total RNA input versus ΔCt displayed a slope lower than 0.1 (Y=1.3895 - 0.0137X, R^2=0.0566), indicating that the efficiencies of the two amplicons were approximately equal.

Analysis of the microbiota and bioinformatics of the *16S rRNA* gene sequencing data. After extraction, 400 ng of RNA were reverse-transcribed into cDNA with random primers using RETROscript (Life Technologies), according to the manufacturer's instructions. The midgut microbiota was assessed by sequencing of the amplified V3-V4 region of the *16S rRNA* gene as recently described (65). Demultiplexed, forward and reverse reads were joined by using FLASH (66). Joined reads were quality trimmed (Phred score < 20) and short reads (< 250 bp) were discarded by using Prinseq (67). High quality reads were then imported in QIIME1 (68). Operational Taxonomic Units (OTUs) were picked through *de novo* approach and uclust method and taxonomic assignment was obtained by using the RDP classifier and the Greengenes database (69), following a pipeline previously reported (65). To avoid biases due to different sequencing depth, OTU tables were rarefied to the lowest number of sequences per sample. Statistical analyses and visualization were carried out in R environment (https://www.r-project.org). Alpha-diversity analysis was carried out in QIIME on rarefied OTU tables. Kruskal-Wallis and pairwise Wilcoxon tests were used to determine significant differences in alpha diversity parameters, weighted Unifrac

distance or in OTU abundance. Permutational Multivariate Analysis of Variance (non-parametric MANOVA) based on Bray Curtis distance matrix was carried out to detect significant differences in the overall microbial community composition among the different parts of the midgut or as affected by the type of diet, by using the *adonis* function in R *vegan* package.

The *16S rRNA* gene sequences produced in this study are available at the Sequence Read Archive (SRA) of the National Centre for Biotechnology Information (NCBI), under accession number SRP064613.

Statistical analysis. Data were analyzed using Prism (GraphPad Software Inc. version 6.0b, San Diego, CA, USA) software using One-Way ANOVA with Tukey's multiple comparison test to compare bacterial load and parameters of larval performances within any single diet treatment. Two-Way ANOVA analysis followed by Bonferroni's post-hoc tests, when significant effects were observed (P value<0.05), was carried out on bacterial load as affected by different diet treatment and different midgut trait. When necessary transformation of data was carried out, to meet assumptions of normality. Levene's test was carried out to test the homogeneity of variance.

REFERENCES

1. Wang YS, Shelomi M (2017) Review of Black Soldier Fly (*Hermetia illucens*) as animal feed and human food. Foods. 6: 91. doi:10.3390/foods6100091.

2. Lord WD, Goff ML, Adkins TR, Haskell NH (1994) The black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) as a potential measure of human postmortem interval: observations and case histories. J Forensic Sci. 39: 215-22.

3. Turchetto M, Lafisca S, Costantini G (2001) Postmortem interval (PMI) determined by study sarcophagous biocenoses: three cases from the province of Venice (Italy). Forensic Sci Int. 120: 28-31.

422 4. Čičková H, Newton GL, Lacy RC, Kozánek M (2015) The use of fly larvae for organic waste treatment.
423 Waste Manag. 35: 68-80.
424
425 5. Makkar HPS, Tran G, Heuzé V, Ankers P (2014) State-of-the-art on use of insects as animal feed. Anim
426 Feed Sci Technol. 197:1-33. doi.org/10.1016/j.anifeedsci.2014.07.008.
427
428 6. Müller A, Wolf D, Gutzeit HO (2017) The black soldier fly, *Hermetia illucens* - a promising source for
429 sustainable production of proteins, lipids and bioactive substances. Z Naturforsch. 72(9-10)c: 351-363.
430 doi:10.1515/znc-2017-0030
431
432 7. Cammack JA, Tomberlin JK (2017). The impact of diet protein and carbohydrate on select life-history
433 traits of the black soldier fly *Hermetia illucens* (L.) (Diptera: Stratiomyidae). Insects 8: 56.
434 doi.org/10.3390/insects8020056.
435
436 8. Harnden LM, Tomberlin JK (2016) Effects of temperature and diet on black soldier fly, *Hermetia illucens*
437 (L.) (Diptera: Stratiomyidae), development. Forensic Sci Int. 266: 109-116. doi:
438 10.1016/j.forsciint.2016.05.007.
439
440 9. Jucker C, Erba D, Leonardi MG, Lupi D, Savoldelli S (2017) Assessment of Vegetable and Fruit
441 Substrates as Potential Rearing Media for *Hermetia illucens* (Diptera: Stratiomyidae) Larvae. Environ
442 Entomol. 46: 1415-1423. doi: 10.1093/ee/nvx154.
443
444 10. Ma J, Lei Y, Rehman KU, Yu Z, Zhang J, Li W, Li Q, Tomberlin JK, Zheng L (2018) Dynamic Effects
445 of Initial pH of Substrate on Biological Growth and Metamorphosis of Black Soldier Fly (Diptera:
446 Stratiomyidae). Environ Entomol. 47: 159-165. doi: 10.1093/ee/nvx186.
447

448 11. De Smet J, Wynants E, Cos P, Van Campenhout L (2018) Microbial community dynamics during rearing
449 of black soldier fly larvae (*Hermetia illucens*) and impact on exploitation potential. Appl Environ Microbiol.
450 84:e02722-17. doi.org/10.1128/AEM.02722-17.
451
452 12. Rehman KU, Cai M, Xiao X, Zheng L, Wang H, Soomro AA, Zhou Y, Li W, Yu Z, Zhang J (2017)
453 Cellulose decomposition and larval biomass production from the co-digestion of dairy manure and chicken
454 manure by mini-livestock (*Hermetia illucens* L.). J Environ Manage. 196: 458-465.
455
456 13. Erickson MC, Islam M, Sheppard C, Liao J, Doyle MP (2004) Reduction of *Escherichia coli* O157:H7
457 and *Salmonella enterica* serovar *Enteritidis* in chicken manure by larvae of the black soldier fly. J Food Prot.
458 67: 685-690.
459
460 14. Liu Q, Tomberlin JK, Brady JA, Sanford MR, Yu Z (2008) Black soldier fly (Diptera: Stratiomyidae)
461 larvae reduce *Escherichia coli* in dairy manure. Environ Entomol. 37: 1525-1530.
462
463 15. Lalander C, Diener S, Magri ME, Zurbrügg C, Lindström A, Vinnerås B (2013) Faecal sludge
464 management with the larvae of the black soldier fly (*Hermetia illucens*)-from a hygiene aspect. Sci Total
465 Environ. 458-460: 312-318.
466
467 16. Lalander C, Fidjeland J, Diener S, Eriksson S, Vinnerås B (2015) High waste-to-biomass conversion and
468 efficient Salmonella spp. reduction using black soldier fly for waste recycling. Agron Sustain Dev. 35: 261-
469 271. doi:10.1007/s13593-014-0235-4
470
471 17. Broderick NA (2016) Friend, foe or food? Recognition and the role of antimicrobial peptides in gut
472 immunity and *Drosophila*-microbe interactions. Phil Trans R Soc B. 371: 20150295.
473 doi:10.1098/rstb.2015.0295
474

475 18. Zdybicka-Barabas A, Bulak P, Polakowski C, Bieganowski A, Waśko A, Cytryńska M (2017) Immune
476 response in the larvae of the black soldier fly *Hermetia illucens*. ISJ 14: 9-17.
477
478 19. Jeon H, Park S, Choi J, Jeong G, Lee S-B, Choi Y, Lee S-J (2011) The intestinal bacterial community in
479 the food waste-reducing larvae of *Hermetia illucens*. Curr Microbiol. 62: 1390-1399.
480 doi.org/10.1007/s00284-011-9874-8.
481
482 20. Zheng L, Crippen TL, Singh B, Tarone AM, Dowd S, Yu Z, Wood TK, Tomberlin JK (2013) A survey
483 of bacterial diversity from successive life stages of black soldier fly (Diptera: Stratiomyidae) by using 16S
484 rDNA pyrosequencing. J Med Entomol. 50: 647- 658. doi.org/10.1603/ME12199.
485
486 21. Lemos FJA, Terra WR (1991) Digestion of bacteria and the role of midgut lysozyme in some insect
487 larvae. Comp Biochem physiol. 100B: 265-268.
488
489 22. Pimentel AC, Barroso IG, Ferreira JMJ, Dias RO, Ferreira C, Terra WR (2018) Molecular machinery of
490 starch digestion and glucose absorption along the midgut of *Musca domestica*. J Insect Physiol. 109: 11-20.
491 23. Shanbhag S, Tripathi S (2009) Epithelial ultrastructure and cellular mechanisms of acid and base
492 transport in the *Drosophila* midgut. J Exp Biol. 212: 1731-1744.
493
494 24. Buchon N, Osman D (2015) All for one and one for all: regionalization of the *Drosophila* intestine.
495 Insect Biochem Mol Biol. 67: 2-8. doi: 10.1016/j.ibmb.2015.05.015.
496
497 25. Lehane M, Billingsley P (1996) Biology of the insect midgut. Lehane and Billingsley Eds.
498
499 26. Terra W (1990) Evolution of digestive systems in insects. Annual Rev Entomol. 35: 181-200.
500
501 27. Broderick NA, Buchon N, Lemaitre B (2014) Microbiota-induced changes in *Drosophila melanogaster*
502 host gene expression and gut morphology. mBio. 5: e01117-14. doi:10.1128/mBio.01117-14

503

504 28. Buchon N, Osman D, David FPA, Fang HY, Boquete JP, Deplancke B, Lemaitre B (2013)
505 Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*. Cell
506 Rep. 3: 1725-1738.

507

508 29. Capo F, Charroux B, Royet J (2016) Bacteria sensing mechanisms in Drosophila gut: Local and systemic
509 consequences. Dev Comp Immunol. 64: 11-21. doi: 10.1016/j.dci.2016.01.001.

510

511 30. Marianes A, Spradling AC (2013) Physiological and stem cell compartmentalization within the
512 *Drosophila* midgut. eLife. 2:e00886. DOI: 10.7554/eLife.00886.

513

514 31. Hogsette JA (1992) New diets for production of house-flies and stable flies (Diptera, Muscidae) in the
515 laboratory. J Econ Entomol. 85: 2291-2294.

516

517 32. Bulak P, Polakowski C, Nowak K, Waśko A, Wiącek D, Bieganowski A (2018) *Hermetia illucens* as a
518 new and promising species for use in entomoremediation. Sci Total Environ. 633: 912-919.

519 33. Terra WR, Espinoza-Fuentesa FP, Ribeiro F, Ferreira C (1988) The larval midgut of the housefly (*Musca*
520 *domestica*): ultrastructure, fluid fluxes and ion secretion in relation to the organization of digestion. J Insect
521 Physiol. 34: 463-472.

522

523 34. McNulty M, Puljung M, Jefford G, Dubreuil RR (2001) Evidence that a copper-metallothionein complex
524 is responsible for fluorescence in acid-secreting cells of the *Drosophila* stomach. Cell Tissue Res. 304: 383-
525 389. doi:10.1007/s004410100371

526

527 35. Dubreuil RR (2004) Copper cells and stomach acid secretion in the *Drosophila* midgut. Int J Biochem
528 Cell Biol. 36: 745-752.

529

530 36. Lemos FJA, Ribeiro AF, Terra WR (1993) A bacteria-digesting midgut-lysozyme from *Musca domestica*
531 (diptera) larvae. Purification, properties and secretory mechanism. Insect Biochem Mol Biol. 23: 533-541.
532
533 37. Vogel H, Müller A, Heckel DG, Gutzeit H, Vilcinskas A (2018) Nutritional immunology: diversification
534 and diet-dependent expression of antimicrobial peptides in the black soldier fly *Hermetia illucens*. Dev
535 Comp Immunol. 78: 141-148.
536
537 38. Fan W, Tang Y, Qu Y, Cao F, Huo G (2014) Infant formula supplemented with low protein and high
538 carbohydrate alters the intestinal microbiota in neonatal SD rats. BMC Microbiol. 14: 279. doi:
539 10.1186/s12866-014-0279-2.
540
541 39. Rothe M, Blaut M (2013) Evolution of the gut microbiota and the influence of diet. Benef Microbes. 4:
542 31-37. doi: 10.3920/BM2012.0029
543
544 40. Zhao J, Zhang X, Liu H, Brown MA, Qiao S (2018) Dietary protein and gut microbiome composition
545 and function. Curr Protein Pept Sci. doi:10.2174/1389203719666180514145437.
546
547
548 41. Behmer ST (2009) Insect herbivore nutrient regulation. Annu Rev Entomol. 54: 165-87.
549
550 42. Raubenheimer D, Simpson SJ (1997) Integrative models of nutrient balancing: application to insects and
551 vertebrates. Nutr Res Rev. 10: 151-179.
552
553 43. Ponton F, Wilson K, Holmes AJ, Cotter SC, Raubenheimer D, Simpson SJ (2013) Integrating nutrition
554 and immunology: a new frontier. J Insect Physiol. 59: 130-137.
555
556 44. Azad AK, Sarker M, Li T, Yin J (2018) Probiotic species in the modulation of gut microbiota: an
557 overview. BioMed Res Int. 2018: ID 9478630.

45. Gareau MG, Sherman PM, Walker WA (2010) Probiotics and the gut microbiota in intestinal health and disease. Nat Rev Gastroenterol Hepatol. 7: 503-514.

46. Sri Vinusha K, Deepika K, Sudhakar Johnson T, Agrawal GK, Rakwal R (2018) Proteomic studies on lactic acid bacteria: a review. Biochem Biophys Rep. 14: 140-148.

47. Chen SW, Hsu JT, Chou YA, Wang HT (2018) The application of digestive tract lactic acid bacteria with high esterase activity for zearalenone detoxification. J Sci Food Agric. 98: 3870-3879. doi: 10.1002/jsfa.8904.

48. Daisley BA, Trinder M, McDowell TW, Collins SL, Sumarah MW, Reid G (2018) Microbiota-mediated modulation of organophosphate insecticide toxicity by species-dependent interactions with *Lactobacilli* in a *Drosophila melanogaster* insect model. Appl Environ Microbiol. 84: e02820-17. doi: 10.1128/AEM.02820-17.

49. Trinder M, Bisanz JE, Burton JP, Reid G (2015) Probiotic *lactobacilli*: a potential prophylactic treatment for reducing pesticide absorption in humans and wildlife. Benef Microbes. 6: 841-847. doi: 10.3920/BM2015.0022.

50. Trinder M, McDowell TW, Daisley BA, Ali SN, Leong HS, Sumarah MW, Reid G (2016) Probiotic *Lactobacillus rhamnosus* reduces organophosphate pesticide absorption and toxicity to *Drosophila melanogaster*. Appl Environ Microbiol. 82:6204-6213.

51. Gibiino G, Lopetuso LR, Scaldaferri F, Rizzatti G, Binda C, Gasbarrini A (2018) Exploring Bacteroidetes: Metabolic key points and immunological tricks of our gut commensals. Dig Liver Dis. 50: 635-639. doi: 10.1016/j.dld.2018.03.016.

586 52. Poff KE, Stever H, Reil JB, Seabourn P, Ching AJ, Aoki S, Logan M, Michalski JR, Santamaria J,
587 Adams JW, Eiben JA, Yew JY, Ewing CP, Magnacca KN, Bennett GM (2017) The native Hawaiian insect
588 microbiome initiative: a critical perspective for Hawaiian insect evolution. Insects. 8: 130.
589
590 53. Zhou J, Huang H, Meng K, Shi P, Wang Y, Luo H, Yang P, Bai Y, Zhou Z, Yao B (2009) Molecular and
591 biochemical characterization of a novel xylanase from the symbiotic *Sphingobacterium* sp TN19. Appl
592 Microbiol Biotechnol. 85: 323-333.
593
594 54. Arias-Cordero E, Ping L, Reichwald K, Delb H, Platzer M, Boland W (2012) Comparative evaluation of
595 the gut microbiota associated with the below- and above-ground life stages (Larvae and Beetles) of the
596 Forest Cockchafer, *Melolontha hippocastani.* PLoS ONE. 7: e51557. doi:10.1371/journal.pone.0051557.
597
598 55. Galac MR, Lazzaro BP (2011) Comparative pathology of bacteria in the genus *Providencia* to a natural
599 host, *Drosophila melanogaster*. Microbes Infect. 13: 673-683.
600
601 56. Varotto Boccazzi I, Ottoboni M, Martin E, Comandatore F, Vallone L, Spranghers T, Eeckhout M,
602 Mereghetti V, Pinotti L, Epis S (2017) A survey of the mycobiota associated with larvae of the black soldier
603 fly (*Hermetia illucens*) reared for feed production. PLoS ONE 12(8):e0182533.
604 https://doi.org/10.1371/journal.pone.0182533
605
606 57. Engel P, Moran NA (2013) The gut microbiota of insects - diversity in structure and function. FEMS
607 Microbiol Rev. 37: 699-735.
608
609 58. Caccia S, Di Lelio I, La Storia A, Marinelli A, Varricchio P, Franzetti E, Banyuls N, Tettamanti G,
610 Casartelli M, Giordana B, Ferrè J, Gigliotti S, Ercolini D, Pennacchio F (2016) Midgut microbiota and host
611 immunocompetence underlie *Bacillus thuringiensis* killing mechanism. PNAS USA. 113: 9486-9491.
612

613 59. Renoz F, Noël C, Errachid A, Foray V, Hance T (2015) Infection dynamic of symbiotic bacteria in the
614 pea aphid *Acyrthosiphon pisum* gut and host immune response at the early steps in the infection process.
615 PLoS ONE. 10: e0122099. doi:10.1371/journal.pone.0122099
616
617 60. Rodgers FH, Gendrin M, Wyer CAS, Christophides GK (2017) Microbiota-induced peritrophic matrix
618 regulates midgut homeostasis and prevents systemic infection of malaria vector mosquitoes. PLoS Pathog.
619 13(5): e1006391.
620
621 61. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative
622 PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 25: 402-408.
623
624 62. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic
625 Acids Res. 29: 2002-2007.
626
627 63. Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST(C)) for group-wise
628 comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res.
629 30:e36.
630
631 64. Franzetti E, Romanelli D, Caccia S, Cappellozza S, Congiu T, Rajagopalan M, Grimaldi A, de Eguileor
632 M, Casartelli M, Tettamanti G (2015) The midgut of the silkmoth *Bombyx mori* is able to recycle molecules
633 derived from degeneration of the larval midgut epithelium. Cell Tissue Res. 361: 509-528.
634
635 65. Berni Canani R, De Filippis F, Nocerino R, Laiola M, Paparo L, Calignano A, De Caro C, Coretti L,
636 Chiariotti L, Gilbert JA, Ercolini D (2017) Specific signatures of the gut microbiota and increased levels of
637 butyrate in children treated with fermented cow's milk containing heat-killed *Lactobacillus paracasei* CBA
638 L74. Appl Environ Microbiol. 83: e01206-17.
639

640 66. Magoc T, Salzberg SL (2011) FLASH: Fast length adjustment of short reads to improve genome
641 assemblies. Bioinformatics. 27: 2957-2963.
642
643 67. Schmieder R, Edwards R (2011) Quality control and preprocessing of metagenomic datasets.
644 Bioinformatics. 27: 863-864.
645
646 68. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG,
647 Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald
648 D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T,
649 Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat
650 Methods. 7: 335-336.
651
652 69. McDonald D, Price MN, Goodrich J, Nawrocki EP, DeSantis TZ, Probst A, Andersen GL, Knight R,
653 Hugenholtz P (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary
654 analyses of bacteria and archea. ISME J. 6: 610-618.
655

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660 S.C., M.C., D.E. and G.T. designed the research; M.B., D.B., F.D.F. and I.D.L. performed
661 the experiments; F.D.F. and I.D.L. analyzed data and contributed figures and tables; S.C., M.C.,
662 D.E. and G.T. wrote the article.

663 **Tables**

664

665 **Table 1.** Length of BSF larval cycle and maximum weight at pupation* for the different diets used
666 in this study.

Diet	Larval period (days)	Maximum weight (mg)	Day of sample collection for microbiota analysis
Standard	18 ± 1 (5)[a]	218 ± 8 (5)[a]	16
Veg Mix	24 ± 2 (5)[a]	195 ± 5 (5)[b]	22
Fish	36 ± 3 (5)[b]	173 ± 3 (5)[c]	30

667 *Data are expressed as mean ± standard error, number of experiments in parenthesis. For each experiment at
668 least 20 larvae have been monitored for development time and weight. Different letters denote statistical
669 differences (One-Way ANOVA).
670

671 **Table 2.** Short summary of the data on microbiota composition of *H. illucens* larvae from present
672 work and published studies[a] (19, 20[b]).

Study	Sample	Feeding substrate	Major Phyla	%[c]
19 (Jeol et al., 2011)	larval gut	Food waste	Bacterioidetes	67.4
			Proteobacteria	18.9
			Firmicutes	9.4
			Fusobacteria	2.0
			Actinobacteria	1.9
		Cooked rice	Proteobacteria	54.0
			Firmicutes	47.3
			Unclassified	3.5
		Calf forage	Proteobacteria	31.1
			Actinobacteria	24.6
			Firmicutes	23.5
			Bacterioidetes	20.5
20 (Zheng et al., 2013)	whole larvae	Gainesville diet[d]	Bacterioidetes	54.4
			Firmicutes	20.0
			Proteobacteria	16.0
			Actinobacteria	9.0
Present study	larval midgut	Standard diet	Bacterioidetes	41.5 (A:65.9, M:54.4, P:41.1)
			Proteobacteria	28.2 (A:25.9, M:33.7, P:25.2)
			Firmicutes	13.6 (A:4.7, M:5.6, P:30.4)
			Actinobacteria	3.9 (A:3.2, M:5.3, P:3.1)
		Veg Mix	Bacterioidetes	65.4 (A:85.2, M:61.2, P:49.8)
			Proteobacteria	19.1 (A:12.2, M:28.9, P:16.2)
			Firmicutes	15.7 (A:2.0, M:28.9, P:16.2)
			Actinobacteria	11.6 (A:0.1, M:3.8, P:30.8)
		Fish diet	Proteobacteria	55.5 (A:37.1, M:30.8, P:98.6)
			Firmicutes	43.0 (A:59.1, M:68.6, P:1.4)

673 [a]In all the studies the microbiota composition was obtained by *16S rRNA* gene sequencing.
674 [b]Estimated on the basis of the histogram presented in the paper.
675 [c]Only percentages >1% are reported. For the present study the % reported is the average of the % in the three different
676 midgut portions (A: anterior, M: middle, P: posterior) that are specified in parenthesis.
677 [d]Gainesville diet is composed by 20% corn meal, 30% alfalfa meal, and 50% wheat bran, saturated with water.
678

679 **Figure legends**

680

681 **Figure 1. Determination of pH value in BSF larvae midgut lumen (A and B) and definition of**
682 **the midgut portions for the microbiota analysis (C).** In (A) and (B) the anatomy of the larval
683 BSF gut is visible. The short foregut is followed by a very long midgut. The beginning of the
684 hindgut (which extends out of the field of view) is easily recognizable by the insertion of
685 Malpighian tubules (MT), structures involved in excretion in insects and that deliver the primary
686 urine into hindgut lumen. The whole gut isolated from *H. illucens* larvae fed with diet containing
687 bromophenol blue (A) or phenol red (B) pH indicators shows the presence of different pH values
688 along the midgut lumen. (C) Image of the midgut, that is subdivided in a relatively short and thick
689 anterior midgut, a middle midgut characterized by an enlarged highly acidic portion (stomach), and
690 the posterior midgut. Bars of different color highlight the position of the cuts for the isolation of the
691 portions used for microbiota analyses. Bars: 2 mm.

692

693 **Figure 2. Relative quantification of bacterial load by qRT-PCR in the different tracts of the**
694 **midgut of BSF larvae reared on different diets.** The values reported are the mean ± standard
695 error (n=5 for each sampling point containing pools of 5 midgut portions each) of the relative
696 expression of the *16S rRNA* gene normalized to that of the *Hi RPL5* gene (see "qRT-PCR for
697 relative bacterial load determination" in Materials and Methods). Different letters denote significant
698 differences for each diet (One-Way ANOVA).

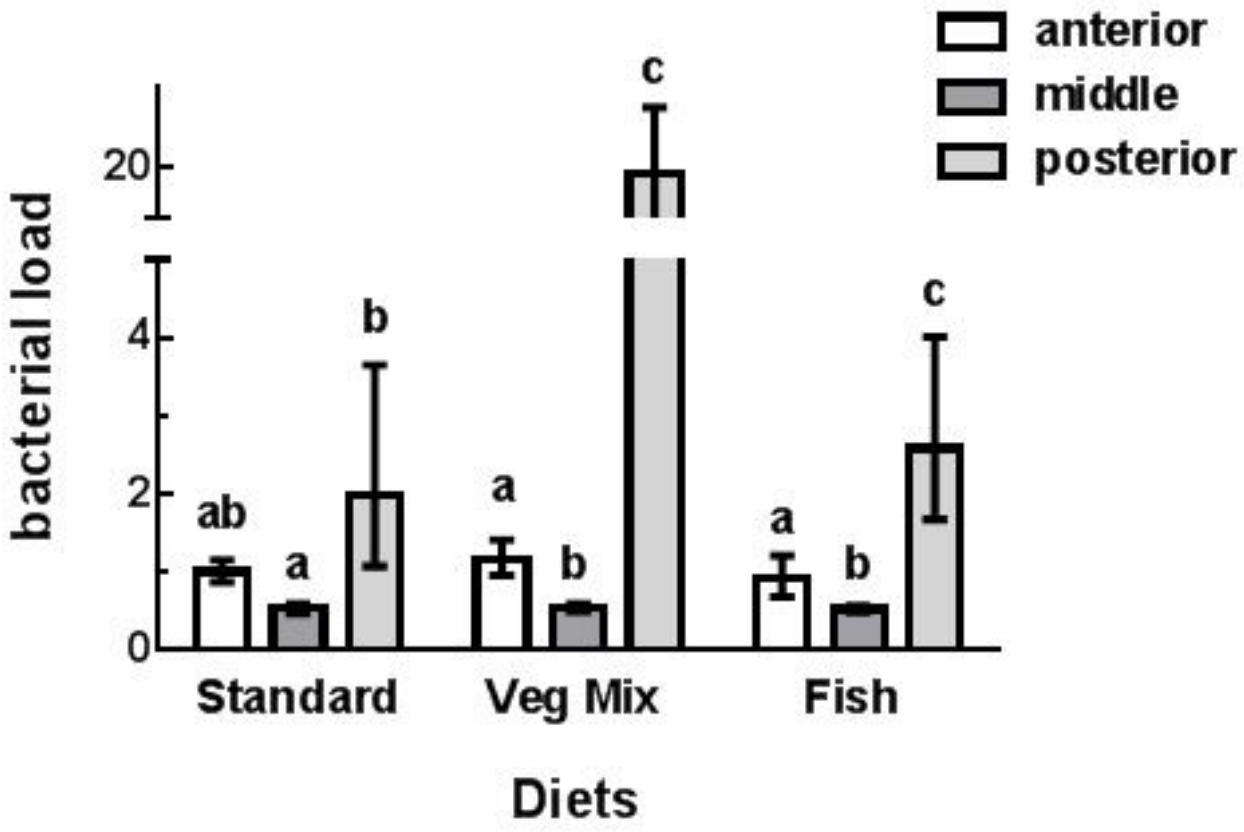
699

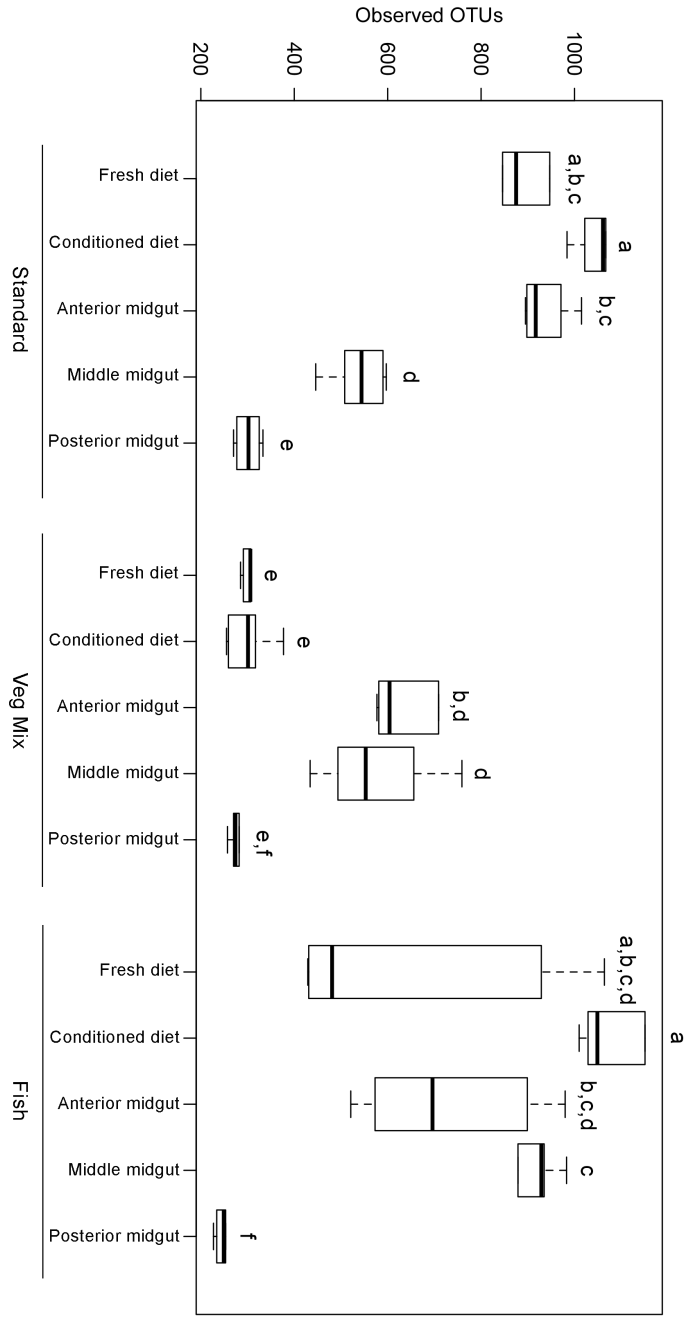
700 **Figure 3. Microbial diversity.** Box plot showing the number of Observed OTUs in the different
701 samples, as detected by high-throughput sequencing of the *16S rRNA* gene. Boxes represent the
702 interquartile range (IQR) between the first and third quartiles, and the line inside represents the
703 median (second quartile). Whiskers denote the lowest and the highest values within 1.5×IQR from
704 the first and third quartiles, respectively. Different letters indicate a significant difference (P<0.05)

as obtained by pairwise Wilcoxon's tests. "Fresh diet" and "conditioned diet" refer to the analysis of the microbiota of the feeding substrates just after preparation and after larval feeding, respectively.

Figure 4. Incidence of the major bacterial taxonomic groups. The stacked bar chart shows the relative abundance of bacterial phyla (A) and genera (B) identified in midgut and diet samples analyzed. The order of the taxa in each bar is the same provided in the legend. Values are the average of 5 replicates. Genera and phyla with abundance < 2% in at least 5 samples are summed up and showed as "others".

Figure 5. Heatplot based on microbiota composition at genus level. Hierarchical Ward-linkage clustering based on the Spearman's correlation coefficient of the microbial taxa abundance. Column bar is color-coded according to the type of diet and the midgut region. Row bar is colored according to the taxa assignment at phylum level. The color scale represents the scaled abundance of each variable, denoted as Z-score, with red indicating high abundance and blue indicating low abundance.





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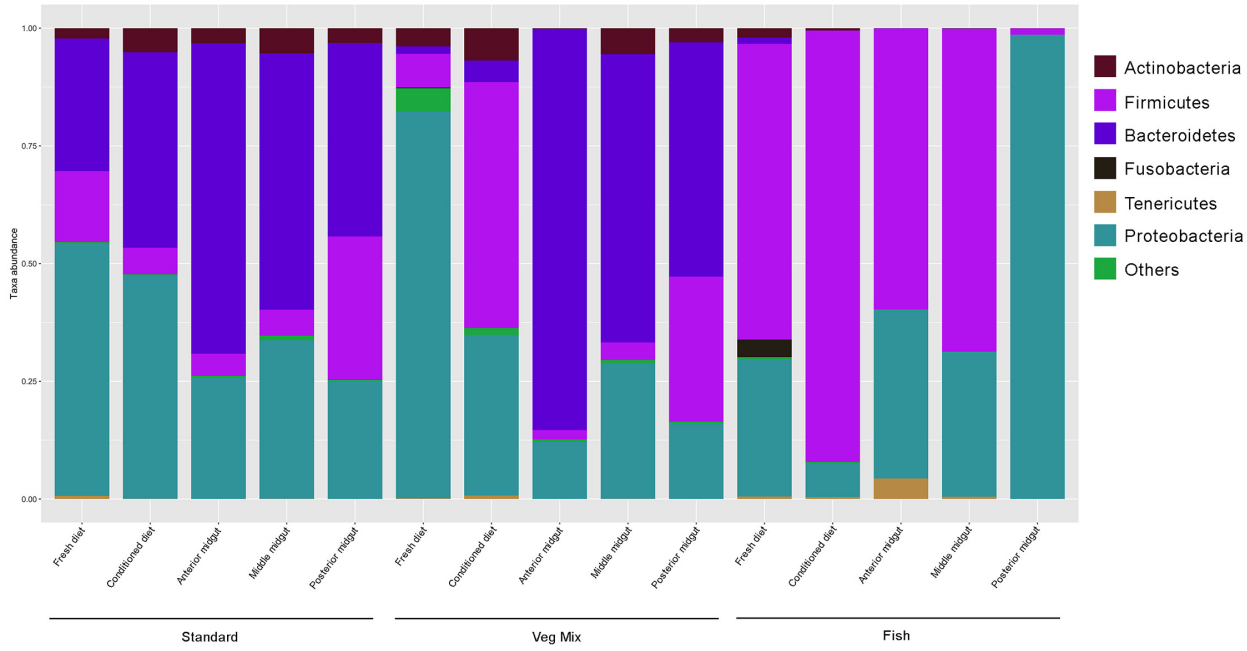

B