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Microbial influences on black soldier fly reproduction: A focus on egg surface colonization

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1 **Microbial influences on black soldier fly reproduction: A focus on egg surface colonization**

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Abstract

Background

The growing global population increases the demand for protein, while the management of organic waste is becoming more challenging. Alternative protein sources are needed to reduce the negative environmental impact of food production. Lately, the black soldier fly (BSF) has been proposed as an ideal animal protein substitute due to its ability to consume and reduce diverse organic waste, thus solving two problems at the same time. Mass-rearing of BSF depends on flourishing reproduction, which is influenced by environmental and physiological factors. BSF females oviposit egg clutches near decomposing organic matter and conspecific eggs, with studies highlighting the crucial role of microorganisms in oviposition. In this study, we focus on the surface microbiota of the egg and its origin. We investigated if the microbiota are inoculated before, during, or actively after oviposition. For this purpose, we analysed the microbiota in the haemolymph and gut of larvae raised on sterilized and non-sterilized feed, the pupal cell pulp, the wash of the egg-laying apparatus and the eggs directly collected after oviposition, the ovarian eggs and the empty female abdomen, the eggs with contact to adult BSF, and sterilized eggs to assess the stage in BSF development during which the microbial colonization of the egg surface occurs.

Results

Our analysis revealed distinct bacterial profiles across life stages, indicating a shift from larval dominance of Enterobacteriaceae to a dominance of Burkholderiaceae on all analysed eggs. On genus level, larval stages were characterized by *Morganella* sp., *Escherichia* sp., and *Proteus* sp.,

transitioning later to less diverse communities in egg samples predominated by *Burkholderia-Caballeronia-Paraburkholderia* sp. While eggs from clutches and directly collected from the ovipositor generated viable offspring, surface sterilized eggs and eggs dissected from the ovary turned out to be nonviable. In microbiological cultivation trials, the established sterilization protocol was shown to be effective in removing viable microorganisms from the egg's surface.

Conclusion

Our study reveals that while a predominant microbiota persists throughout all life stages, its composition undergoes a progressive transformation during maturation, particularly before oviposition. Gaining deeper insights into egg surface microbiota and the cues guiding oviposition has the potential to boost egg production and simplify mass harvesting of BSF larvae.

Keywords: microbial communities, oviposition, attractants, interkingdom communication, circular economy, insect farming, bio-economy, microbial colonization, insect immunity, alternative protein

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Introduction

Rapid population growth, accelerating urbanization, and rising incomes lead to increased demand for food and feed and to a growing challenge in managing organic wastes [1-3]. In turn, this leads to an intensification of environmental stress through deforestation, overfishing of the oceans, and greenhouse gas emissions on account of the production of animal proteins such as beef, pork, and chicken [4]. To meet the demands of animal protein resources, meat production is estimated to increase by more than 75% until 2050, and global fish production is projected to be 30 million tons higher in 2030 compared with 2016 [1, 3]. As a consequence, the demand for animal and/or plant-based protein-rich animal feed like fish- or soybean meal will surge.

In 2021, 20% of wild-caught fish was processed into fishmeal for aquaculture purposes [5]. The current food system is responsible for 80% of deforestation and 29% of greenhouse gas (GHG) emissions. Additionally, agriculture uses 34% of all land globally and withdraws 70% of the freshwater used globally for watering. As a result, the current food system is estimated to be responsible for 68% of biodiversity loss, which effect is predicted to grow even further [5, 6]. Moreover, about 1.7 billion tons of all food produced is wasted [7]. The resulting organic waste is mostly not properly managed and has a significant negative impact on the environment (3.3 billion tons of GHG emissions, per year) and the economy (losses of 1.2 trillion dollars per year) [7-9].

To reduce the environmental impact of food production, alternative sources of animal protein are needed. Insect biomass as an alternative animal protein source has been reported to have a lower environmental impact than other sources of animal protein [10]. Insect farming emits less

71 GHG, uses less land, and requires lower inputs of feed and water due to higher conversion
72 efficiencies [10, 11].

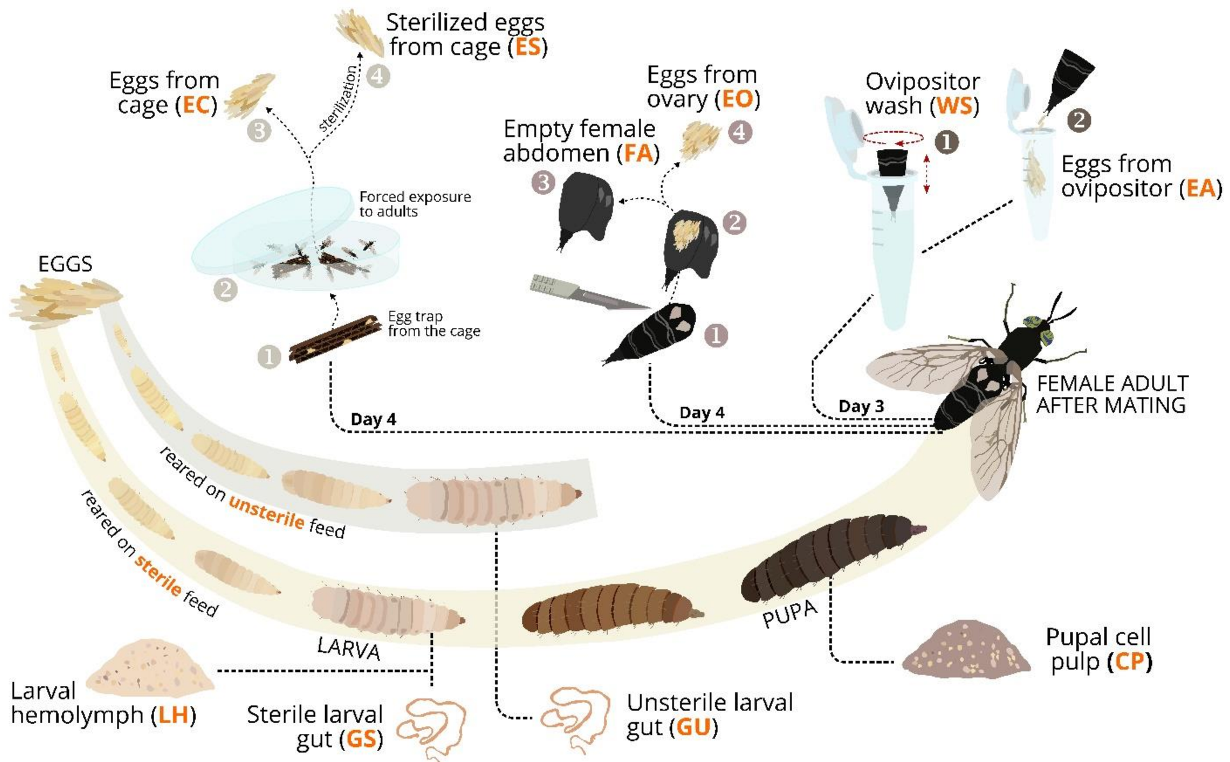
73 In the last years, the black soldier fly (*Hermetia illucens*, L., BSF) has been identified as a key
74 player to substitute animal protein for food and feed [12, 13]. The BSF adults have a reduced
75 digestive tract; they primarily survive on reserves accumulated during the larval stage. Hence,
76 BSF larvae (BSFL) are high in protein (40-44% dry matter base) and fat (up to 49% dry matter
77 base) and contain several micro-and macronutrients important for livestock health and
78 development [14]. The larvae are able to digest a large variety of organic matter such as food
79 waste, faecal sludge, manure, and agro-industrial by-products [12]. The undigested residues
80 mixed with BSFL excrements find use as organic fertilizer, capable to substitute or replace mineral
81 fertilizers [15]. By converting organic wastes into nutrient-rich insect biomass suitable for
82 feedstock production with organic fertilizer as the main process by-product, the BSF can
83 contribute to circular economy goals [16, 17]. It even has been reported that BSFL can reduce the
84 methane production of swine manure by 86% and that the direct GHG emissions are lower
85 compared to conventional composting [11, 18].

86 For these and many more reasons, the BSF is an ideal candidate for the industrialization of
87 insect farming [19]. Successful large-scale rearing of BSF depends on a flourishing reproduction
88 of adults [20]. The reproductive performance of BSF is influenced by physiological and
89 environmental factors as well as technological parameters [21]. The female adults oviposit single
90 eggs in clutches (up to 900 eggs) close to decomposing organic matter and are attracted by
91 conspecific eggs [22]. Zheng, et al. (2013)[23] showed that bacteria isolated from conspecific eggs

92 attract gravid females, presumably by emissions of volatile organic compounds. A better
93 understanding of the interkingdom communication between microbes and BSF females, and in
94 particular regulation of oviposition, can significantly increase the egg production and mass
95 harvesting of larvae [21].

96 In this study, we focused on the bacterial communities on the egg surface and their origin. We
97 hypothesized that the microbiota on the egg surface will either be inoculated before oviposition,
98 during oviposition, or actively after oviposition by adult flies. We sampled the microbiota in ten
99 approaches, that is, from the larval haemolymph (LH) and the larval guts fed with sterilized (GS)
100 and non-sterilized feed (GU), the pupal cell pulp (CP), the wash of the egg-laying apparatus (WS)
101 and the eggs directly collected after oviposition (EA); the ovarian eggs (EO) and the empty female
102 abdomen (FA); eggs of the fly cage with contact to adult BSF (EC), and sterilized eggs (ES).
103 Additionally, we screened the egg cytoplasmic microbiota, investigating the possible presence of
104 microorganisms within. The microbiota was identified through 16S rRNA gene sequencing to
105 assess the stage in BSF development when the microbial colonization of egg surface occurs.

106



107 *Figure 1. Illustration of the sampling procedure to assess egg surface microbiome of black soldier fly and its origin: Larval fed with*
108 *unsterile (GU) and sterile (GS) feed and the larval haemolymph of GS (LH), the pupal cell pulp (CP), and from the female adults*
109 *after mating, a wash of the egg-laying apparatus (WS) and the afterwards placed eggs of the egg-laying apparatus (EA), eggs*
110 *collected from the ovary (EO) and the empty female abdomen (FA), eggs collected from a fly cage after forced exposure to adults*
111 *(EC) and sterilized (ES).*

112 ***Material and Methods***

113 *Breeding of black soldier flies*

114 The BSF colony was reared in an ICH750eco climate chamber (Memmert, Schwabach,
115 Germany) at a temperature of 27 °C (± 0.5 °C), 60% relative humidity, and a 16:8 h (L:D)
116 photoperiod [24]. BSFL were kept in black polypropylene boxes (180 × 120 × 80 mm) sealed with
117 plastic lids with integrated nets to allow aeration. Larvae were fed twice a week *ad libitum* with

118 a 40:60 (w/v) mixture of ground chicken feed (Grünes LegeKorn Premium, Landwirtschaftliche
119 Genossenschaft, Klagenfurt, Austria) and tap water until prepupation [25]. When the transition
120 to the pupal stage started, migrating prepupae were guided into collection boxes via a ramp
121 installed on the inner side and a pipe attached on the outer side of the larva boxes, thereby
122 allowing their self-harvesting. Thereafter, pupae were kept in white plastic cups (50 ml) and
123 covered with wood shavings (Dehner Terra, Rain, Germany). After eclosion, flies were collected
124 manually into transparent polypropylene cages (390 × 280 × 280 mm) with a net (fibreglass; 200
125 × 300 mm, mesh size 2 × 2 mm) for aeration integrated into the lid of the cage. The fly cage was
126 illuminated with light-emitting diode panels (Y51515227 184210, Barthelme, Nuremberg,
127 Germany) in a 16:8 (L:D) photoperiod [26]. On two opposite walls of the fly cage, one piece of
128 corrugated black polypropylene cardboard each (henceforth termed flutes) was installed for
129 oviposition by using magnets. A glass test tube filled with tap water and plugged with a piece of
130 cellulose paper was provided as a water source. On Day 4 after manually transferring the flies
131 into the cages, the eggs were harvested from inside the flutes.

132 *Experimental setup and sampling*

133 A flute containing ten egg clutches was transferred into larva boxes, placed above chicken feed
134 freshly mixed with water, and held in place using toothpicks. To prevent larvae from escaping,
135 dry ground chicken feed was spread along the inner edges of the box as described in Addeo, et
136 al. (2022)[27]. To avoid the introduction of exogenous microorganisms into the feed, the larvae
137 used in this study were fed with ground, autoclaved chicken feed mixed 60% (w/v) with distilled
138 water *ad libitum* every other day. Ten distinct sampling approaches were applied to assess when,

139 where, and which bacterial and fungal communities occurred during BSF development, and to
140 determine the source of microbial colonization. Additionally, a control treatment with larvae
141 raised on unsterilized chicken feed was included. Samples for subsequent DNA extraction were
142 collected in triplicates; for each replicate, five individuals were pooled, except for WS, where ten
143 individuals were pooled due to low amounts of DNA; for details, see section "DNA extraction".

144 *Collection of larval haemolymphs (LH) and guts from non-sterilized (GU) and sterilized diet (GS)*

145 Nine days after hatching (DAH) about 50 larvae were collected and stored at -20 °C, in
146 estimation of half-life at 18 days until first prepupation through self-observation and literature
147 [28]. Frozen larvae were thawed for two min in a solution of 50:50 5% bleach (Danklorix, CP
148 GABA, Hamburg, Germany) and Milli-Q (Merck, Darmstadt, Germany) water for surface
149 sterilization [29, 30] and then placed in a 50-ml tube filled with pure Milli-Q water. To assure
150 complete extraction, the gut was extracted by pulling out the anus using sterile forceps and then
151 transferring it into a sterile microcentrifuge tube (at least 0.05 g/replicate) [31]. The remaining
152 haemolymph was placed in an empty and sterile microcentrifuge tube (at least 0.10 g/replicate).
153 The same procedure was used to collect the gut of the larvae fed with the non-sterilized diet.

154 *Collection of pupal cell pulp (CP)*

155 About 50 pupae were collected 19 DAH and stored at -20 °C. Frozen pupae were thawed for
156 two min in a solution of 50:50 5% bleach and Milli-Q water for surface sterilization, and
157 afterwards, pupae were placed in a 50 ml tube filled with Milli-Q water. Each pupa was cut along
158 both lateral sides with sterile scissors and the cell pulp was scraped out with a sterile spatula. All

159 five pupa replicates were collected into an empty and sterile microcentrifuge tube (at least 0.10
160 g/ replicate).

161 *Collection of wash of the egg-laying apparatus (WS) and eggs immediately after oviposition (EA)*

162 To assure easy handling and to prevent flies from escaping while collecting gravid females, the
163 number of flies per cage was limited to a density of 0.0033 flies/cm^3. Fifty females and 50 males
164 aged 24 h were released into the fly cages. A total of three fly cages were used to obtain enough
165 females for all sampling approaches, and sampling of individuals occurred randomly across all
166 cages. The fly cages were kept under the same conditions (see section "Breeding of black soldier
167 flies"). To obtain enough gravid females and assuming that females start oviposition on Day 4
168 after transferring flies to the cages [26], the collection of females for this treatment was set on
169 Day 3 after transferring flies to the cages. Gravid females were collected manually one at a time.
170 Each female was held above a sterile microcentrifuge tube filled with 700 µl lysis buffer SL1
171 (NucleoSpin Soil kit, Macherey-Nagel, Düren, Germany), and the ovipositor was dipped into the
172 liquid and moved in circles for one min to wash microbes off of the ovipositor's surface (WS).
173 Thereafter, the female was decapitated to induce oviposition. Each female was held above a
174 sterile microcentrifuge tube to allow oviposition into the tube (EA; at least 0.05 g/replicate).

175 *Collection of the ovarian eggs (EO) and the empty female abdomen (FA)*

176 Approximately 20 gravid females were collected on Day 4 after transferring flies to the fly
177 cages and stored at -20 °C. Frozen females were thawed for two min in a 50:50 solution of 5%
178 bleach and Milli-Q water for surface sterilization and placed in a 50 ml tube filled with Milli-Q. To
179 access the ovary, each female was cut along both lateral sides of the abdomen with sterile

180 scissors, and the ovary was collected into a sterile microcentrifuge tube using a sterile spatula (at
181 least 0.05 g/replicate). The remaining abdomen of the female was separated from the thorax and
182 collected into a sterile microcentrifuge tube (at least 0.05 g/replicate).

183 *Collection of eggs from the fly cage after contact with adult BSF (EC)*

184 The remaining flies in the fly cages were allowed to oviposit. On Day 6 after transferring the
185 flies into the fly cage, the flutes were collected into a sterile Petri dish. Five females and five
186 males were introduced into the Petri dish and left for one h at 27 °C and 60% RH. This procedure
187 assured contact between the eggs and adults, and enabled inoculation of eggs with adult-derived
188 microbes. Thereafter, five egg clutches were collected into sterile microcentrifuge tubes (at least
189 0.05 g/replicate).

190 *Collecting of sterilized eggs (ES)*

191 Five egg clutches were collected into sterile microcentrifuge tubes (at least 0.05 g/replicate).
192 The tubes were filled with 700 µl of a 50:50 mixture of 5% bleach and Milli-Q Water, vortexed for
193 10 s, and incubated for 2 min. The tubes were centrifuged (30 s at 11,000 × g), and the
194 supernatant was removed. The pellet was washed following these steps: 700 µl of Milli-Q water
195 was added; vortexed for 10 s; centrifuged (1 min /11,000 × g), the supernatant was removed, and
196 these steps were repeated at least five times until the smell of bleach was no longer noticeable
197 but before the egg surface started to break.

198 *Viability of sampled eggs*

199 To assess the viability of the eggs after the sampling procedures, additionally, three egg
200 clutches were collected for each of the sampling procedures and placed in a sterile Eppendorf
201 tube above non-sterilized feed and controlled daily for hatching larvae.

202 *Differences in larval growth on sterilized and non-sterilized feed*

203 To assess whether there are differences in growth for larvae reared on sterilized and non-
204 sterilized feed, we performed a separate feeding trial. BSFL were reared in triplicates (90 larvae
205 per replicate) on sterilized and non-sterilized chicken feed 40:60 (w/v) in 90 x 90 x 40 mm boxes
206 covered with cellulose paper. The BSFL were fed a fresh weight of 100 mg/larva/day. Every other
207 day, three times five larvae were randomly selected from each box (without replacement) and
208 weighed to track their biomass gain.

209 *Extraction of the egg cytoplasm*

210 The extraction of egg cytoplasm was performed using a micromanipulator (M-152, Narishige)
211 equipped with a capillary (BF100-78-10, Sutter Instrument) and connected to an inverted
212 microscope (CKX53, Olympus). We extracted the egg cytoplasm from two different groups, each
213 in three replicates: one consisting of a single individual and the other of five individuals.
214 Immediately after extraction, the cytoplasm was immersed in 25 µL of Milli-Q water.

215 *Cultivation and isolation of microorganisms*

216 Egg cytoplasm solutions (25 µL) were plated on Standard I Nutrient Agar prepared from 15 g
217 L^{-1} peptone, 3 g L^{-1} yeast extract, 6 g L^{-1} NaCl, 1 g L^{-1} glucose, and 12 g L^{-1} agar (pH 7.5). The plates

218 were incubated at 27 °C for up to 72 h. Single colonies were picked using a heat-sterilized loop
219 and transferred onto fresh agar plates via dilution plating.

220 *Colony PCR and Sanger sequencing*

221 A master mix consisting of 12.5 µL Taq 2X Master Mix with 1.5 mM MgCl2 (VWR, Radnor, PA,
222 USA), 0.5 µL 27f primer (Eurofins Genomics, Ebersberg, Germany), 0.5 µL 1492r primer (Eurofins
223 Genomics, Ebersberg, Germany), 0.5 µL 2% BSA (Thermo Fisher Scientific, Waltham, MA, USA)
224 and 11 µL PCR-grade water (Carl Roth, Karlsruhe, Germany) per reaction was prepared for colony
225 PCR. Following the aliquoting of the master mix into 0.2 mL reaction tubes, bacterial isolates were
226 carefully picked by gently scratching the colonies using sterilized pipette tips. The tip of the
227 pipette tip was directly submerged in the PCR reaction mix and stirred to detach the harvested
228 bacterial biomass. The following protocol was used for PCR amplification: Step 1: initial
229 denaturation at 95 °C, 5 min; Step 2: denaturation at 95 °C, 30 s; Step 3: annealing at 53 °C, 30 s;
230 Step 4: elongation at 72 °C, 45 s; Step 5: final elongation at 72 °C, 10 min; Step 6: storage at 12 °C
231 until termination; Steps 2-4 were cycled 30 times. The PCR products were then purified using a
232 GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA) and eluted using the enclosed
233 elution buffer. The purified DNA was quantified via UV-Vis spectrophotometry (NanoDrop 2000c,
234 Thermo Fisher Scientific, Waltham, MA, USA) and the quality was assessed via gel
235 electrophoresis. DNA passing the quality control was sent to Eurofins Genomics (Ebersberg,
236 Germany) for overnight Sanger sequencing using the 27f primer. The returned sequences were
237 aligned to the 16S ribosomal RNA sequences (Bacteria and Archaea) database via nucleotide
238 BLAST® search and using the megablast program for highly similar sequences.

239 *DNA extraction*

240 DNA was extracted using the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) following
241 the manufacturer's protocol with some modifications: the lysed sample of the larval
242 haemolymph was vortexed for 10 min, and the other samples for 5 min. Prior to precipitation
243 with SL3 Buffer, the supernatant was moved to a new collection tube. DNA was eluted twice
244 using 20 µl SE Elution Buffer each. DNA concentration and quality were checked via gel-
245 electrophoresis and UV-Vis spectrophotometry (NanoDrop 2000c, Thermo Fisher Scientific,
246 Waltham, MA, USA). DNA was stored at -20 °C until further processing.

247 *16S rRNA gene amplicon sequencing*

248 The UV-Vis spectrophotometry showed low DNA concentrations for some samples. Therefore,
249 an enrichment PCR of all samples was performed by the sequencing provider. The enrichment
250 was performed by diluting (2.5 ng/µl) the samples, followed by an enrichment PCR with locus-
251 specific primers (V34:IlluminaF
252 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNCCTACGGGNGGCWGCAG; IlluminaR
253 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNGACTACHVGGGTATCTAATCC, red =
254 locus-specific sequences). Then, a 1st-step PCR with locus-specific primer and Illumina overhang
255 and a cleanbead purification were performed, followed by a 2nd-step PCR with index primer and
256 another cleanbead purification. The final libraries were pooled, and a final cleanbead purification
257 of the pool was carried out. Illumina MiSeq amplicon 16S genetic sequencing was performed by
258 Microsynth AG (Balgach, Switzerland) using a 2 × 250 bp paired-end approach with the universal
259 bacterial primers 341f (5′-CCTACGGGRSGCAGCAG-3′) and 802r (5′-TACNVGGGTATCTAATCC-3′)

260 targeting the V3-4 region on the 16S rRNA gene. Library preparation was performed by the
261 sequencing provider based on a Nextera two-step PCR including purification, quantification, and
262 equimolar pooling. In addition, the ITS2 genetic region was sequenced using the ITS3f (5'-
263 GCATCGATGAAGAACGCAGC-3') and ITS4r (5'-TCCTCCGCTTATTGATATGC-3') primer pair.
264 However, due to the low quality of the reads resulting from this sequencing job, we decided to
265 exclude the data on fungal communities from further analyses and interpretation.

266 *Processing and analysis of sequencing data*

267 Raw reads generated by targeting the V3-V4 genetic region were filtered, trimmed, and
268 dereplicated in DADA2 v1.8 following the standard operating procedure [32]. After inferring
269 amplicon sequence variants (ASVs), the paired forward and reverse reads were merged, and
270 chimeras were removed. Taxonomy was assigned using the reference databases SILVA (v.132;
271 Quast, et al. (2012)[33]). The data were visualized using ampvis2 (v.2.7.4; Andersen, et al.
272 (2018)[34]) and ggplot2 (v.3.3.5; Wickham (2016)[35]). Venn diagrams were created using the
273 MicEco package (v.0.9.17; Russel (2022)[36]).

274 Reproducible documentation of sequence processing and data analysis as well as download
275 options for relevant data can be accessed via
276 https://tklammsteiner.github.io/eggsurfacemicrobiome.

277 *Statistical analysis*

278 Alpha diversity (Shannon index) and linear discriminant analysis of effect size (LEfSe; threshold
279 at LDA score (log10) >= 2) were calculated using the microbiome (v.1.16.0; Lahti, et al. (2017)[37]

280 and microbiomeMaker (v. 1.0.1; Yang (2021)[38] package, respectively. Differences between
281 means of alpha diversity indices were calculated via Wilcoxon test. Permutational analysis of
282 variance (PERMANOVA) was calculated based on Bray-Curtis dissimilarity values using the adonis
283 function (permutations = 1000) in vegan (v.2.5-7; Oksanen, et al. (2020)[39]). Pairwise differences
284 in microbial community composition of treatment groups were assessed using the
285 pairwise.perm.manova function (nperm = 1000) with subsequent Bonferroni correction in
286 RVAideMemoire package (v.0.9.81; Hervé (2022)[40]). The results were considered statistically
287 significant when their p-value was < 0.05.

288 ***Results***

289 *An extensive shift in family-level relative abundance during BSF development*

290 An average of 31,698 ± 11,232 raw reads per library were generated by Illumina MiSeq
291 amplicon sequencing. After filtering, denoising, and chimera removal, 26,125 ± 8,914 high-quality
292 reads remained, which were further rarefied to the smallest sample size (16,167 reads) before
293 subsequent biostatistical analysis. Due to an inadequately low read number (679 reads), sample
294 EA1 (replicate 1 of eggs from ovipositor) was removed as an outlier in the process of subsampling.
295 The highest relative abundance for bacteria in all larval stages was Enterobacteriaceae, though
296 less dominant for LH, followed by Enterococcaceae (Fig 2A, Fig S1). In LH, the families
297 Aerococcaceae, Bacillaceae, and Burkholderiaceae were also highly abundant. In terms of genus-

298 level representatives of Enterobacteriaceae, *Morganella* sp. was most abundant for all larval

299 stages (GU, GS, and LH), followed by *Escherichia* sp. and *Proteus* sp. (Fig 2B). The abundance of

300 Enterobacteriaceae decreased during the prepupal stage and was similarly abundant as

301 Burkholderiaceae in the CP samples. Within the family of Enterobacteriaceae, Providencia was

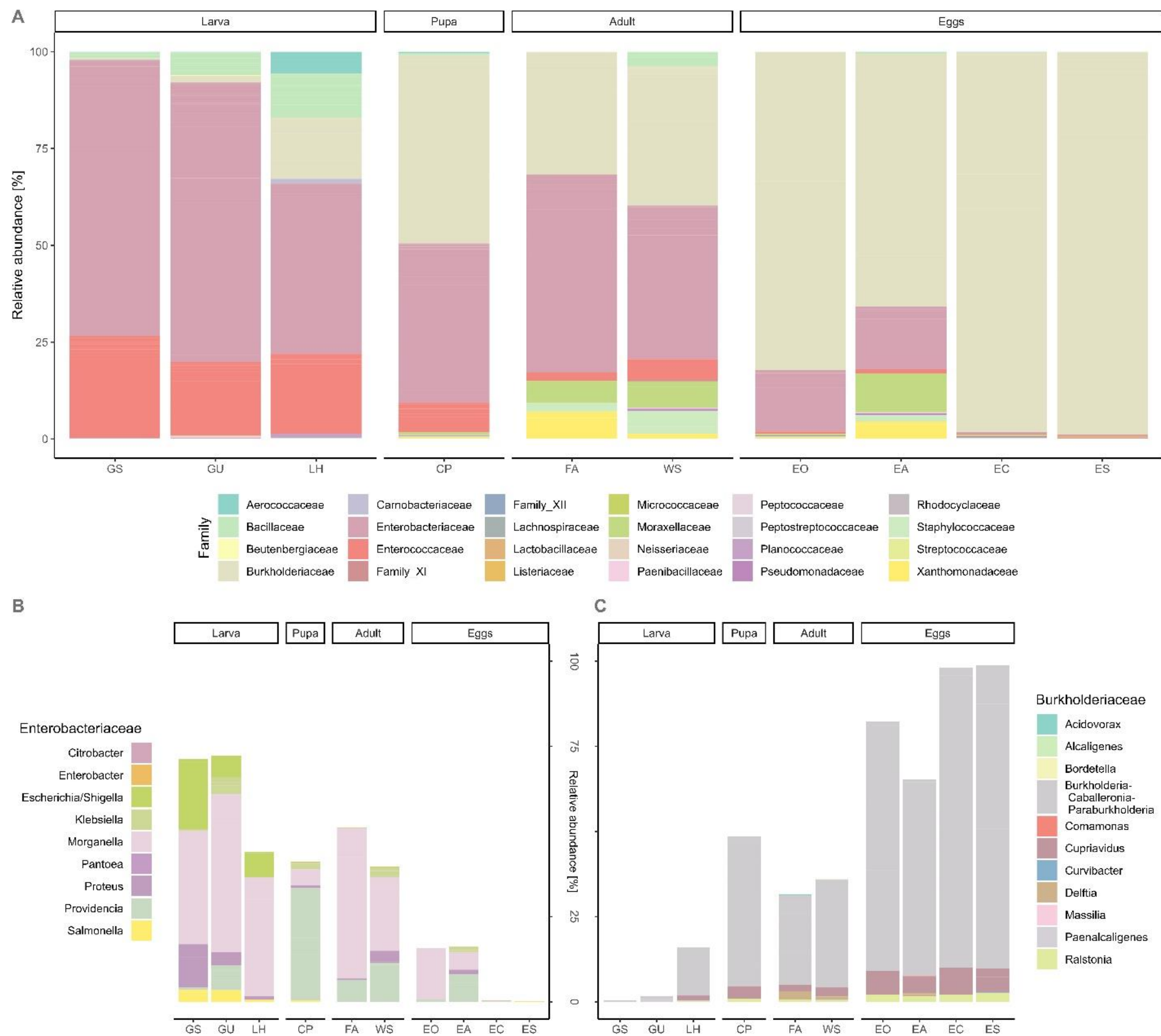


Figure 2. A) Community composition at family level. Genus-level composition of the families B) Enterobacteriaceae and C) Burkholderiaceae in different life stages of the black soldier fly: Larval fed with unsterile (GU) and sterile (GS) feed and the larval haemolymph of GS (LH), the pupal cell pulp (CP), and from the female adults after mating, a wash of the egg-laying apparatus (WS) and the afterwards placed eggs of the egg-laying apparatus (EA), eggs collected from the ovary (EO) and the empty female abdomen (FA), eggs collected from a fly cage after forced exposure to adults (EC) and sterilized (ES).

302 the most abundant genus in CP. This trend shifted slightly in the adult stage where the abundance

303 of Enterobacteriaceae increased again, but Burkholderiaceae were still highly present. In all the

304 egg stages, Burkholderiaceae accounted for most of the classified sequences, with the group of

305 *Burkholderia-Caballeronia-Paraburkholderia* sp. as the most abundant genera dominating EC and

306 ES samples. Similarities were found in microbiota composition between the FA, WS, and EA

307 samples, showing the presence of Xanthomonadaceae, Micrococcaceae, and Staphylococcaceae.

308 *Diversity in the egg-surface microbiome during BSF development and unique and shared ASVs*

309 As expressed by the Shannon diversity index (Fig 3), the egg stage had a significantly lower
310 diversity compared with the larval (p = 0.020) and pupal stages (p = 0.038) as well as the adult
311 stage (p = 0.004). In the larval stage, GU had the highest diversity (H' = 2.6 ± 0.9) and LH the
312 lowest (H' = 2.2 ± 1.1). For the adult stage, the WS had a higher diversity (H' = 2.8 ± 0.3). For the
313 egg stage, the highest diversity was observed in the EA samples (H' = 2.2 ± 0.8), while all of the
314 ES samples had a relatively low diversity (H' = 1.1 ± 0.1). The highest distance in the network

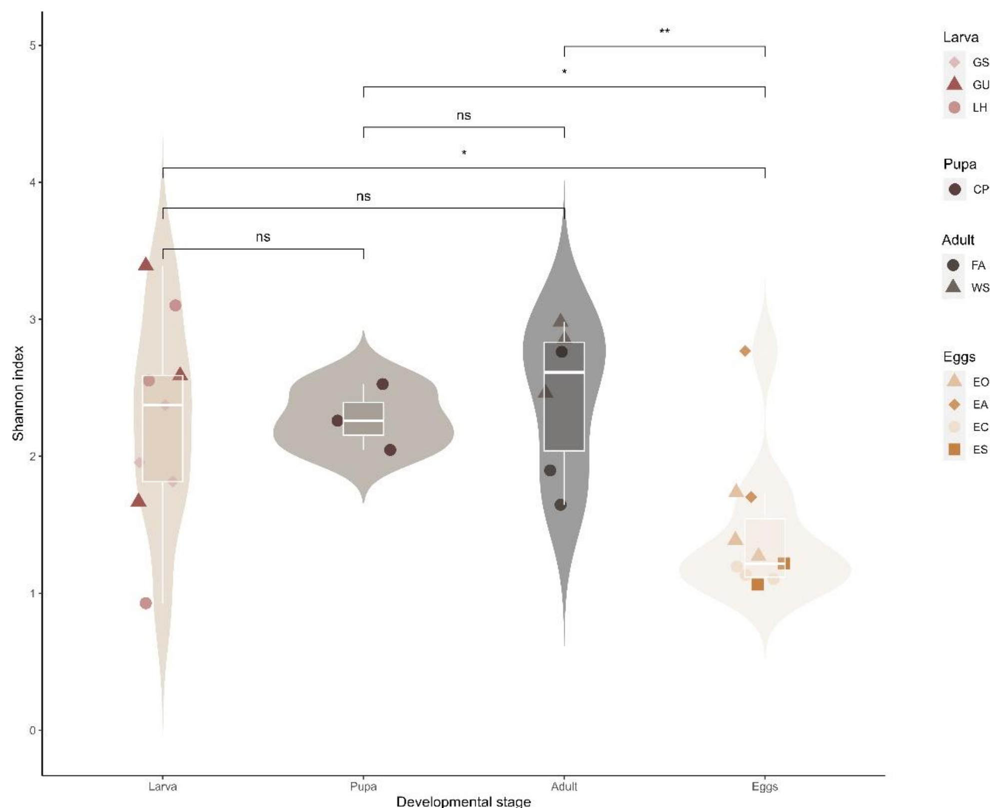


*Figure 3. Shannon diversity index (NS: p = 1, ns: p> 0.05, *: p<= 0.05; **: p <= 0.01) for the microbial communities of various black soldier fly life stages: Larval fed with unsterile (GU) and sterile (GS) feed and the larval haemolymph of GS (LH), the pupal cell pulp (CP), and from the female adults after mating, a wash of the egg-laying apparatus (WS) and the afterwards placed eggs of the egg-laying apparatus (EA), eggs collected from the ovary (EO) and the empty female abdomen (FA), eggs collected from a fly cage after forced exposure to adults (EC) and sterilized (ES).*

315 analysis between communities can be observed from LH and GS to EC and ES (Fig S2).

316 The highest number of unique ASVs (ASVs not shared with other life stages) was found in the
317 larval stage (118 ASVs, Fig 4A), while the lowest number of unique ASVs was found in the pupal
318 stage (30 ASVs). The most shared ASVs were found between the adult and egg stages (28 ASVs)
319 and the lowest shared ASVs were between larva and egg, and pupa and adult (2 ASVs). Among
320 the sampling approaches WS, EA, FA, and EO (Fig 4B), the highest unique ASVs for adults were
321 found via WS (61 ASVs), whilst via FA only 6 unique ASVs were found. Originating from the same
322 treatment, the highest number of ASVs for the egg stage was found in the EA (35 ASVs), and
323 similarly low to FA was the number of ASVs found in EO (7 ASVs). The highest number of shared
324 ASVs was found between WS and EA (22 ASVs), while FA and EO only shared 3 ASVs. Between EO
325 and WS, and FA and EO, the shared ASVs were 0. However, WS and FA shared 6 ASVs.

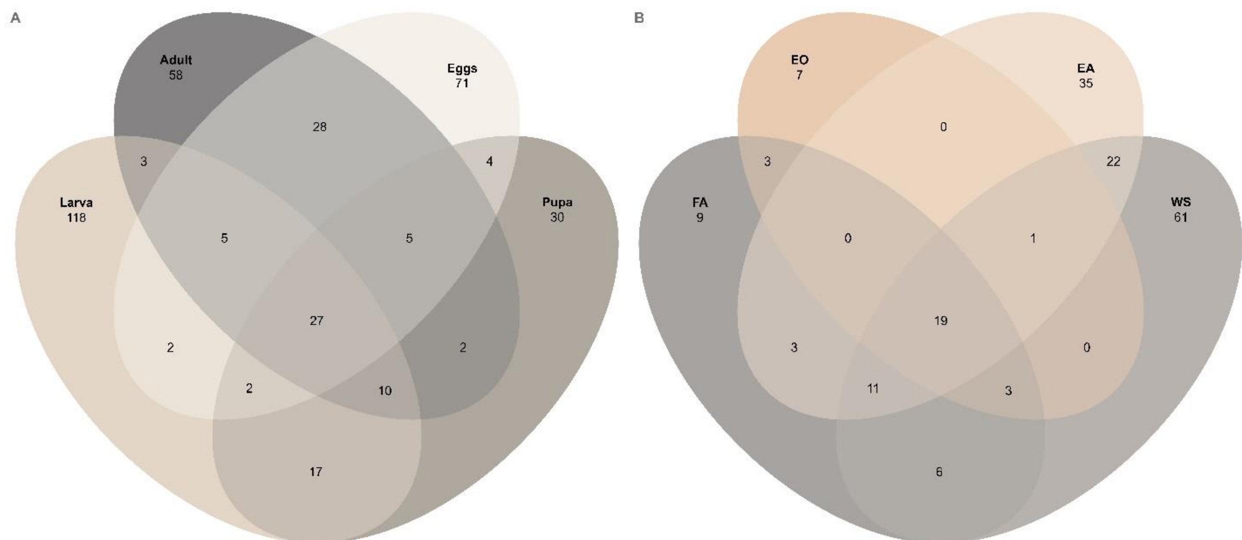


Figure 4. Venn diagram showing unique and shared ASVs for A) different life stages of black soldier fly and B) comparison between adult and egg stages collected from the female adults after mating, a wash of the egg-laying apparatus (WS) and the afterwards placed eggs of the egg-laying apparatus (EA), eggs collected from the ovary (EO) and the empty female abdomen (FA).

326 Biomarker analysis based on LEfSe further distinguished significantly overrepresented
327 bacterial genera for larval, pupal, adult, and egg developmental stages. The *Burkholderia-*
328 *Caballeronia-Paraburkholderia* group was found to be characteristic for egg samples, while the

329 genera *Acinetobacter*, *Staphylococcus*, and *Stenotrophomonas* were similarly overabundant in

330 adult samples. *Providencia*, which also showed high relative abundances in adults and eggs, was

331 by far the most overrepresented genus in pupal samples. Among the investigated BSF life stages,

332 the

333 complexity of the larval gut microbiota put forth the most biomarker taxa passing the threshold

334 of LDA >= 2, with *Morganella*, *Melissococcus*, and *Escherichia/Shigella* scoring highest.

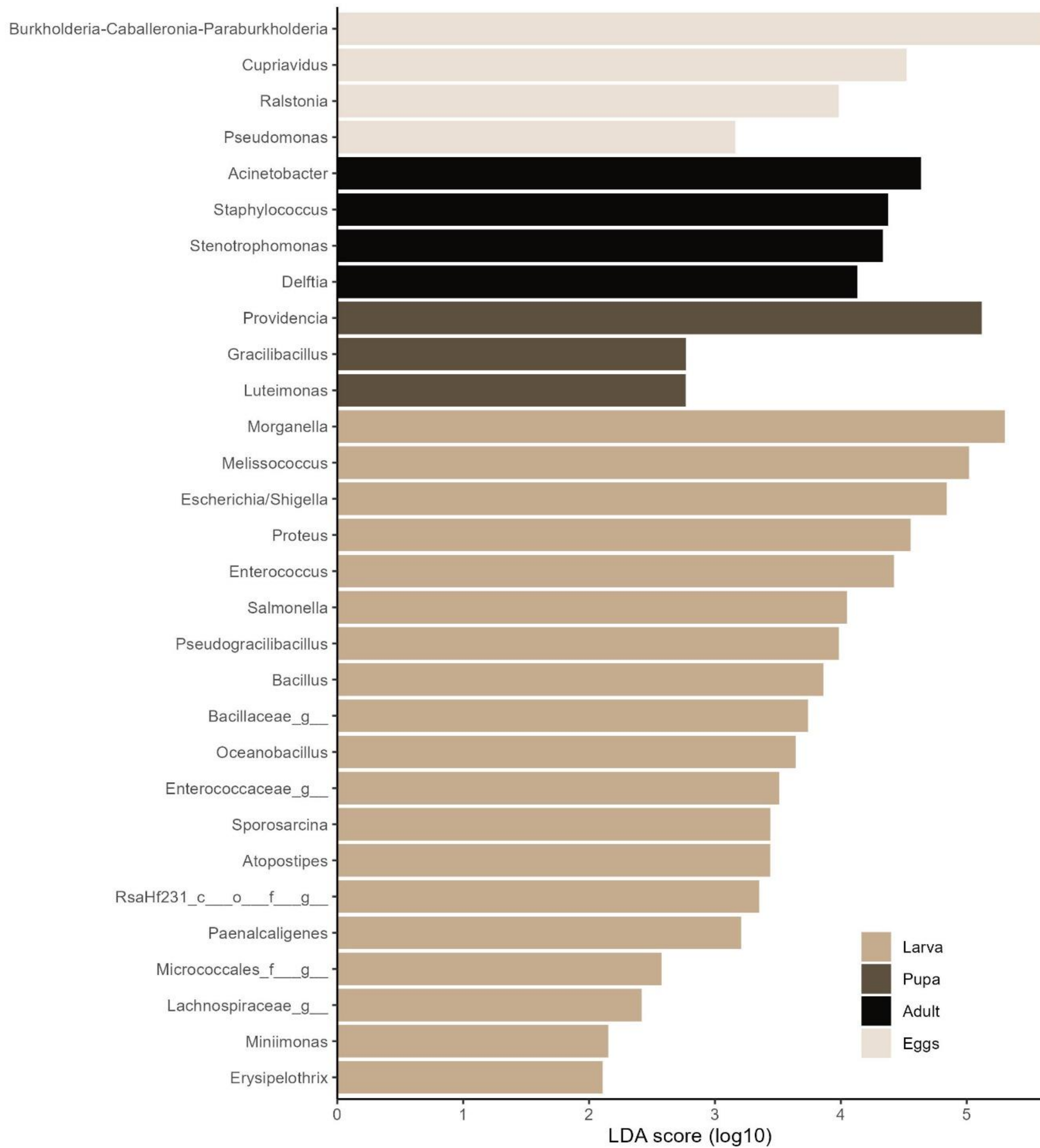


Figure 5. Linear discriminant analysis of effect size (LEfSe) identified characteristic bacterial genera for each developmental stage. Samples were grouped based on their life stage, whereas Larva consisted of GU, GS, and LH samples; Pupa consisted of CP samples, Adult consisted of FA and WS samples; Eggs consisted of EO, EA, EC, and ES samples. The threshold for biomarker identification was set to LDA score (log10) >= 2.

335

336 PERMANOVA based on Bray-Curtis distances confirmed that there were significant differences
337 in microbial communities across stages (p = 0.001; larvae, pupae, adult, egg), as well as across
338 tissue samples (p = 0.001; larval guts, larval haemolymph, adult haemolymph, ovipositor,
339 ovarium, and eggs) (Table S1). Pairwise significant differences were shown in microbial
340 community composition across all developmental stages, except between pupae and adults, and
341 across all tissue samples, except between tissue of ovarium and tissue of haemolymph and
342 ovipositor (Table S2).

343 *Viability of eggs and larval growth*

344 Only for the EA and EC sampling procedure hatching larvae could be observed and showed
345 successful growth (Figure S3). The eggs from the EO and ES sampling procedures were not viable.
346 No significant differences could be found in the growth of larvae fed with sterilized and non-
347 sterilized feed (Figure S4).

348 *Bacterial isolates from the egg cytoplasm*

349 Five bacterial isolates were obtained from the microbiological cultivation of wash solutions from
350 BSF eggs, which were divisible into two morphologically distinct groups. Notably, bacterial
351 growth was observed only on two plates containing cytoplasm extracted from a single egg.
352 Sanger sequencing of all five isolates confirmed the morphological distinctions and resulted in
353 the taxonomical identification of *Bacillus zanthoxyli* (Isolates 1 and 2) and *Dermacoccus*
354 *nishinomiyaensis* (Isolates 3, 4, and 5) (Table S3).

355 ***Discussion***

356 This study aimed to decrypt the microbiota on the surface of the BSF eggs and to assess at
357 which developmental stage and how the egg's inoculation occurred. Our results indicate that a
358 gradual shift in bacterial community structure occurs during BSF development, from an
359 Enterobacteriaceae-dominated community in larval stages to a Burkholderiaceae-dominated
360 community in egg stages. Furthermore, the results indicate that inoculation of the dominant
361 bacterial community on the eggs surface occurs before oviposition, as the relative abundance in
362 the EO samples shows.

363 The composition of bacterial communities in the gut of the GU and GS larvae was similar,
364 indicating little to no influence on sterilization of the feed. In studies where BSFL were fed with
365 unsterilized chicken feed, *Gammaproteobacteria* (Enterobacteriaceae, *Morganella sp*.) and
366 *Bacilli* (*Enterococcus* sp., *Lactococcus* sp., Fig 2A, 2B and 5) were the most abundant phyla, thus
367 supporting our results [31, 41, 42]. Genera such as *Morganella* sp., *Enterococcus* sp., and
368 Providencia sp. were significantly overrepresented in larval and pupal samples and have
369 previously been found to play a key role in the BSFL core gut microbiome (Fig. 5; [31]). In LH, the
370 relative abundance of Enterobacteriaceae decreased, though still dominant, while *Bacilli* and
371 Burkholderiaceae increased. *Bacilli* have often been associated with BSF larvae; they promote
372 their growth by fermenting their food [31, 43, 44], whereas Burkholderiaceae have been rarely
373 detected, and their role in the BSF life cycle is unknown. In a study performed by Zheng, et al.
374 (2013)[23], Burkholderiaceae were identified through all life stages of BSF, though classified as a
375 minor component of the fly's microbiome. In the CP samples, Enterobacteriaceae were almost
376 as abundant as Burkholderiaceae. This trend shifted in the adult stage, in which
377 Enterobacteriaceae made up the major share of reads, though Burkholderiaceae were still

378 heavily represented. In addition, the groups of Xanthomonadaceae, Micrococcaceae and
379 Staphylococcaceae had an increased presence in the adult stage. These families have previously
380 been associated with BSFL [23, 31, 45]. In other insect species such as the pumpkin fruit fly
381 (*Zeugodacus tau*), the same bacterial families have been reported to possibly serve as a source
382 of vitamins, nitrogen, and amino acids, and some of them can be transmitted vertically from the
383 parents to their offspring [46]. A similar population structure of bacteria was found in the EA
384 treatment, indicating that an inoculation through the egg-laying apparatus occurs, as this
385 treatment consisted of freshly oviposited eggs. Furthermore, the EO samples showed a heavy
386 dominance of Burkholderiaceae and a strong presence of Enterobacteriaceae, but unlike EA, no
387 other bacterial groups were highly represented, indicating an inoculation at the time of
388 oviposition. Interestingly, most other bacterial groups on the older eggs (EC and ES) were
389 outcompeted by Burkholderiaceae. Some members of the Burkholderiaceae can function as
390 opportunistic pathogens and some have the capacity to degrade chlororganic pesticides [23]. A
391 study has shown that a stinkbug (*Saccharum officinarum*) may harbour some fenitrothion-
392 resistant *Burkholderia* from the environment, and these bacteria can be an easy way for the
393 insect to detoxify insecticides [47]. *Burkholderia-Caballeronia-Paraburkholderia* sp. were the
394 most dominant genera found among the Burkholderiaceae throughout all life stages and were
395 identified as characteristic colonizers of BSF eggs (Fig 2C, 5). These taxa can interact with their
396 host by supplementing specifically required forms of nitrogen and other nutrients to ensure
397 normal development of, for example, xylophagous insects [48]. The features of bacterial
398 communities across the life stages of BSF indicate that some important microbes are tightly
399 linked to the fly's development and growth, which includes maturation of the immune system,

400 resistance to chemical substances (e.g., insecticides), supplementation of nutrients, and
401 digestion [49].

402 Similarly, the Shannon diversity index indicates that bacterial community composition
403 experiences changes in community complexity during the BSF life cycle (Fig 3). The bacterial
404 diversity was higher during larval and adult stages and decreased for the pupal stage and reached
405 a significantly low level for egg stages. This is not surprising, considering BSF eggs are immobile
406 and pupae are semi-immobile and do not feed anymore and, therefore, have less exposure to
407 environmental and transient microbes [23]. The lowest diversity was observed for the ES
408 treatment indicating that sterilisation of the egg surface can be considered effective. However,
409 to assess if trans-generational immune priming occurs via bacterial injection into the developing
410 egg, further analyses testing the sterilization should be performed [50]. Zheng, et al. (2013)[45]
411 showed that sterilized eggs lead to a lower percentage of oviposition. This implies that the
412 bacteria present in unsterilized eggs may attract conspecific gravid females, a phenomenon also
413 observed in other dipterans such as mosquitoes [51].

414 A subset of 27 bacterial ASVs was present in all life stages (Fig 4A), whereas 28 ASVs were
415 shared between adults and eggs and additional 17 ASVs between larvae and pupae. This similarity
416 in taxonomic features indicates the transmission of bacteria across life stages. The larval and egg
417 stages had 118 and 71 unique ASVs, respectively, that were not present in other life stages,
418 indicating a distinct community structure. To assess if and to what degree an inoculation of eggs
419 by adult females might take place, constellations of unique and shared ASVs between FA, EO,
420 WS, and EA samples were calculated in a Venn diagram (Fig 4B). These samples consist of eggs
421 sampled directly from the female abdomen and the emptied female abdomen, a wash of the egg-

422 laying apparatus and the eggs that were directly oviposited afterwards. From this analysis, a
423 profile of shared bacterial groups associated with females and eggs post- and pre-oviposition was
424 derived. The results show that with 22 shared bacterial ASVs between EA and WS, it is likely that
425 an inoculation occurs during the oviposition process. Furthermore, the EA has zero shared
426 features with the EO eggs, indicating no similarities before and after oviposition. However, the
427 FA samples shared only three ASVs with EA, indicating that the bacterial community of adult
428 females is not similar to that of freshly oviposited eggs before oviposition starts. This could mean
429 that females accumulate certain bacteria during oviposition to inoculate eggs with a specific
430 community. A similar progression can be derived from the diagrams showing community
431 compositions (Fig 2).

432 In the context of this study, we analysed the egg cytoplasm of BSF to explore the potential
433 presence of intraovular microbial communities. The extracted cytoplasm was cultivated on
434 standard nutrient agar with the intention of providing a broad nutrient source for the growth of
435 aerobic microorganisms, while recognizing that this approach might exclude the growth of
436 bacteria requiring more specific environmental conditions and nutrient supply. Notably, we
437 identified *Bacillus zanthoxyli* in a single egg sample, a finding previously unreported in BSF, most
438 likely suggesting a potential contamination source. *Dermacoccus nishinomiyaensis* has been
439 weakly observed in the larval guts in a study performed by Klüber, et al. (2022)[52], hinting at its
440 potential association with the BSF life cycle. Nonetheless, we must exercise caution in
441 conclusively suggesting the presence of *Dermacoccus* inside the BSF eggs, as neither of these
442 species was consistently detected in any of the treatments analysed in depth. Especially, despite
443 implementing a bleach treatment to eliminate cuticular bacterial DNA, the detection of the major

444 bacterial DNA associated with BSF eggs yielded results similar to those reported by Binetruy, et
445 al. (2019)[53], which focused on the removal of external bacterial DNA to analyse internal
446 bacterial DNA of ticks. These findings highlight the complexities in accurately characterizing the
447 egg cytoplasm microbiota of BSF and emphasize the need for more comprehensive studies to
448 analyse its true composition and significance during BSF development.

449 This study aimed at analysing the microbiota on the egg surface and their origin. Our results
450 show that the microbial diversity significantly decreases across the sampling stages and is lowest
451 for the egg stage. This early stage, however, is strongly dominated by members of the
452 Burkholderiaceae. The presence of this family of bacteria through all life stages points at a
453 transmission across stages, which becomes especially noticeable between the adult and egg
454 stages during oviposition (EA and WS, Fig 4B). After oviposition, the Burkholderiaceae
455 outcompeted other taxa and according to the surface-sterilized eggs, this development seems
456 independent from the adult flies (Fig 2A and C, Fig 3), indicating no active inoculation. To further
457 understand this process in detail, eggs separated from adults right after oviposition should be
458 analysed. Also, different egg surface sterilization methods and their effectiveness should be
459 evaluated.

460 ***Conclusion***

461 The characterization of bacteria associated with the surface of BSF eggs contributes to
462 understanding the important role of microorganisms in interkingdom interaction and the
463 attraction of conspecific gravid females [45]. Our results indicate a high abundance of
464 Burkholderiaceae in the ES treatment as well as in the EC treatment. Further investigations

465 should be performed to analyse if Burkholderiaceae is mainly inside the egg or on its surface.
466 Decrypting this interkingdom interaction could help to manipulate and optimize the process of
467 oviposition via artificial inoculation of a substrate with microbial attractants. This might be a
468 milestone for BSF industrial rearing, as larval loss caused by uncoordinated oviposition could be
469 minimized. Furthermore, by analysing bacterial communities of BSF life stages, this study
470 improves the understanding of the transfer of possible pathogens between life stages and
471 generations.

472

473 ***Abbreviations***

474 GHG – Greenhouse gas

475 BSF – Black Soldier Fly

476 BSFL – Black Soldier Fly larvae

477 LH – Larval haemolymph

478 GS – Guts from larvae reared on sterilized diet

479 GU – Guts from larvae reared on non-sterilized diet

480 CP – Pupal cell pulp

481 WS – Wash of egg-laying apparatus

482 EA – Egg oviposited directly after the wash

483 EO – Egg extracted out of the ovary

484 FA – Females abdomen after extraction of ovaries

485 EC – Eggs of the fly cage after forced contact with adults

486 ES – Sterilized eggs

487 DAH – Days after hatching

488

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639 3517-5.

640

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645 ***Contributions***

646 CDH, TK, BCSS, and FMS contributed to the study design. CDH performed all experiments,
647 sampling and laboratory works. KTS performed the feeding trial of larvae. TK performed
648 bioinformatics, statistical analyses, and data visualization. FMS acquired funding. CDH wrote
649 the first draft of the manuscript. All the authors read, revised, and approved the final version of
650 the manuscript.

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653 ***Ethics declarations***

654 Not applicable

655 ***Consent for publication***

656 Not applicable

657 ***Availability of data and materials***

658 Raw sequence data supporting this study are openly available from the European Nucleotide
659 Archive (ENA) under the BioProject accession number PRJNA809118 and can be accessed under

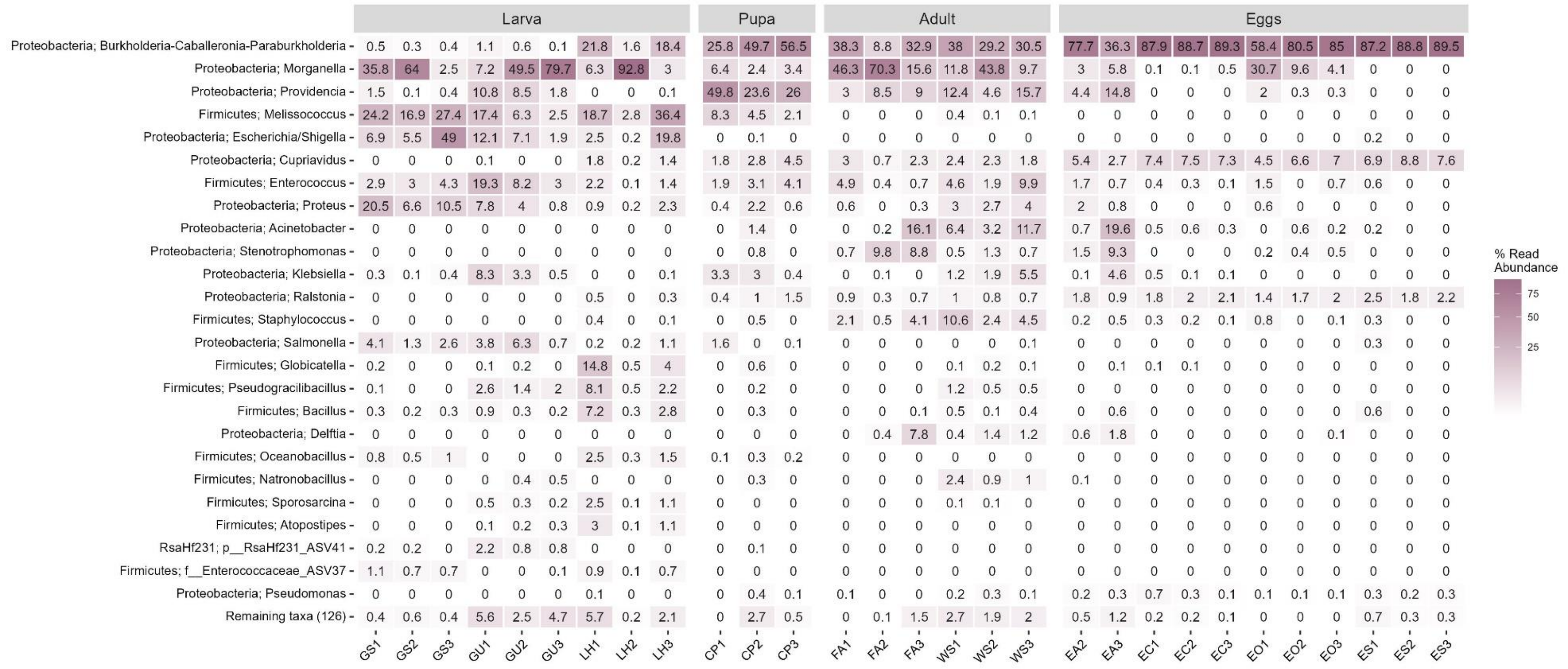
660 the following URL: https://www.ebi.ac.uk/ena/browser/view/PRJNA809118. Detailed
661 documentation on sequence preprocessing, statistical analysis, and visualization is available
662 under the following URL: https://tklammsteiner.github.io/eggsurfacemicrobiome.

663 ***Competing interests***

664 The authors declare that they have no competing interests.

665

666 ***Supplementary Material***

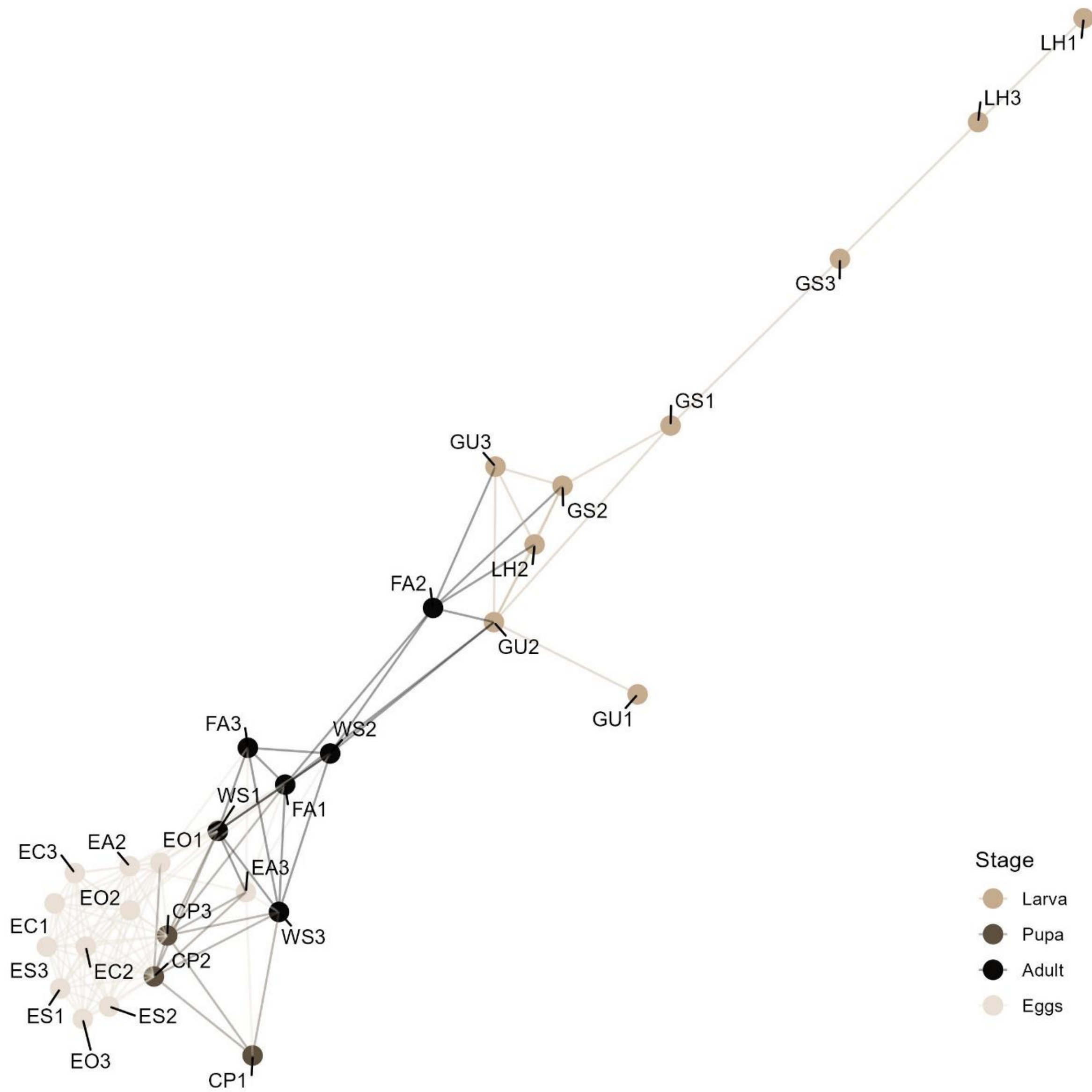


667 *Figure S1. Heatmap of microbial communities in different life stages (A) and tissue samples (B) of the black soldier fly: Larval fed with unsterile (GU) and sterile (GS) feed and the*

668 *larval haemolymph of GS (LH), the pupal cell pulp (CP), and from the female adults after mating, a wash of the embryo-laying apparatus (WS) and the afterwards placed embryos*

669 *of the embryo-laying apparatus (EA), embryos collected from the ovary (EO) and the empty female abdomen (FA), embryos collected from a fly cage after forced exposure to*

670 *adults (EC) and sterilized (ES)*



671 *Figure S2. Network analysis of microbial communities in different life stages (A) and tissue samples (B) of the black soldier fly:*
672 *Larval fed with unsterile (GU) and sterile (GS) feed and the larval haemolymph of GS (LH), the pupal cell pulp (CP), and from the*
673 *female adults after mating, a wash of the egg-laying apparatus (WS) and the afterwards placed eggs of the egg-laying*
674 *apparatus (EA), eggs collected from the ovary (EO) and the empty female abdomen (FA), eggs collected from a fly cage after*
675 *forced exposure to adults (EC) and sterilized (ES)*

676

677 *Table S1. Permanova based on Bray- Curtis analysis in different life stages (A) and tissue samples (B) of the black soldier fly:*
678 *Larval fed with unsterile (GU) and sterile (GS) feed and the larval haemolymph of GS (LH), the pupal cell pulp (CP), and from the*
679 *female adults after mating, a wash of the egg-laying apparatus (WS) and the afterwards placed eggs of the egg-laying*
680 *apparatus (EA), eggs collected from the ovary (EO) and the empty female abdomen (FA), eggs collected from a fly cage after*
681 *forced exposure to adults (EC) and sterilized (ES). Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

682 A)

683	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
684 ## Stage	3	3.9545	1.31815	12.676	0.60335	0.000999 ***
685 ## Residuals	25	2.5997	0.10399		0.39665	
686 ## Total	28	6.5541			1.00000	

687 B)

688	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
689 ## Tissue	4	3.6108	0.90270	7.3607	0.55092	0.000999 ***
690 ## Residuals	24	2.9433	0.12264		0.44908	
691 ## Total	28	6.5541			1.00000	

692

693

694 *Table S2. Pairwise Permanova analysis with Bonferroni correction in different life stages (A) and tissue samples (B) of the black*
695 *soldier fly: Larval fed with unsterile (GU) and sterile (GS) feed and the larval haemolymph of GS (LH), the pupal cell pulp (CP),*
696 *and from the female adults after mating, a wash of the egg-laying apparatus (WS) and the afterwards placed eggs of the egg-*
697 *laying apparatus (EA), eggs collected from the ovary (EO) and the empty female abdomen (FA), eggs collected from a fly cage*
698 *after forced exposure to adults (EC) and sterilized (ES). Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

699 A)

	Larva	Pupa	Adult
Pupa	0.042	-	-
Adult	0.012	0.156	-
Eggs	0.006	0.048	0.006

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704 B)

	Eggs	Gut	Haemolymph	Ovarium
Gut	0.02	-	-	-
Haemolymph	0.01	0.04	-	-
Ovarium	0.16	0.15	0.52	-
Ovipositor	0.06	0.03	1.00	0.87

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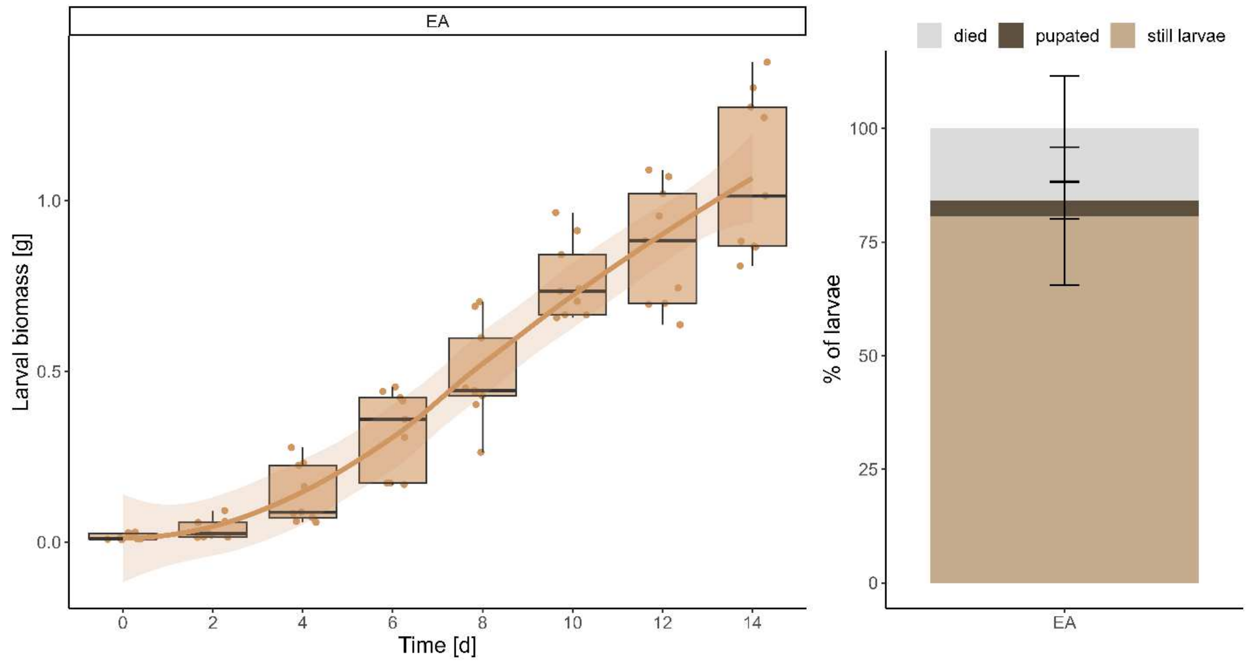

711 *Figure S3. The growing performance of black soldier fly larvae freshly hatched from forced oviposited eggs into sterile Eppendorf*

712 *tubes, fed sterilized feed (chicken feed 40:60 w/v) and percentage of pupated larvae and larvae that died.*

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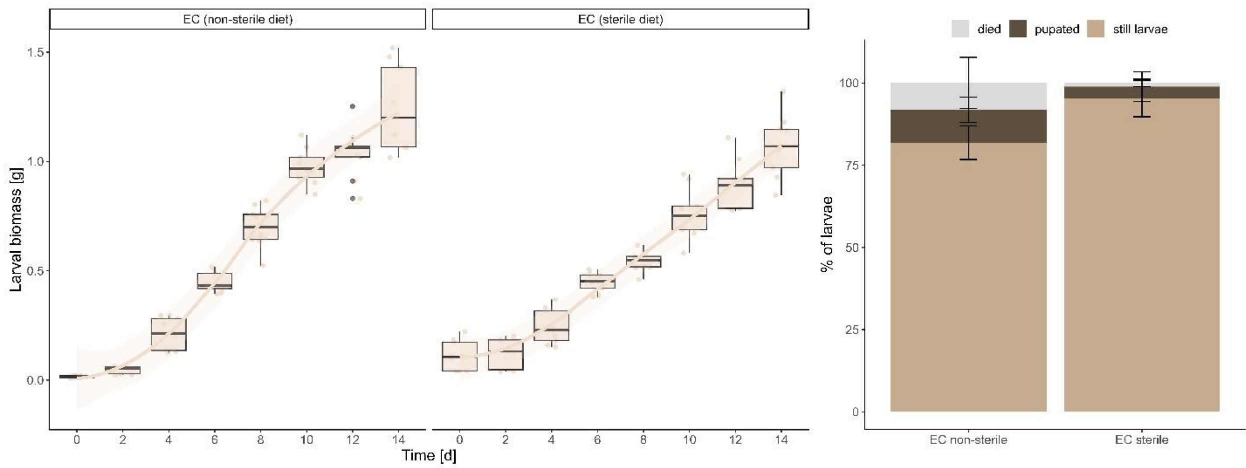
714



715 *Figure S4. Differences in black soldier fly growth performance reared on a sterilized and non-sterilized feed (chicken feed 40:60*

716 *w/v) and percentage of pupated larvae and larvae that died.*

717 *Table S3 Sanger sequencing and nucleotide BLAST® search results of the isolates gained during the analysis of single individual egg cytoplasm of BSF.*

Isolate	Sequence	NCBI Hit
1	CCAATCCAAAGCCAAACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTGGAGATAGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTAGTTGCCAGCATTAGTTGGGCACTCTAAGGTGACTGCCGTGACAACCGGAGAAAGGTGGGGATAA	*Bacillus zanthoxyli* Acc. Nr.: 164882.1 Per. Ident.: 99.33%
2	CTGCTATACATGCAAGTCGAGCGAACTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAATACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACTCTAGAGATAGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCCGTGAC	*Bacillus zanthoxyli* Acc. Nr.: 164882.1 Per. Ident.: 99.73%
3	GCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCGCTAGGTGTGGGACTCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCCGCATGCTACATGGCCGGGACAGAGGGTTGCGAAACCG	*Dermacoccus nishinomiyaensis* Acc. Nr.: 044872.1 Per. Ident.: 99.42%

4	TGCTTAACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCTGGGATAAGCCTGGGAAACTGGGTCTAATA CTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGA GAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC GGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGG CGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGA AATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTT GGGCGCTAGGTGTGGGACTCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGC GGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTCGT GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAAT CATCATGCCCCTTATGTCTTGGGCTTCCCGCATGCTACAAG	*Dermacoccus nishinomiyaensis* Acc. Nr.: 044872.1 Per. Ident.: 99.57%
5	GGCTGCTTACACATGCAAGTCGAACGATGAAGCACCAGCTTGCTGGTGTGGATTAGTGGCGAACGGGTGAGTAACACGTGAGTAACCTGCCCCTCACTCTGGGATAAGCCTGGGAAACTGGGTCTAA TACTGGATACGACCGATCTCCGCATGGAGTGTTGGTGGAAAGTTTTTGTGGTGGGGGATGGACTCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCT GAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCT TCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCTAACGTGACGGTACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTG GGCGTAAAGAGCTTGTAGGCGGTTTGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAGTGTGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGT GAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACGCTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACG TTGGGCGCTAGGTGTGGGACTCATTCCACGAGTTCCGTGCCGCAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG GCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAATCATGCAGAGATGTGTGCGTCTTCGGACTGGTGTACAGGTGGTGCATGGTTGTCGTCAGCTC GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCATGTTGCCAGCACTTCGGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAA ATCATCATGCCCCTTATGTCTTGGGCTTCCGCATGCTACAATGGCCGGTACAGAGGGTTGCGAAACCGTGA	*Dermacoccus nishinomiyaensis* Acc. Nr.: 044872.1 Per. Ident.: 99.42%

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