1   **Influence of the lab adopted natural diet and environmental and parental**

2   **microbiota on life history and metabolic phenotype of *Drosophila***

3   ***melanogaster* larvae.**

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18 ## Abstract:

19   Obesity is an increasing worldwide epidemic and contributes to physical and mental health

20   losses. The development of obesity is caused by multiple factors including genotype, hormonal

21   misregulation, psychological stress, and gut microbiota. Our project investigated the effects

22   produced by microbiota community, acquired from the environment and horizontal transfer, on

23   traits related to obesity. The study applied a novel approach of raising *Drosophila melanogaster*

24   from ten, wild-derived genetic lines (DGRP) on naturally fermented peaches, thereby preserving

25   genuine microbial conditions. Our results indicated that larvae raised on the natural and standard

26   lab diets were significantly different from each other in every tested phenotype. In addition,

27   sterilized larvae raised on the autoclaved peach diet, therefore exposed to natural nutritional

28   stress but lacking natural microbiota community, were associated with adverse phenotypes such

29   as low survival rate, longer developmental time, smaller weight, and elevated triglyceride and

30   glucose levels. Our findings suggested that frozen peach food provided nutritional conditions

31   similar to the natural ones and preserved key microbial taxa necessary for survival and

32   development of *Drosophila* larvae. The presence of parental microbiota did not produce a

33   significant effect on any of the tested phenotypes when larvae were raised on the lab diet.

34   Contrarily, on the peach diet, the presence of parental microbiota increased the weight and

35   development rate, even if the original peach microbiota were still present. In addition, we found

36   that larvae raised on the peach diet formed a microbial community distinctive from larvae raised

37   on the lab or peach autoclaved diets. The effect that individual microbial taxa produced on the

38   host varied significantly with changing environmental and genetic conditions, occasionally to the

39   degree of opposite correlations.

<u>Introduction</u>

The holobiont theory states that a host and its commensal microbiota possess a metagenome that expresses a synergistic phenotype, which is subjected to evolutionary forces as one complex organism (Rosenberg et al., 2010). A phenotype of this unit could be varied by genome modifications of the host, as well as its commensal bacteria. Metagenomic changes induced by bacteria have more potential for genetic variability and could arise by altering dominant species of bacteria, as well as acquisition of new strains of microorganisms from an environment (Bordenstein and Theis, 2015).

Obesity is a worldwide epidemic that continues to grow and contributes to the development of various diseases, including but not limited to: type two diabetes mellitus, stroke, asthma, arthritis, coronary heart disease, arterial hypertension, all components of metabolic syndrome (Seganfredo et al., 2017, Wahba and Mak, 2007). Obesity does not only induce health risks but also is an important social factor. Stereotypically, the major cause of obesity is considered to be overeating and lack of exercise. Obese individuals are often stigmatized as lazy and unsuccessful, making them vulnerable to discrimination (Puhl and Heuer, 2010). Contrary to the stereotypic view, the development of obesity and metabolic syndrome is caused not only by excessive calorie intake and lack of physical activity but also by genotype, epigenetic factors, sleep deprivation, malfunction of endocrine system, psychological stress, and gut microbiota (Seganfredo et al., 2017, Han and Lean, 2016).

Gut microbiota is one of the most important factors shaping metabolic phenotype and, as the consequence is a key element in the development of metabolic and autoimmune diseases, cancer, and asthma (Read and Holmes, 2017, Leitão-Gonçalves et al., 2017). Alterations in gut

62   microbiota biodiversity and community structure are correlated with the development of the

63   obese phenotype (Turnbaugh et al., 2006, Flint et al., 2017). Transfer of the microbiota from an

64   obese to a lean, axenic (microbiota free) individual significantly increases weight gain and

65   adiposity, compared to axenic mice colonized with microbiota community from a lean organism

66   (Ridaura et al., 2013, Tilg and Moschen, 2016, Turnbaugh et al., 2006). These results suggest

67   that obesity can be transferred from one individual to another; therefore, exhibiting some

68   characteristics of an infectious disease (Tilg and Moschen, 2016).

69   During colonization of a fruit, *Drosophila* inoculates the substrate with their microbiota (Morais

70   et al., 1995). In addition, when females deposit embryos on a substrate, parental microbiota

71   resides on the chorion of the egg (Ryu et al., 2008). The initial microbiota is gained by the first

72   instar larvae through consumption of the chorion (Ryu et al., 2008). Later, during feeding, larvae

73   acquire additional microbiota from the environment, representatives of which remain in the

74   larval and pupal intestine until eclosion of the adult fly (Ridley et al., 2012). Wong et. al (2015)

75   showed that parental microbiota transferred with the chorion of the egg could modify the

76   microbial community composition in a food substrate and in the offspring. In addition, the

77   transfer of axenic *Drosophila* on food substrate would change the food microbial community to

78   resemble the symbiotic microbiota composition that would develop in the host (Wong et al.,

79   2015).

80   Symbiotic microbiota play an important role in *D. melanogaster* development and metabolic

81   phenotype. Axenic flies have a longer development time, lower weight, protein, and glycogen

82   content but higher free glucose and triglyceride levels (Newell and Douglas, 2014b, Dobson et

83   al., 2015, Huang and Douglas, 2015). There are similar negative phenotypic effects on axenic *D.*

84   *melanogaster* and on those being raised on harmful diets such as high sugar and high fat (Newell

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85 and Douglas, 2014a, Huang and Douglas, 2015, Reed et al., 2014, Birse et al., 2010, Dew-Budd

86 et al., 2016). Although axenic *Drosophila* consume less food, their energy storage indices

87 (triglyceride, glucose, glycogen, and trehalose levels) stay significantly higher than that of

88 conventional flies (Wong et al., 2014). In addition, the presence of the commensal microbiota

89 allows *Drosophila* to maximize their lifespan and reproductive output (Leitão-Gonçalves et al.,

90 2017). Dobson et. al (2015) showed that the abundance of specific bacterial taxa is associated

91 with a change in metabolic phenotype: *Acetobacter*, *Gluconobacter* and *Komagataibacter* are

92 negatively correlated with a fly's energy storage index. *Lactobacillus* is positively associated

93 with triglyceride concentration and *Achromobacter* and *Xanthomonadaceae* have a positive

94 correlation with glycogen levels (Chaston et al., 2014, Chaston et al., 2016). In addition,

95 *Drosophila* microbiota varies across genetic backgrounds which makes it possible to establish

96 associations between a host's genes and microbiota dependent metabolic responses as well as

97 particular symbiotic species (Chaston et al., 2016, Dobson et al., 2015, Early et al., 2017).

98 Symbiotic microbiota allow *Drosophila* to overcome the nutritional limitation of their diet. Shin

99 et. al (2011) showed that axenic larvae raised on a casamino acid diet experienced a 90% body

100 size reduction and were not able to survive to form pupae. However, the presence of only one

101 bacterial species, *Acetobacter pomorum,* could restore survival and the normal rate of larval

102 development via induction of the hosts' insulin-like growth factor signaling (Shin et al., 2011).

103 Leitão-Gonçalves et. al (2017) demonstrated that axenic flies express a strong preference for

104 yeast-rich food due to their demand for essential amino acids. *A. promorum* and several

105 *Lactobacilli* species are able to suppress the yeast appetite in *Drosophila* and shift the flies'

106 nutritional preference toward high-sugar concentration diets (Leitão-Gonçalves et al., 2017).

107 Their change in nutritional preference may be explained by competition between the host and its

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108   symbiotic microbiota for available sugars and through production of essential amino acids by the

109   microbial community (Leitão-Gonçalves et al., 2017).

110   Microbiota composition of lab dwelling *Drosophila* primarily consists of Acetobacteraceae,

111   Enterococcaceae, and Lactobacillaceae families (Chandler et al., 2011, Early et al., 2017).

112   Within these families, the influence of *Acetobacter tropicalis*, *Enterococcus faecalis*,

113   *Lactobacillus brevis*, and *Lactobacisul plantarum* on a host's metabolic phenotype have been

114   studied more than others (Early et al., 2017, Shin et al., 2011, Huang and Douglas, 2015).

115   However, the microbiota of wild fly populations may differ in the diversity and the abundance of

116   dominant species (Chandler et al., 2011). In the lab, *Drosophila* raised on fruits still exhibit a

117   more complex and diverse community of symbiotic microbes compared to conventionally raised

118   flies (Vacchini et al., 2017). Lab food preservatives, especially methylparaben sodium salt

119   (moldex), largely contribute to the difference between natural and laboratory associated

120   microbiota communities (Tefit et al., 2017). Therefore, studying the evolutionary relationship of

121   *Drosophila* and its microbiota, as well as the symbiont's influence on fly's metabolic phenotype

122   only on standard lab microbiota, may be insufficient to understand the natural relationship and

123   co-evolution of the fly and its microbiota.

124   With this work we wanted to address a series of specific questions and hypotheses:

125   **1)** How does a natural diet with a naturally occurring and/or maternally inherited community of

126   microbes influence the life history and metabolic phenotypes of flies relative to a standard lab

127   diet? Is there genetic variation in the phenotypic response to nutritional change and dietary and

128   parental microbiota availability?

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129   **1.1** We hypothesized that larvae raised on a natural diet will exhibit different life history

130   traits and metabolic phenotypes, comparing with larvae raised on a standard lab diet.

131   **1.2** We hypothesized that the presence of a maternally transmitted microbiota will

132   significantly impact larvae phenotypes, and that this impact may vary across dietary

133   treatments.

134   **1.3** Given prior findings on the roles of genetic variation on metabolic phenotypes, we

135   hypothesized that there is genetic variation in phenotype that interacts with the dietary

136   conditions and the availability of maternally transmitted microbiota.

137   **2)** Will the symbiotic microbiota community of the larvae raised on the natural diet be different

138   from the lab food raised larvae? Will the presence of maternally inherited microbiota influence

139   the formation of microbial communities? Is the microbiota community variable across host

140   genotypes?

141   **2.1** We hypothesized that the gut microbial community composition and diversity will

142   vary substantially across both dietary and parental microbiota conditions.

143   **2.2** We hypothesized that the maternally transmitted microbiota will have "founder

144   effects" in the formation of the larval gut microbiome.

145   **2.3** We hypothesized that the composition of the microbial community will exhibit

146   variation with host genotype.

147   **3)** Will specific microbial taxa and/or microbiota communities as a whole influence the larvae

148   phenotypes differently across diets and genotypes?

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149　　**3.1** We hypothesized that some microbial taxa will have consistent correlations with host

150　　phenotype across diets and treatments while others will have a diet or treatment specific

151　　relationship.

Materials and Methods

153　**Diets preparation**

154　On August 28th, 2017, we put approximately 200 peaches outdoors and allowed them to decay

155　for six days. On September 3rd 2017, the fruits were collected, manually ground, and stored in

156　freezers at -20 °C. Peach food (PR) preparation protocol was the following: we allowed

157　approximately one liter of the peach food to thaw, homogenized it with an immersion blender,

158　and distributed it into vials, with approximately 10 ml of food per vial. In order to prepare

159　autoclaved peach food (PA), vials containing peach food were autoclaved for 25 min at 121 °C.

160　Regular *Drosophila* lab food (R) was cooked according to the protocol described in previous

161　works (Dew-Budd et al., 2016, Mendez et al., 2016).

162　To ensure that the autoclaved peach food did not contain any live microorganisms, we used 1g of

163　autoclaved and non-autoclaved peach materials and diluted them in 9 ml of sterile Phosphate-

164　buffered saline solution (PBS) (Leitão-Gonçalves et al., 2017, Carvalho et al., 2005). Then, we

165　performed a serial dilution in PBS to get food dilutions (Leboffe and Pierce, 2012). We mixed 1

166　ml of each dilution with standard methods agar (Criterion) via the pour plate method (Leboffe

167　and Pierce, 2012). The agar was prepared according to the manufacturer's directions. Samples

168　were incubated at 35 °C for 48 hours (Maturin et al., 2001). The independent variable for the diet

169　component will be referred to as D.

Drosophila stocks and husbandry

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171   We used 10 naturally derived genetic lines created by the DGRP2 project: 142, 153, 440, 748,

172   787, 801, 802, 805, 861, and 882 (Mackay et al., 2012, Huang et al., 2014). Stocks were

173   maintained at constant temperature, humidity and light/dark cycle on a molasses-based lab diet

174   as described in previous works (Reed et al., 2014, Dew-Budd et al., 2016, Mendez et al., 2016).

175   The independent variable for the genetic component will be referred to as G.

176   **Drosophila embryos sterilization**

177   In order to remove parental microbiota, we sterilized ~12-hour old embryos with subsequent

178   two-minute washes in 2.5% active hypochlorite solution, 70% ethanol solution, and sterilized

179   distilled water (Leitão-Gonçalves et al., 2017, Carvalho et al., 2005). After sterilization, embryos

180   were placed on the apple agar plates, and incubated until the first instar stage under fly-rearing

181   conditions described above (Ashburner, 1989). The non-sterilized control embryos (NS) were

182   allowed to develop for ~24 hours (until the 1st instar larvae stage) on the apple agar plates, on

183   which they had been deposited. In order to demonstrate that sterilized embryos did not possess

184   parental microbiota, 20 sterilized 1st instar larvae were collected and grinded in 200 ul of the

185   sterile PBS using a mechanical homogenizer. The resulting mixtures were plated on nutrient and

186   standard method agars (Criterion). The agars were prepared according to the manufacturer's

187   directions. Plates were incubated for 48 hours at 35 °C (Maturin et al., 2001). NS larvae were

188   used as the positive control according to the same procedure. The independent variable for the

189   sterilization treatment component will be referred to as T.

190   **Larvae rearing and collection**

191   In three separate time periods (~ 30 days apart), we put 50 sterilized and non-sterilized larvae of

192   each genetic line in at least three vials of PA, PR and R food, each. The independent variable for

193   the time period (round) component will be referred to as R. Larvae were allowed to develop until

194   the late third instar wandering stage (when they stopped moving but before the pupation started)

195   and then collected in micro centrifuge tubes with sterile Ringer's solution (Ashburner, 1989).

196   Each vial was checked for the presence of larvae, at the right developmental stage, four times per

197   day at 9 am, 11 am, 2:30pm, and 5 pm for 18 days after larvae colonization. Larvae were

198   inspected for the presence of any damage (damaged ones were sorted out), cleaned with at least

199   two washes in a sterile Ringer's solution, and stored in the Ringer's solution at -20 °C in

200   Eppendorf tubes with 10 larvae per tube.

201   **Measuring Experimental Phenotypes**

202   **Survival.** The number of larvae collected per vial was summed and used to evaluate the number

203   of larvae that survived till the late 3rd instar stage. **Developmental rate**: The developmental rate,

204   in days, was calculated for each larva individually, from the day it was put in the food vial to the

205   collection date. We then calculated median developmental time per vial and used it in our

206   statistical analysis (Ridley et al., 2012). **Weight**: In order to measure the dry weight, larvae were

207   taken from the -20 °C freezer, allowed to reach room temperature and placed in a VWR standard

208   oven at 37 °C overnight. After drying, each larva was weighed individually using Mettler Toledo

209   XS 105 microbalance. Weights were recorded with LabX direct software v. 2.2. **Triglyceride**:

210   With the exception of four samples (due to low survival of larvae of certain D/G/T/R

211   combinations), we homogenized 10 larvae per sample to determine total triglyceride

212   concentration using the Sigma Triglyceride Determination Kit (Clark and Keith, 1988, De Luca

213   et al., 2005, Reed et al., 2010, Dew-Budd et al., 2016). Results were adjusted to represent the

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214   average triglyceride level per mg of dry larval weight. **Protein**: Protein levels were quantified

215   using the Bradford's method with 10 homogenized larvae per sample (with the exception of 3%

216   of the samples in which we used one to nine larvae, due to especially low survival rates of the

217   specific groups) (Bradford, 1976, Dew-Budd et al., 2016). Protein levels were averaged to

218   represent the protein concentration per mg of dry larvae weight. **Glucose**: For most of the

219   samples, combined trehalose and glucose concentrations were quantified via homogenization of

220   10 larvae (with the exception of 5% of the samples in which we used four to nine larvae) with

221   subsequent overnight incubation in 1 μg/mL trehalase solution and further application of the

222   Sigma Glucose Determination Kit (Rulifson et al., 2002, Reed et al., 2014, Dew-Budd et al.,

223   2016). Glucose levels were averaged and adjusted to represent the amount of glucose per mg of

224   dry weight.

225   To assess the triglyceride, protein and glucose levels in the diets, we used freshly unfrozen food

226   and unfrozen food that was incubated in the food vials for seven days at 25 °C, in the same

227   incubator as the experimental fly stocks, where larvae development was taking place. For the

228   food assays, we used the same procedure as for the larvae but with ~12.35 mg of the food

229   sample. For the glucose assay, instead of incubating samples in trehalase, we incubated them in

230   100μl of 1 mg/μl solution of invertase (to convert sucrose into glucose) (Ward's Natural Science)

231   overnight at 37 °C. For analysis, the results were adjusted to represent the amount of the

232   measured compound per mg of the sample.

233   **DNA extraction and sequencing**

234   DNA was extracted from 10 larvae with the Qiagen blood and tissue DNA extraction kit

235   according to the standard protocol, with overnight incubation of the samples in proteinase K at

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236   56 °C. DNA extractions were used for sequencing the V4 region of the 16S ribosomal RNA

237   subunit (16S rRNA), which was performed in the Microbiome Core Facility of The University of

238   Alabama in Birmingham, AL according to the previously published method on the Illumina

239   MiSeq platform (Kumar et al., 2014).

240   Trimmomatic-0.36 (Bolger et al., 2014) was used to process demultiplexed DNA sequences.

241   Standard Illumina-specific barcode sequences, all sequences with less than 36 bases, and leading

242   and trailing low-quality bases were all removed using the default settings of the Trimmomatic-

243   0.36 program. The USEARCH-fastq_mergepairs tool was used to combine forward and reverse

244   readings. All reads with an expected error greater than 1 were removed, as well as chimeric reads

245   and singletons. Combined readings without a merging pair were filtered using fastq_filter

246   command. The -cluster_otus tool was used to cluster readings into operational taxonomic units

247   (OTUs) with 97% identity. The -unoise3 tool was used to cluster readings into zero-radius OTU

248   (ZOTU) 100% identity. OTUs were then designated with the lowest taxonomic rank using the

249   UCULT algorithm implemented in QUIME 1.9.1 (Caporaso et al., 2010b, Edgar, 2010) along

250   with SILVA reference database version 132 (Quast et al., 2012). Using SILVA v. 132 database,

251   PyNAST (Caporaso et al., 2010a) with default options was used for sequence alignment. The

252   phylogenetic trees of ZOTUs and OTUs were assembled using the default options of QUIIME

253   1.9.1 with the FastTree program (Price et al., 2009). Alpha and beta diversities were rarefied

254   with QUIIME 1.91 -single_rarefaction.py using the --subsample_multinomial option in order to

255   subsample the replacements. Rarefaction for all samples was performed to the depth of 4,500

256   readings. This was the lowest possible number of readings between samples.

257   **Statistical Analysis**

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258   **Data transformation.** Normality tests, data transformations and statistical models were done

259   with JMP Pro 14.0. Phenotype measurements were tested for normality with the Shapiro-Wilk

260   test and an outlier box plot. Only larvae survival (**ST. 1**) showed normal distribution. Therefore,

261   all other phenotypic measurements data were transformed. We performed a cube root

262   transformation on the data for development rate and glucose by weight, a square root

263   transformation on data for weight and protein by weight, and a log transformation on data for

264   triglyceride by weight levels. The microbial abundance was $\log(x+1)$ transformed for all

265   parametric analyses.

266   **Microbial Diversity**. Alpha and beta diversities were computed in QIIME v. 1.9.1. To estimate

267   alpha diversity, we used Shannon, Simpson, and PD Whole Tree metrices. As all of the alpha

268   diversity indices were not normally distributed, we performed a pairwise comparison of them

269   with a Wilcoxon rank-sum test in R v. 3.5.1 with "matrixTests" package v. 0.17 (Bruno et al.,

270   2019). Beta diversity was estimated with Bray-Curtis and Weighted Unifrac distances. The

271   similarity between each sample's beta diversity distance was evaluated via hierarchical

272   clustering, applying a ward method for distance calculation, and visualized with a constellation

273   plot in JMP v. 14.0.

274   **Statistical modeling:** In order to evaluate the contribution of each variable and their interactive

275   effect on each phenotypic development, we used standard least squares model with model effects

276   to include Diet (D), Genotype (G), Sterilization Treatment (T) and their specific interactive

277   effect: D*G (diet by genotype), D*T (diet by treatment), G*T (genotype by treatment), D*G*T

278   (diet by genotype by treatment). In order to verify that the built model fits the data, a Lack of Fit

279   test was performed. If the time period of the experiment (R) and/or the variance between the

280   colorimetric assay runs (triglyceride, protein, and glucose) (P) produced a significant effect,

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281   these variables were included in the model's effects, unless their addition caused the model to

282   fail the lack of fit test. Thus, the models for larvae survival, development time, and weight were

283   the following:

284    $$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D * G)_{4i} + \beta_5 (D * T)_{5i} + \beta_6 (G * T)_{6i}$$

285    $$+ \beta_7 (D * G * T)_{i7} + \varepsilon_i$$

286   For triglyceride by weight:

287    $$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D * G)_{4i} + \beta_5 (D * T)_{5i} + \beta_6 (G * T)_{6i}$$

288    $$+ \beta_7 (D * G * T)_{i7} + \beta_8 P_{8i} + \varepsilon_i$$

289   And for protein and glucose by weight:

290    $$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D * G)_{4i} + \beta_5 (D * T)_{5i} + \beta_6 (G * T)_{6i}$$

291    $$+ \beta_7 (D * G * T)_{i7} + \beta_8 P_{8i} + \beta_9 R_{9i} + \varepsilon_i$$

292   Where y_i is the response, β values are constants, and ε_i is a random error term.

293   All models for interactive effects of diet, genotype, and treatment were done with all 10 genetic

294   lines, with the exception of glucose which was done without 861 due to the low survival rate of

295   this genetic line.

296   In order to verify that the built model fits the data, a Lack of Fit test was performed. Only the

297   models with non-significant lack of fit p-value were kept and used for an evaluation. To assess

298   the pairwise difference between diets (R vs PR and PR vs PA) and treatments (NS vs S), we used

299   the least square model with one main explanatory variable of interest (Diet or Treatment). We

300   also included time period and assay plate variance as additional explanatory variables if they

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301   produced a significant effect. We then used Post-Hoc pairwise comparisons to evaluate the

302   Student's t-test.

303   **Microbial abundance.** In order to evaluate if the diet and treatment could serve as categorical

304   predictors for classification of the larvae microbial samples, we performed discriminant analysis

305   at phylum, class, order, family, and genus taxonomic levels, as well as at the level of individual

306   ZOTUs. The results were visualized with a canonical plot in JMP v. 14.0 (JMP manual). For the

307   10 most abundant representatives of each taxonomic level, we applied the linear covariance

308   method for the discriminant analysis which allowed us to visualize the covariates in the form of

309   rays. Using this method allowed us to represent which of them drove the separation of the

310   clusters (JMP manual). When we ran the analysis with all identified taxa, we applied a wide

311   linear method for the discriminant analysis. To compare the abundance of microbial taxa

312   between the diets and treatments, we performed a Wilcoxon test as described above. The p

313   values were adjusted for the false discovery rate with Benjamini-Hochberg correction and added

314   to data tables as FDR p. The threshold for the value of FDR p that should be considered

315   significant could be subjective and vary from 0.25 to 0.05 among microbiology studies (Wu et

316   al., 2011, Bruce-Keller et al., 2015). To evaluate the interactive effect of the variables on the

317   abundance of each identified microbial taxa, we used the three-way interaction model.

318
$$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D*G)_{4i} + \beta_5 (D*T)_{5i} + \beta_6 (G*T)_{6i}$$

319
$$+ \beta_7 (D*G*T)_{i7} + \beta_8 R_{8i} + \varepsilon_i$$

320   In order to identify the correlations between phenotypes and microbial abundances, we found the

321   average phenotype for each combination of diet, genetic line, treatment, and round and aligned it

322   with the microbial sequences of the corresponding combination of independent variables.

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323   Spearman's rank correlation between the microbial abundances and tested phenotypes was

324   calculated with Hmisc v. 4.3-0 in R v. 3.5.1, with the adjustment of p values for FDR p as

325   described above. We also tested the possible interactive effect of each identified microbial taxa

326   and one of the independent variables on the formation of the tested phenotypes according to the

327   formula:

328    $y_i = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_3 (x_{1i} * x_{2i})_{3i} + \beta_4 R_{4i} + \varepsilon_i$

329   where, x_1 was the abundance of the microbial taxa, x_2 was one of the independent variables

330   (D, G, or T) and R was time component. Development, weight, triglyceride, protein, and

331   glucose were normalized with log, square root, log, log, and cube root, transformations

332   respectively.

333   ## **Results**

334   **1) The influence of a natural diet with a naturally occurring and/or maternally inherited**

335   **community of microbes on the life history and metabolic phenotypes of flies relative to a**

336   **standard lab diet**

337   **1.1** Larvae raised on a natural diet exhibited different life history traits and metabolic phenotypes

338   compared to larvae raised on a standard lab diet. **Survival.** The number of larvae that survived

339   on the lab diet was significantly higher than the larval survival on a natural peach diet regardless

340   of sterilization (NS: p= 0.001, S: p= 0.0079) (Fig. 1A, Table 1.1, Sup. Table 1.1). The

341   undisturbed microbial community of the peach diet produced a significant positive effect on

342   larvae survival, when compared with the autoclaved peach diet for both sterilized and non-

343   sterilized larvae (NS: p= 0.0001, S: p< 0.0001) (Fig. 1A, Table 1.1, Sup. Table 1.1).

344   **Development rate.** Within both controlled and sterilized treatments, larvae developed faster on

345   the regular lab diet compared to the natural diet (NS: p= 0.0002, S: p< 0.0001) (Fig. 1B, Table

346   1.1, Sup. Table 1.1) and faster on the original peach food compared to the autoclaved diet (NS:

347   p< 0.0001, S: p< 0.0001) (Fig. 1B, Table 1.1, Sup. Table 1.1). **Weight.** Larvae raised on the lab

348   food were significantly heavier than those that were raised on the natural diet (NS and S: p<

349   0.0001) (Fig. 1C, Table 1.1, Sup. Table 1.1). Among the peach diets, larvae consuming the

350   autoclaved diet were significantly lighter (NS and S: p< 0.0001) (Fig. 1C, Table 1.1, Sup. Table

351   1.1), suggesting that under natural nutritional conditions, microbiota in the food substrate

352   facilitate growth and weight gain of the larvae. **Triglyceride.** Although fresh and incubated R

353   food had higher triglyceride levels than the PR food (both p< 0.0001) (Sup. Table 1.2, 1.3),

354   larvae raised on the PR diet had significantly higher triglyceride concentrations by weight than

355   those that were raised on a lab diet, independent of sterilization treatment (NS and S: p< 0.0001)

356   (Fig. 1D, Table 1.1, Sup. Table 1.1). Incubated autoclaved peach food had significantly higher

357   triglyceride content (p< 0.0001) comparing with regular peach food (Sup. Table 1.3) and

358   produced larvae with higher triglyceride by weight levels (NS and S p< 0.0001) (Fig. 1D, Table

359   1.1, Sup. Table 1.1). **Protein.** Independent of the treatment, larvae raised on the regular food had

360   higher protein by weight levels compared to larvae raised on peach food (NS: p< 0.0045, S: p<

361   0.0001) (Fig. 1E, Table 1.1, Sup. Table 1.1). Larvae raised on the PA diet had significantly

362   higher protein by weight levels compared to PR raised larvae, but only in the absence of parental

363   microbiota (NS: p= 0.099, S: p< 0.0001) (Fig. 1E, Table 1, Sup. Table 1.1). Evaluating the

364   difference in fresh food protein content, we found significantly higher protein concentration in

365   the regular lab diet compared to the peach diet (p< 0.0001) (Sup. Table 1.2). Between fresh

366   peach diets there was no significant difference (Sup. Table 1.2). However, after incubation,

17

367   autoclaved peach food had significantly less protein than regular peach food (p= 0.0389) (Sup.

368   Table 1.3). **Glucose.** Larvae raised on a lab food diet had significantly higher glucose levels than

369   larvae raised on the peach food diet (NS: p< 0.0001, S: p< 0.0001) (Fig. 1F, Table 1, Sup. Table

370   1.1). Larvae that consumed PR food had lower glucose by weight levels compared with larvae

371   raised on the autoclaved version, which was consistent with our 1.1 hypothesis (NS: p= 0.0089,

372   S: p< 0.0001) (Fig. 1F, Table 1, Sup. Table 1.1). Interestingly, the fresh peach food had a

373   significantly higher glucose concentration than the lab food (p< 0.0001) (Sup. Table 1.2).

374   However, after incubating the peach food, the concentration of glucose was lower in PR than in

375   both R (p<0.0001) and PA (p< 0.0001) diets (Sup. Table 1.3), suggesting a strong impact from

376   the live microbial community.

377   **1.2** We observed that the presence of a maternally transmitted microbiota significantly impacted

378   larvae phenotypes, and that impact varied across dietary treatments. **Survival.** The parental

379   microbiota did not produce a significant effect on the larvae's survival on the lab diet or the non-

380   autoclaved peach diet (Fig. 1A, Table 1.2, Sup. Table 1.4), but its presence significantly

381   enhanced the overall survival of larvae on the autoclaved peach diet (p< 0.0001) (Fig. 1A, Table

382   1.2, Sup. Table 1.4). This suggested the existence of microbial taxa that were necessary for a

383   successful transition of the larvae through the instar stages under natural nutritional conditions,

384   and that these microbial species could be picked up from the food substrate if available and/or

385   inherited maternally. **Development rate.** Presence of parental microbiota on the peach diet

386   reduced the number of days necessary for larvae to reach the third instar stage (p= 0.0002) and

387   autoclaved peach diet (p< 0.0001) but not on a regular lab diet (Fig. 1B, Table 1.2, Sup. Table

388   1.4), suggesting that under natural nutritional conditions, maternal microbes might influence the

389   developmental rate independent of the microbiota acquired from the food substrate. **Weight.**

390   Maternally inherited microbiota produced a significant positive effect on larval weight on all of

391   the tested diets (R: p= 0.005, PR: p= 0.0003, PA: p< 0.0001) (Fig. 1C, Table 1.2, Sup. Table

392   1.4). This indicated the universality of their influence on larval growth across food substrates.

393   **Triglyceride.** Parental microbiota did not influence the triglyceride levels significantly on any

394   diet (Fig. 1D, Table 1.2, Sup. Table 1.4). **Protein.** Evaluating the role of parental microbiota, we

395   observed that sterilized larvae had higher protein by weight levels but only on the PA diet (p=

396   0.0014) (Fig. 1E, Table 1.2, Sup. Table 1.4). This suggested that the core microbiota involved in

397   a natural metabolic phenotype formation might be inherited or acquired from the environment.

398   **Glucose.** The parental microbiota reduced the glucose by weight levels only on PA food (p=

399   0.001) (Fig. 1F, Table 1.2, Sup. Table 1.4), indicating that both parental and environmental

400   microbial taxa might be sufficient to reduce glucose levels in larvae.

401   **1.3** Evaluating the contribution of tested independent variables on larvae phenotypes, we

402   observed a genetic variation in most of the tested life history traits and phenotypes that interacted

403   with the dietary conditions and the availability of maternally transmitted microbiota. **Survival.**

404   All of the independent variables included in the model produced a significant effect on the

405   larvae's ability to survive until the late third instar stage (for diet, genetic line, treatment, diet by

406   genotype, and the interactive effect of diet by genetic line p< 0.0001, genetic line by treatment

407   p= 0.0374, and the interactive effect of diet, genetic line and treatment p< 0.0009). Out of the

408   tested variables, diet was the strongest predictor of survival (variance explained (VE) 28.4%),

409   followed by the interactive effect of the diet by treatment (VE= 8.02%) and genetic line (VE=

410   5.13%) (Table 1.3, Sup. Table 1.5). **Development.** The development rate of the larvae was

411   significantly influenced by diet (p< 0.0001), genotype (p< 0.0001), and treatment (p< 0.0001)

412   (Table 1.3). Among the specific interaction of these variables, only D*T (p< 0.0001) and G*T

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413   (p= 0.0374) produced a significant effect on development (Table 1). Diet was the key factor that

414   influenced the time necessary for the larvae to reach the late third instar stage and explained

415   almost half of all variance (VE= 47.5%) followed by the genetic line (VE= 4.98 %, Table 1). The

416   combination of the rest of the variables was responsible only for 8.42 % of variation in

417   developmental time (Table 1.3, Sup. Table 1.5).

418   **Weight.** All of the independent variables, with the exception of D*T, produced a significant

419   effect on dry larval weight (p< 0.0001) with diet being the best predictor (VE= 31.4%), followed

420   by genotype (VE= 7.44%), and D*G interaction (VE= 2.88%) (Table 1.3, Sup. Table 1.5).

421   **Triglyceride.** Once again, diet explained the largest portion of variance (VE= 41.4%) across all

422   independent variables. The interactive effect of D*G was a better predictor of triglyceride levels

423   than the genotype (VE= 5.03% and 2.36%, respectively) (Table 1.3, Sup. Table 1.5). **Protein.** In

424   contrast with other measured phenotypes, the variance explained by the model was

425   predominantly evenly distributed across the independent variables, with genotype having the

426   highest predicting power (VE= 5.71%) followed by D*G interactive effect (VE= 5.09%) (Table

427   1.3, Sup. Table 1.5). **Glucose.** Diet was the strongest predictor of larvae glucose levels (VE=

428   17.8 %, p< 0.0001) (Table 1.3, Sup. Table 1.5). Other variables that produced a significant effect

429   on glucose levels were treatment (p= 0.0009), D*G (p = 0.05), and D*T (p= 0.0005) (Table 1.3,

430   Sup. Table 1.5).

431   **2) The symbiotic microbiota community composition of the larvae raised on the natural**

432   **diet was different from the lab food raised larvae and was influenced by maternally**

433   **inherited microbiota and the host's genotype.**

20

434 **2.1** The gut microbial community composition and diversity varied substantially across dietary

435 and treatment conditions. **Alpha diversity.** We characterized a total of 6,763 unique ZOTUs

436 across the whole dataset with the number of ZOTUs per sample ranging from 55 to 886. The

437 total number of reads per sample ranged from 4,685 to 908,308. Of the ZOTUs that could be

438 assigned a taxonomic classification, we identified 134 classes, 218 families, and 394 genera

439 represented across the samples. The response of alpha diversity to changing diets varied with the

440 larval sterilization treatment. For NS larvae, we found that the Shannon index of larvae raised on

441 PR and R diets was significantly higher than those raised on the PA diet (p= 0.0478 and p=

442 0.0252) (Sup. Table 2.1). All other comparisons were not significant. However, if the embryos

443 were subjected to sterilization, the microbial species richness of larvae raised on the PA diet was

444 significantly higher than larvae raised on the PR diet (p= 0.0371) (Sup. Table 2.1). In addition,

445 there was no significant difference in microbial species richness between larvae raised on regular

446 or peach regular diets (Sup. Table 2.1). We observed the exact same pattern for the PD whole

447 tree index. Larvae raised on any diet were not significantly different in Shannon's index (Sup.

448 Table 2.1).

449 **Beta diversity**. The hierarchical clustering of the Bray-Curtis distances indicated that the most

450 distant microbial communities were formed between larvae raised on the R and PR diet (Fig.

451 2A). This pattern held true for both sterilized and non-sterilized larvae (Fig. 2A). Clustering

452 Weighted Unifrac distances suggested that PR and R diets may produce symbiotic microbial

453 communities that were phylogenetically distant from each other, especially if the parental

454 microbiota had been removed (Fig. 2B).

455 **Taxa composition**. Applying discriminant analysis on the ten most abundant microorganisms at

456 each taxonomic level revealed which organisms were largely responsible for the differentiation

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457   of the microbial composition on the canonical plot, based on diet. Thus, PR food is largely

458   defined by the abundance of Cyanobacteria at the phylum level (Sup. Fig. 1),

459   Epsilonproteobacteria at the class level (Sup. Fig. 2), Streptophyta at the order level (Sup. Fig.

460   3A, 3B), Leuconostocaceae sequences at the family level (Sup. Fig. 4), and *Leuconostoc* at the

461   genera level (Fig. 3A, Sup. Fig. 5). In turn, the lab diet was defined by Firmicutes, Bacilli,

462   Lactobacillales, Lactobacillaceae, and *Lactobacillus* respectively (Sup. Fig. 1-5, Fig. 3A).

463   Interestingly, when we considered only the 10 most abundant organisms at each taxonomic level,

464   we did not see a full separation between R and PA diets unless the larvae were sterilized (Sup.

465   Fig. 1-5). If parental microbiota were removed, the differentiation of the PA diet was led by

466   Actinobacteria, Actinobacteria and Alphaproteobacteria, Clostridiales and Rickettsiales,

467   Lachnospiraceae and Rickettsiaceae, and *Bacteroides* and *Wolbachia* (Sup. Fig. 1B-5B, Fig. 3A).

468   Including all identified bacterial groups in the discriminant analysis revealed that diet was a good

469   predictor of bacterial taxa composition at phylum, class, order, family, genus, and even

470   individual ZOTU levels (Fig 3B, Sup. Fig.6-11).

471   Overall, for non-sterilized larvae the abundance of eight phyla, 12 classes, 20 orders, 27 families,

472   40 genera, and 141 ZOTUs were significantly different between PR and R food (Sup. Tables 3.1-

473   3.6). Comparing PR and PA food, we found that the abundance of four phyla, six classes, nine

474   orders, 16 families, 20 genera, and 76 ZOTUs were significantly different (Sup. Tables 3.1-3.6).

475   Lastly, we observed the significant difference for the abundance of four phyla, three classes, five

476   orders and families, three genera, and 27 ZOTUs between R and PA food (Sup. Tables 3.1-3.6).

477   This indicated the minimal difference between microbial communities of these diets to be

478   consistent with the discriminant analysis.

22

479   In larvae lacking parental microbiota, we observed that the abundance of six phyla, 10 classes,

480   18 orders, 34 families, 37 genera, and 114 ZOTUs were significantly different between lab and

481   peach diets (Sup. Tables 3.1-3.6). Comparing PR and PA diets, we found a significant difference

482   in abundance of seven phyla, 14 classes, 32 orders, 48 families, 67 genera, and 200 ZOTUs (Sup.

483   Tables 3.1-3.6). R and PA diets were significantly different in the abundance of five phyla, six

484   classes, nine orders, 13 families, 18 genera, and 87 ZOTUs (Sup. Tables 3.1-3.6).

485   **2.2** The maternally transmitted microbiota influenced the composition of the larvae's symbiotic

486   microbiota communities. **Alpha diversity.** S larvae had higher values for species richness,

487   Shannon, and PD whole tree indexes on the PA diet (Sup. Table 2.2). NS larvae had a

488   significantly higher Simpson index value on the PR diet (Sup. Table 2.2). All other comparisons

489   were not significantly different. **Beta diversity.** When comparing the difference between beta

490   diversity metrics in NS and S treatments for each diet, we observed a distinctive clustering,

491   based on the treatment of samples that were raised on PA food for Bray-Curtis Distance (Fig.

492   4A) and Weighted Unifrac distance (Fig. 4D). For the samples that were raised on R food, we

493   observed the clustering for Weighted Unifrac distance only (Fig 4F).

494   **Taxa composition.** The discriminant analysis indicated that the status of inheritance of the

495   parental microbiota could serve as a good predictor for differentiation of the bacterial community

496   as indicated with the canonical plot on all taxonomic levels (phylum, order, class, family, genus,

497   and ZOTU) (Fig.5 A-C, Sup. Fig. 12A-17C). Among the 10 most abundant phyla that defined

498   the differentiation of the NS community were Firmicutes and Bacterioidetes on the PA diet (Sup.

499   Fig. 18A), Firmicutes on the PR diet (Sup. Fig. 18B), and Proteobacteria, Tenericutes, and

500   Flusobacteria on the R diet (Sup. Fig. 18C). Phyla that were influential for differentiation of the

501   S community were Actinobacteria, Flusobacteria and Cyanobacteria on the PA diet (Sup. Fig.

23

502   18A), Actinobacteria, Tenericutes, and Flusobacteria on the PR food (Sup. Fig. 18B), and

503   Planctomycets and Bacterioides on the R diet (Sup. Fig. 18C). Considering bacterial classes, the

504   NS treatment was strongly defined by Bacilli on the PA (Sup. Fig. 19A) and the PR diets (Sup.

505   Fig. 19B), and Alphaproteobacteria and Bacteroida on the R food diet (Sup. Fig. 19C). The

506   sterilization treatment was mostly separated due to Actinobacteria on the PA diet, Alpha and

507   Beta proteobacteria on the PR diet, and Gammaproteobacteria on the R diet (Sup. Fig. 19A-C).

508   On R food, microbial communities from the sterilized and non-sterilized larvae were not fully

509   separated on the canonical plot (Sup. Fig. 19C). At the order level, the NS community was

510   defined by Lactobcillales on the PA and the PR diets, and Actinomycetales and Rhodospirillales

511   on the R food (Sup. Fig. 20A-C). Sterilized larvae were associated with abundances of

512   Streptophyta on the PA food, as well as Burkholderiales and Rhodospirillales on the PR diet

513   (Sup. Fig. 20A-B). On the R diet, four out of ten tested orders were strongly associated with the

514   S treatment (Sup. Fig. 20C). At the family level, the NS larvae were correlated with

515   Lactobacillaceae on the PA and the PR diets and Acetobacteraceae on the R food diet (Sup. Fig.

516   21A-C). Sterilized larvae were defined by the abundance of Nocardiaceae on the PA food and

517   Leuconostocaceae and Caulobactereceae on the R diet (Sup. Fig. 21A-C). On the genera level,

518   NS was primarily separated by *Lactobacillus* on the PA diet and *Acetobacter* and *Agrobacterium*

519   on the R food (Sup. Fig. 22A, C). On the PR food diet, 95% confidence ellipses almost

520   overlapped, indicating that sterilization status might not be the decisive predictor for abundance

521   of the 10 most common genera (Fig. 5E). The S treatment was primarily defined by the

522   abundance of *Leuconostoc* and *Gluconobacter* on the PA diet and *Lactobacillus* and *Leuconostoc*

523   on the R diet (Fig. 5 A, C, Sup. Fig. 22A, C).

524 Multiple microbial groups were significantly different in their distribution between NS and S

525 treatments across the diets on all taxonomic levels. On regular food, there existed an abundance

526 of four phyla (Sup. Table 4.1), four classes (Sup. Table 4.2), five orders (Sup. Table 4.3), six

527 families (Sup. Table 4.4), nine genera (Sup. Table 4.5), and 41 ZOTUs (Sup. Table 4.6). On the

528 PR diet we saw a significant difference in the abundance of one phylum (Sup. Table 4.1), two

529 classes (Sup. Table 4.2), six orders (Sup. Table 4.3), nine families (Sup. Table 4.4), 11 genera

530 (Sup. Table 4.5), and 61 ZOTUs (Sup. Table 4.6). The highest number of significantly different

531 taxa was observed on the PA diet with seven phyla (Sup. Table 4.1), 12 classes (Sup. Table 4.2),

532 21 orders (Sup. Table 4.3), 34 families (Sup. Table 4.4), 43 genera (Sup. Table 4.5), and 148

533 ZOTUs (Sup. Table 4.6).

534 **2.3** The composition of the microbial community exhibited variation with host genotype, which

535 further exhibited a significant interactive effect with diet and treatment. We also tested the

536 influence of genotype and other variables' interactive effect on the abundance of microbiota. At

537 the phyla level, 14 were significantly influenced by genotype, three by D*G interaction, five by

538 G*T, five by D*T, and six by D*G*T (Sup. Table 5.1). Abundances of 30 classes were

539 significantly influenced by genotype, eight by D*G, G*T, and D*T, and 10 by D*G*T

540 interaction (Sup. Table 5.2). Among the orders, an abundance of 46 was significantly influenced

541 by genotype, 13 by D*G, 15 by G*T, 16 by D*T, and 15 by D*G*T (Sup. Table 5.3). The

542 abundance of 72 families was significantly influenced by genotype, 20 by D*G, 15 by G*T, 18

543 by D*T, and 23 by D*G*T (Sup. Table 5.4). Lastly, genotype significantly influenced 94 genera,

544 D*G influenced 44, G*T influenced 46, D*T influenced 30, and D*G*T influenced 45 genera

545 (Sup. Table 5.5).

25

546   **3.1** We identified microbial taxa that exhibited correlations with host phenotype across diets and

547   treatments, with many that had a diet, treatment or genotype specific relationship. Across the NS

548   larvae, we found four significant interactions on the R food, 14 on PR diet, and 14 on PA diet at

549   the phylum level (Sup. Table 6.1). For the S larvae at the same taxonomic level, we found eight

550   significant interactions on the R diet, five on the PR and two on PA food (Sup. Table 6.1). At the

551   class taxonomic level, nine significant interactions were found on R food, 35 on PR, and 24 on

552   PA diet (Sup. Table 6.2). Considering S larvae, there were 23 significant correlations on the R

553   diet, 11 on PR, and four on the PA diet (Sup. Table 6.2). For NS larvae at the order level, we

554   found 23 significant correlations on the R diet, 57 on PR, and 46 on PA diet (Sup. Table 6.3). For

555   S larvae we found 29 significant correlations on R food, 22 on PR, and nine on PA diet (Sup.

556   Table 6.3). At the family level, we found 34 significant correlations on R food, 88 on PR, and 67

557   on PA diet for NS larvae (Sup. Table 6.4). For S larvae, we observed 51 significant interactions

558   on R food, 29 on PR, and 14 on PA diets (Sup. Table 6.4). Across the genera, we found 40

559   significant interactions on R, 105 on PR, and 64 on PA diets, for NS larvae (Fig. 6-7, Sup. Table

560   6.5). Considering S larvae, we found 76 significant interactions between tested taxa and

561   phenotypes on R, 46 on PR, and 33 on PA diets (Fig. 6-7, Sup. Table 6.5). At the level of

562   individual ZOTUs, for NS larvae, we found 226 significant interactions on R, 283 on PR, and

563   225 on PA diets (Sup. Table 6.6). For S larvae the number of significant interactions between

564   ZOTUs abundances and larvae phenotypes were as follow 313 on R, 164 on PR, and 230 on PA

565   diets (Sup. Table 6.6).

566   We evaluated the interactive effect of the abundance of microbial taxa and diet, genotype, and

567   treatment on forming the tested phenotypes. D*A produced a significant effect in 11 cases at the

568   phylum level (Sup. Table 7.01), in 26 at the class level (Sup. Table 7.02), in 52 at the order level

569   (Sup. Table 7.03), in 72 at the family level (Sup. Table 7.04), and in 95 cases at the genus level

570   (Sup. Table 7.05). We found a significant G*A interaction in eight cases at the phylum level

571   (Sup. Table 7.06), in 10 at the class level (Sup. Table 7.07), in 39 at the order level (Sup. Table

572   7.08), in 57 at the family level (Sup. Table 7.09), and in 105 cases at the genus level (Sup. Table

573   7.10). T*A produced a significant effect in 13 cases at the phylum level (Sup. Table 7.11), in 27

574   at class level (Sup. Table 7.12), in 36 at the order level (Sup. Table 7.13), in 60 at the family

575   level (Sup. Table 7.14), and in 87 cases at the genus level (Sup. Table 7.15).

Discussion

576

577   **1.1: Overall, it appeared that frozen peach food was capable of providing nutritional**

578   **conditions similar to the natural ones and can preserve key microbial taxa necessary for**

579   **survival and development of *Drosophila* larvae.**

580   The reduction in survival, increase in development time and increase in triglyceride levels, as

581   well as decreased weight and protein levels of the larvae raised on the natural food compared

582   with the larvae raised on the R lab food resembles the phenotype generated by a reduced protein

583   diet. These findings correlate with our evaluation of the protein concentrations in different diets.

584   (Klepsatel et al., 2018, Bing et al., 2018, Skorupa et al., 2008, Sang, 1956). In addition, the

585   adaptation of *Drosophila* to the lab environment was connected to increased weight and reduced

586   stress tolerance (Sgro and Partridge, 2000, Hoffmann et al., 2001, Russell et al., 2012).

587   Therefore, nutritional and pathogenic stresses associated with the natural food conditions could

588   further contribute to the decrease in survival and development rate of larvae raised on the PR

589   food compared to the standard diet (Staubach et al., 2013, Bing et al., 2018, Pais et al., 2018,

590   Sang, 1956).

591   The pattern regarding glucose concentration was more interesting. Freshly unfrozen peach food

592   had a higher glucose concentration, but larvae raised on the PR diet had the lowest concentration

593   compared to larvae raised on any other diet. This pattern was likely caused by the activity of

594   naturally acquired microbes since it was shown that the presence of several microbial taxa that

595   naturally associate with *Drosophila,* such as *Acetobacter,* is correlated with decreased sugars in

596   fly food and *Drosophila* itself (Huang and Douglas, 2015, Dobson et al., 2015). In addition,

597   incubation of the PR food, even without the larvae, led to a drastic reduction of glucose levels

598   compared to the R and PA diets. Furthermore, the difference between all phenotypes (with the

599   exception of glucose) increased if the peach food diet was autoclaved and even more (with the

600   exception of triglyceride) if the parental microbiota were not transferred to the autoclaved diet.

601   This suggested that symbiotic microbiota drove the phenotypic change between PR and PA

602   raised larvae.

603   These findings are consistent with previous studies which showed that the presence of naturally

604   associated microbiota was advantageous for *Drosophila melanogaster* and *Drosophila suzukii* on

605   fresh fruit diets, which are also poor in protein content (Pais et al., 2018, Bing et al., 2018). In

606   fact, larvae raised on a PA diet closely resembled the phenotype of axenic larvae and axenic

607   larvae raised under low protein nutritional conditions. Examples of this similar phenotype

608   include lower survival and body size/weight (Shin et al., 2011, Wong et al., 2015, Dobson et al.,

609   2015), longer development time (Shin et al., 2011, Newell and Douglas, 2014a), elevated

610   glucose (Huang and Douglas, 2015) and triglyceride levels (Newell and Douglas, 2014a, Dobson

611   et al., 2015).

612   **1.2 Maternally deposited microbes produced positive effects on larvae that were raised on**

613   **the peach diets.**

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614   Interestingly, the presence of parental microbiota did not produce a significant effect on any of

615   the tested phenotypes, when larvae were raised on the lab diet. Contrarily, on the peach diet, the

616   presence of parental microbota increased the weight and development rate even if the original

617   peach microbiota were still present. These findings are consistent with the reports of beneficial

618   effects, of the maternally deposited microbiota, for larvae on a fruit diet. These results also

619   indicate the importance of considering an organism's natural environmental conditions when

620   addressing the questions about symbiotic relationships and evolutionary patterns (Pais et al.,

621   2018, Bing et al., 2018).

1.3 Genotype was one of the key factors that influenced larvae phenotypes.

623   It is important to note that although the described patterns were observed for the total

624   experimental population of larvae, the genetic component still played a significant role in

625   generating all but the glucose phenotype. In addition, consistent with previous research, we

626   observed that D*G interaction played a significant role in forming metabolic phenotype as well

627   as contributed to the survival of the organism (Reed et al., 2010, Reed et al., 2014). Furthermore,

628   most of the tested phenotypes were significantly correlated with G*T and even D*G*T,

629   indicating the importance of considering multiple factors to understand the development of

630   complex traits.

2.1 Microbiota of the larvae raised on PR food exhibit a distinct community structure and might remind microbiota of wild flies.

633   Multiple studies were performed to evaluate the gut microbiota composition of lab and wild

634   populations of *Drosophila* (Chandler et al., 2011, Adair et al., 2018, Douglas, 2018, Pais et al.,

635   2018, Wong et al., 2013). Although most of them consistently report the prevalence of different

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636   members of Alphaproteobacteria, Bacilli, or Gammaproteobacteria in lab and wild populations,

637   the relative abundance of the taxa, especially at lower taxonomic levels, often varies between

638   studies (Douglas, 2018). In our work, larvae raised on the PR food diet formed a distinct

639   community clearly separated from the larvae raised on the R food diet, as displayed on the

640   canonical plot. We observed a higher prevalence of *Gluconobacter* and *Leuconostoc* and lower

641   abundance of *Lactobacillus* in larvae raised on the PR diet compared with the R food diet (Pais

642   et al., 2018, Staubach et al., 2013, Corby-Harris et al., 2007, Chandler et al., 2011), which is

643   consistent with previous findings performed on natural populations of *Drosophila.*

644   However, it is difficult to judge how well the microbial community of our experimental larvae

645   represent the microbial community of wild population, as the variety of factors could influence

646   gut microbiota composition in flies which certainly complicates the comparison between studies

647   (Douglas, 2018, Staubach et al., 2013, Jehrke et al., 2018). As such, it was shown that the gut

648   microbiota composition of lab reared flies may vary with diet (and even among the standard diets

649   with the major carbohydrate source), genetic line, development stage temperature, and etc.

650   (Jehrke et al., 2018, Douglas, 2018, Wong et al., 2011, Moghadam et al., 2018). The wild

651   populations of gut microbiota in *Drosophila* was shown to vary with collection location and diet

652   (Adair et al., 2018, Wong et al., 2013, Staubach et al., 2013, Martinez-Porchas et al., 2017). In

653   other insects and wild populations of vertebrates, gut microbiota was shown to change even with

654   seasonality (Behar et al., 2008, Ferguson et al., 2018, Tong and Zhang, 2019, Maurice et al.,

655   2015).

656   In addition, the relationship between *Drosophila* gut microbiota during the developmental and

657   adult stages is a subject of controversy between a few studies that compared those relationships

658   (Jehrke et al., 2018, Wong et al., 2011, Vacchini et al., 2017). Furthermore, to the best of our

30

659   knowledge, the gut microbiota of the larvae from the natural populations was not assessed at all.

660   This is likely due to the complexity of identifying *Drosophila* species during the larval stage.

661   Therefore, we hope to provide the methodology for the possibility of exploring the effects of a

662   natural diet, and the microbial community associated with it, in a controlled lab environment.

663   This setting provides the opportunity to work not only with adult flies but also with larvae.

664   **2.2-2.3 Community structure of symbiotic microbiota were correlated with diet, treatment,**

665   **host genotype and their specific interactive effects.**

666   The development of symbiotic microbiota populations was shown to be correlated with the

667   available nutrients present in the diet, the host's genotype and parental microbiota left on the

668   chorion of the egg (Douglas, 2018, Jehrke et al., 2018, Wong et al., 2015). Complementary to the

669   results reported by Jehkre (2018), we also observed that the genotype of the host may influence

670   the abundance of bacterial taxa more than the diet. Wong (2015) reported that the bacterial

671   population deposited on the *Drosophila* embryo may shift the symbiotic microbiota population

672   of the offspring, even in the presence of bacteria that previously colonized the food substrate. We

673   observed similar results in most cases.

674   However, among the 10 most abundant genera on the PR diet, the full separation of the S and

675   NS larvae microbial community compositions was not present on the canonical plot indicating

676   the possibility of a difference in the response of the lab and the natural microbial population to

677   the presence of *Drosophila* parental microbiota. This differentiation was not likely caused by the

678   nutrition composition of the food since the PA separation, represented on the canonical plot,

679   between S and NS treatments was obvious in all cases. In addition, for beta diversity distances,

680   the abundance of individual microbial taxa, as well as the correlations between the abundances

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681   of microbial taxa within the microbial community, represented patterns found in PA food that

682   resembled the ones in the R food raised larvae, if parental microbiota were not removed (Fig 4,

683   5, Sup. Fig.12-23). Overall, consistently with previous studies, our findings indicated the

684   dependency of relative microbial abundances on all of the tested variables and additionally on

685   the interactive effect between them (Wong et al., 2015, Jehrke et al., 2018, Douglas, 2018).

686   **3.1 The influence of individual microbial taxa as well as the influence of the whole**

687   **microbial community on the host may vary with the diet and other environmental and**

688   **genetic conditions.**

689   Genotype and gut microbiota composition are among the major factors that control the

690   development of obesity traits (Parks et al., 2013). Changes in some key microbiota populations

691   are associated with the rapid expansion in the prevalence of metabolic syndrome (Zhang et al.,

692   2010). Alterations in the gut microbiota community can modulate insulin secretion and

693   sensitivity, thus contributing to diabetes susceptibility (Kreznar et al., 2017). Moreover, previous

694   research indicates that genetic variation considerably influences the gut microbiota composition

695   (Zhang et al., 2010, Kreznar et al., 2017, Jehrke et al., 2018). However, most of the studies

696   mentioned above have used less than ten genotypes to study the correlation between gut

697   microbiota and the pathogenesis of obesity in mice. The challenges of using a mouse model

698   involve relatively high expenses for husbandry and logistics (Rosenthal and Brown, 2007, Berger

699   et al., 2005, Martin et al., 2016).

700   *Drosophila melanogaster* is an exceptional model to study the effect of genotype on the

701   phenotype formation, due to the variety of established tools such as Drosophila Genetic

702   Reference Panel and The Drosophila Synthetic Population Resource. These resources offer a

703   variety of diverse genotypes, with sequenced parental genomes, that allow for testing the

704   microbiota effects across various genetic backgrounds and provides potential for studying

705   genetic interaction between host and its symbionts, and even mapping the specific genetic loci

706   responsible for the interactions (Mackay et al., 2012, King et al., 2012, Chaston et al., 2016). The

707   phenotypic response to a diet modification often varies with the genotype (Reed et al., 2010,

708   Reed et al., 2014). In fact, diet by genotype (DxG) interaction may explain more variance than

709   diet alone in the metabolic response of such traits, such as triglyceride and carbohydrate

710   concentrations (Reed et al., 2010). In addition, recent findings showed that genotype by diet

711   interactions significantly influences metabolomic profiles; hence, laying the foundation for

712   explaining the mechanism through which DxG influences metabolic traits (Reed et al., 2014,

713   Williams et al., 2015). Our results are consistent with previous research, in that phenotypic

714   response varied significantly between genetic lines (Reed et al., 2014, Williams et al., 2015).

715   Genotype had a significant effect on survival, development rate, and triglyceride levels, and was

716   the second-best predictor of weight and the best predictor of protein levels.

717   Obesity and type two diabetes are associated with elevated weight, high blood glucose

718   concentrations, and excess accumulation of adipose tissue (Martyn et al., 2008, Akter et al.,

719   2017). Consistent with recent studies linking *Lactobacillus* and *Coprococcus* to obesity in

720   humans, our results show that these genera are positively associated with glucose levels (Million

721   et al., 2012, Ignacio et al., 2016, Murugesan et al., 2015, Armougom et al., 2009). In addition,

722   previous research has shown an overall decrease in the abundance of *Firmicutes* in obese humans

723   (Schwiertz et al., 2010). Similarly, we observed that the total abundance of *Firmicutes* is

724   negatively associated with triglyceride levels. It should be noted that the correlation between

725   metabolic phenotype and particular microbial taxa could vary between studies (Ley et al., 2006,

726   Furet et al., 2010, Turnbaugh et al., 2006).

727   Consistently with previous *Drosophila* research, we observed that the abundance of microbial

728   taxa was correlated with measured phenotypes. Such as *Acetobacteraceae* is negatively

729   correlated with larvae glucose levels (Chaston et al., 2016, Douglas, 2018, Chaston et al., 2014).

730   Additionally, *Acetobacter* increases development time while *Lactobacillus* and *Firmicutes*

731   decreases it (Chaston et al., 2014, Chaston et al., 2016, Newell and Douglas, 2014a, Storelli et

732   al., 2011). Previous work showed that *Acetobacter* species reduced triglyceride levels while most

733   *Lactobacillus* species had no effect (Chaston et al., 2016, Newell and Douglas, 2014a). In

734   contrast, our data shows that *Acetobacter* did not significantly affect triglyceride levels, and

735   *Lactobacillus* showed a negative correlation. Consistent with Newell and Douglas (2014b), we

736   found that *L. brevis* and *L. plantarum* had no significant effect on protein levels, but in addition,

737   our results indicated that the abundance *Acetobacter* was negatively correlated with the protein

738   levels.

739   Consistently with Jehrke (2018), we observed that most of the correlations between the tested

740   phenotype and abundance of microbiota are relatively weak. Weaker correlations observed with

741   large sample sizes in microbiome research, while significant, fail to hold up to the use of stricter

742   FDR values or other conservative p adjustment methods (Jehrke et al., 2018, Wu et al., 2011).

743   Expanding the analysis of the bacterial species abundance for each phenotype beyond the most

744   dominant species, while providing a more complete overview of the correlation between tested

745   phenotypes and microbial abundance, also raises FDR values as a result of increasing sample

746   sizes (Wu et al., 2011). Previous microbiome studies have dealt with high FDR values by

747   accepting higher thresholds, so as to not miss possible correlations (Wu et al., 2011). Since the

748   level of FDR that should be tolerated is poorly defined and often widely variable compared to

749   accepted p-values, its value is often seen as arguable (Pawitan et al., 2005). Considering the large

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750   sample sizes used in our analysis, using a low FDR value may obscure important correlations

751   between the tested phenotypes and their abundance of microbiota (Wu et al., 2011).

752   Some of the inconsistencies between our work and previous studies on the correlation of the

753   abundance of microbial taxa and measured metabolic phenotype, perhaps may be addressed to

754   the interactive effect between the variables included in the experiments. Several studies showed

755   that the contribution of the symbiotic microbiota to the host may be observed only in a diet

756   dependent manner. Such as Shin et al. (2011), showed that axenic *Drosophila* larvae would not

757   be able to develop on a protein poor diet without activation of the insulin signaling pathway by

758   its symbiotic microbe. Wong et al. (2014) found diet-dependent differences in microbiota

759   produced effect, including differences in vitamin microbial sparing on a low-yeast diet and

760   suppression of lipid and carbohydrate storage on a high-sugar diet. Bing et. al (2018) found that

761   symbiotic microbiota of *D. suzukii* are critical for providing proteins for development of flies

762   raised on fresh fruit, but that these microbial proteins are not essential for development of flies

763   raised on a nutrient sufficient diet.

764   In our study, we also observed that the effect that microbial abundance at the level of individual

765   taxa produced on larvae phenotype varied with the diet. In few cases even the direction of the

766   correlation between the abundance of microbial taxa and tested phenotype was opposite on

767   different diets. In addition, using PCA, we observed that correlational effects that microbial

768   abundance (as an example at the family level) produced on measured metabolic (Sup. Fig. 24A)

769   and fitness phenotypes (development rate and survival) (Sup. Fig 24B) varied between the diets.

770   The correlation coefficients for the influence of all microbial taxa on measured metabolic

771   phenotypes clustered together for the PR diet but not for other diets (Sup. Fig 24A). The

772   correlation coefficients between microbiota abundances and fitness phenotypes clustered for all

773   but PA diets (Sup. Fig 24B). Taking into account all of the described findings, perhaps in future

774   studies that will aim to understand the mechanisms for formation of metabolic phenotypes, we

775   will see a consistent control for the gut microbiota composition in a similar fashion as now we

776   can see it for genetic lines and nutritional composition of the food.

777   ## Acknowledgements:

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791 **Figures**
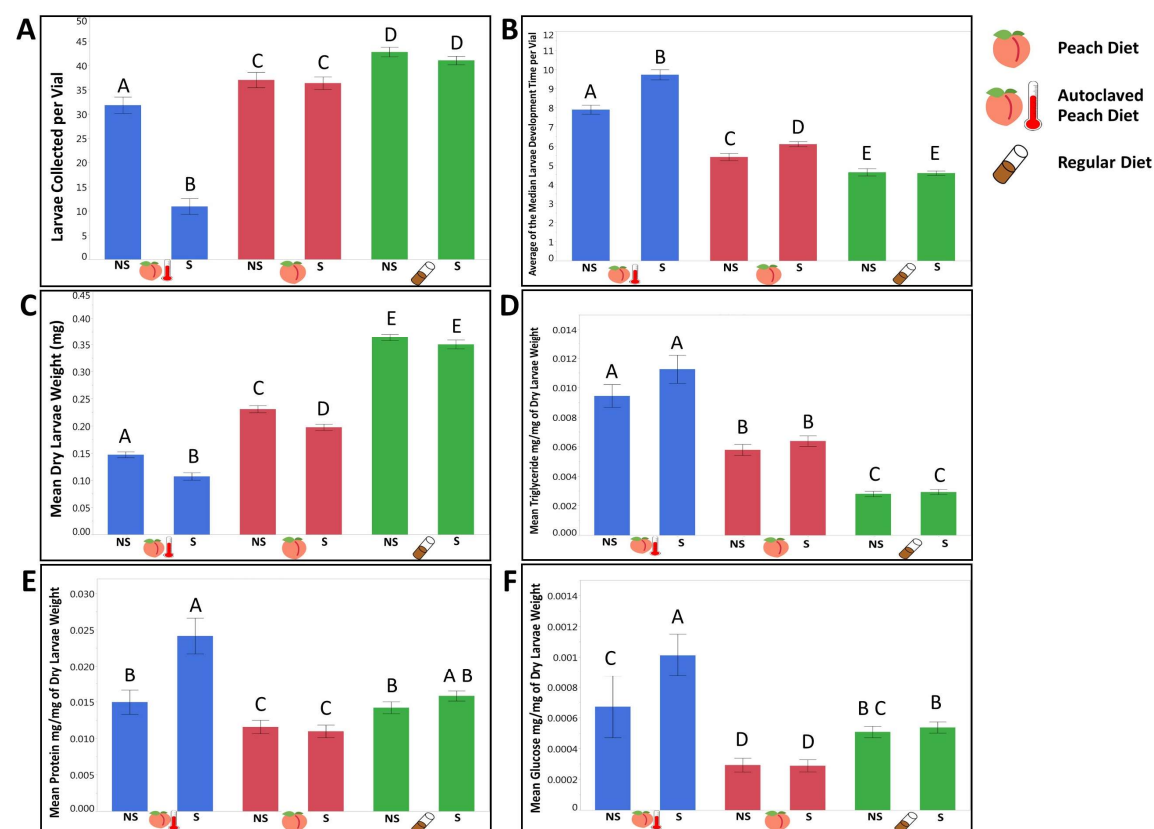
793 **Figure 1: The Influence of diet and treatment on larvae mean** A) survival until late 3rd instar

794 stage B) development time C) weight D) Triglyceride E) Protein F) Glucose per mg of dry larvae

795 weigh. Error bars indicated standard error.
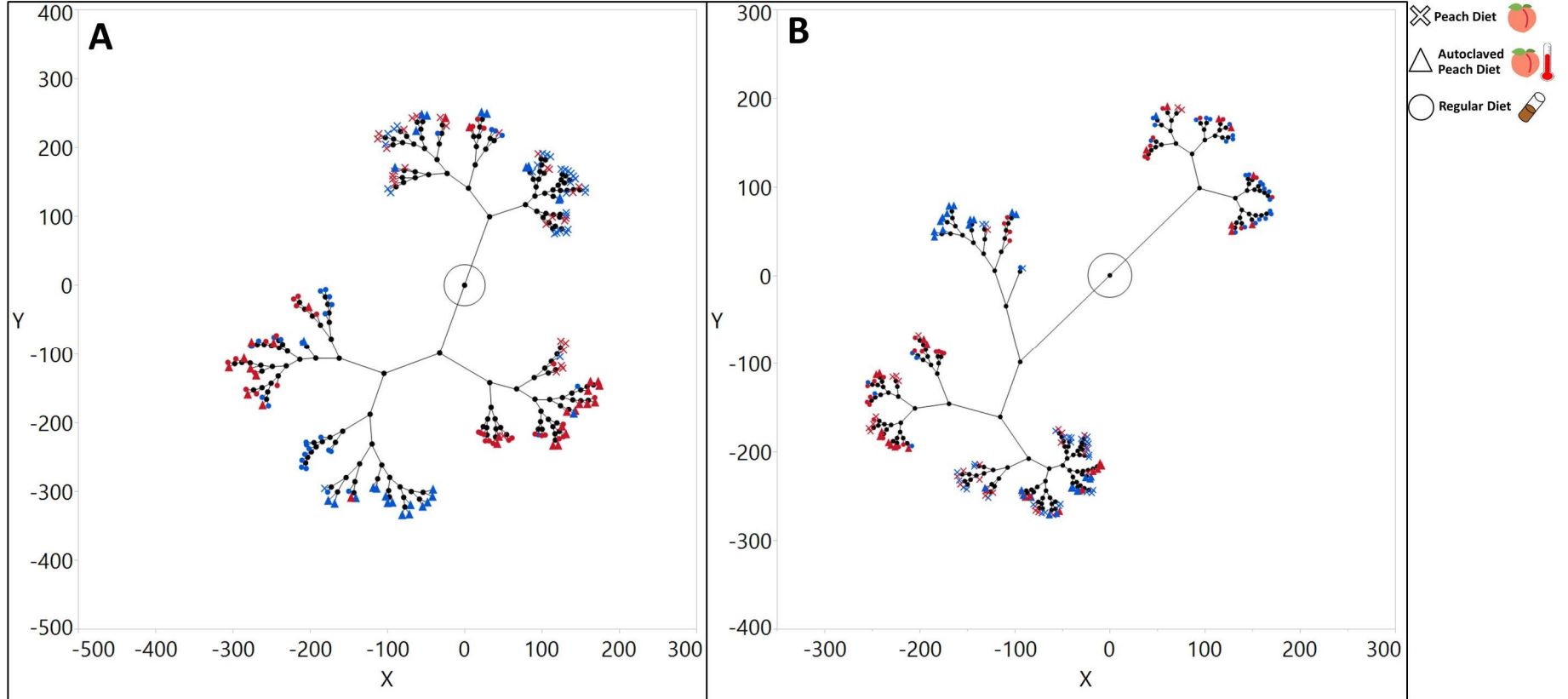
Figure 2: The influence of the diet on beta diversity distances between larvae symbiotic microbiota communities. Constellation plot based on hierarchal clustering of larvae microbiota community beta diversity distances A) Bray-Curtis distances B) Weighed Unifrac distances. Samples from sterilized larvae are marked with a blue color and samples from non-sterilized larvae are marked with the red color. Samples raised on regular lab diet are marked with circled, on a peach diet with crosses and on peach autoclaved diet with triangles.
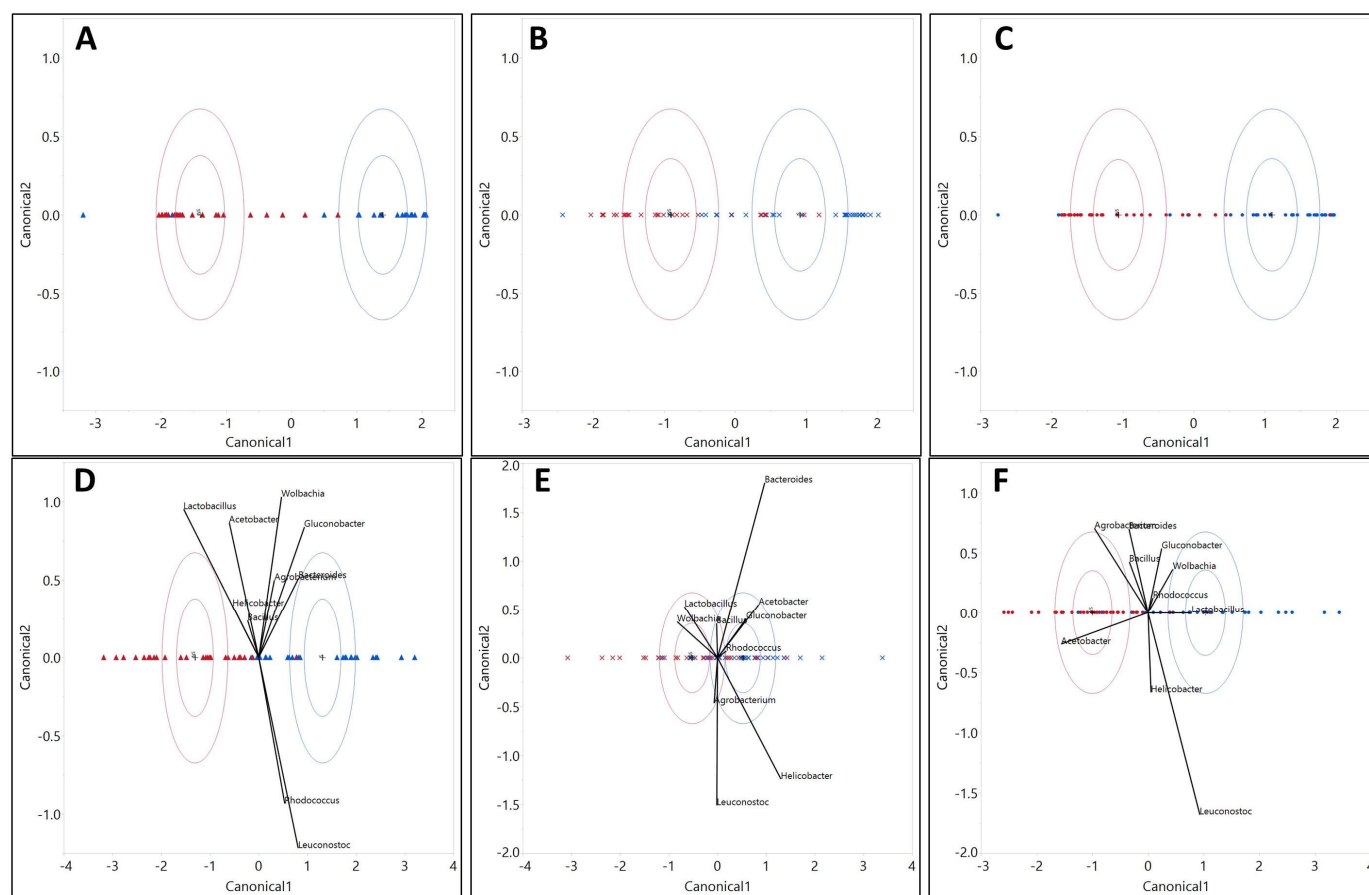
1
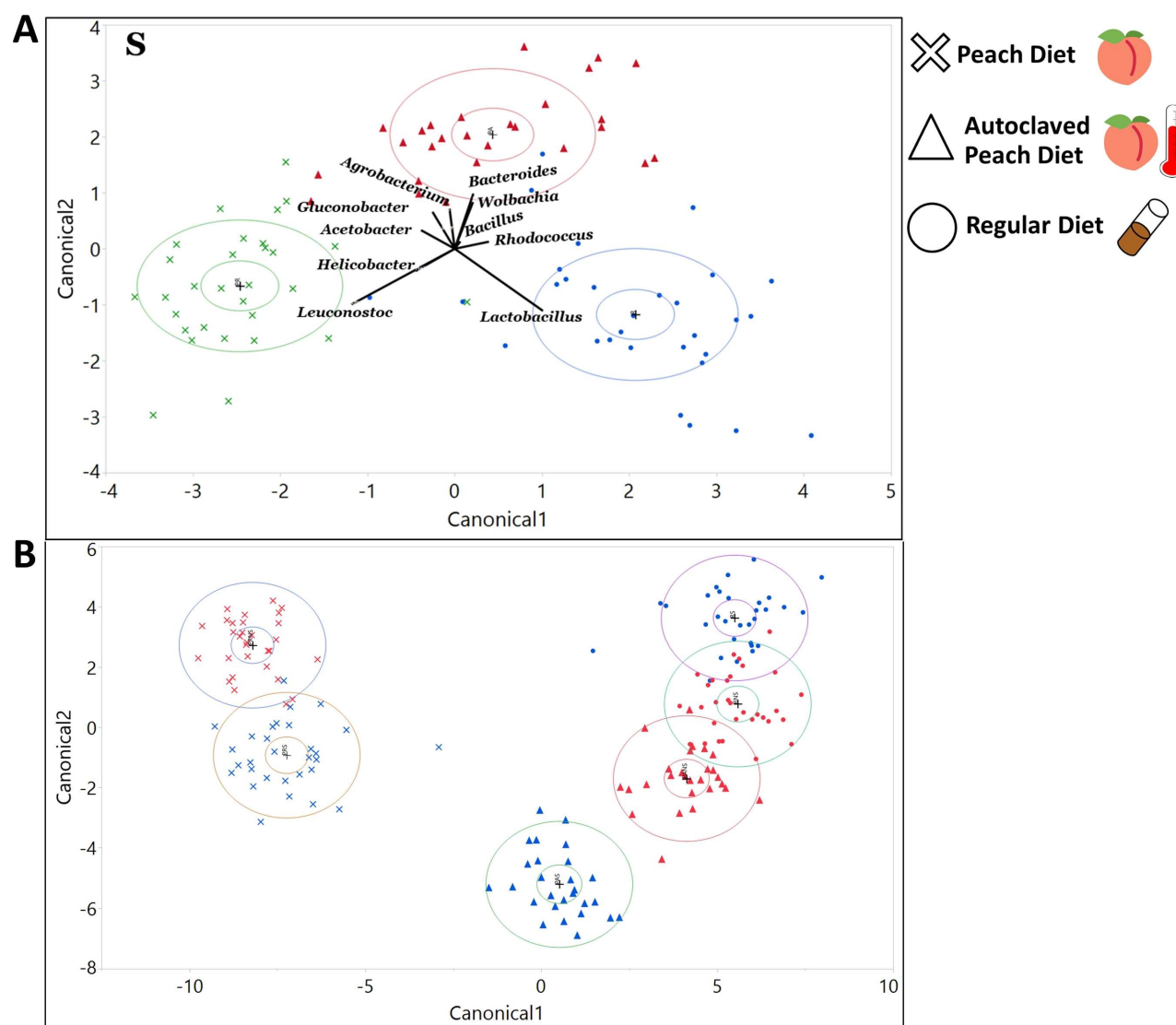
Figure 3: The influence of the diet on larvae symbiotic microbiota communities' composition. Discriminant analysis of symbiotic microbiota community based on taxa relative abundances in larvae from non-sterilized (red) and sterilized (blue) treatments. A) 10 most abundant microbial genera found in sterilized larvae. The length of the vector is correlated with the strength of the impact that it produced for the samples to be separated, in the vector direction, on the canonical plot B) 100 most abundant microbial zotu found in both sterilized and non-sterilized larvae. Non sterilized samples are marked with red color and sterilized larvae are marked with blue color.
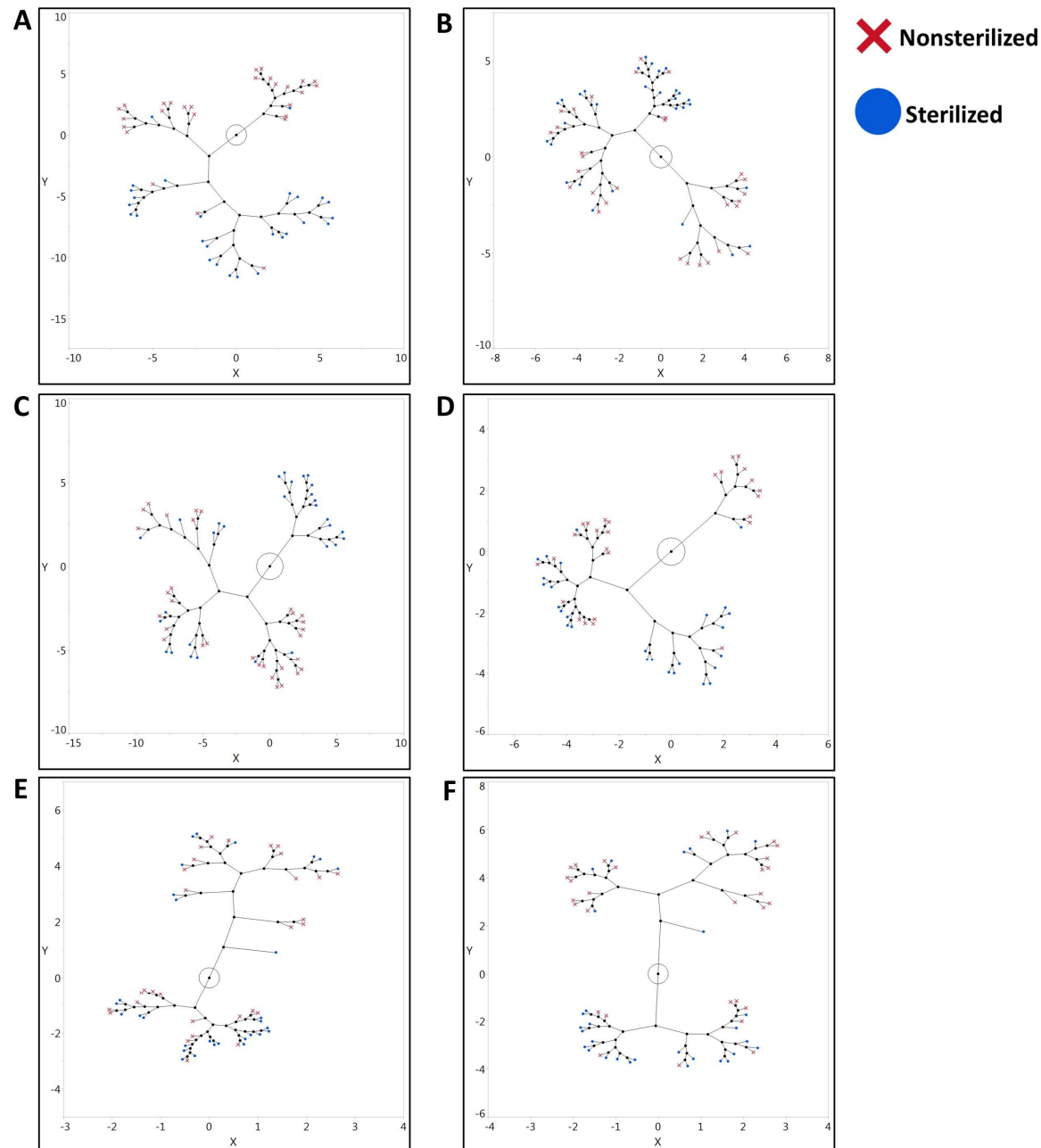
Figure 4: The influence of the treatment on beta diversity distances between larvae symbiotic microbiota communities. Hierarchal clustering of larvae microbiota community beta diversity distances A-C) Bray-Curtis distances between NS and S larvae raised on PA, PR, and R diets, respectively D-F) Weighed Unifrac distances between NS and S larvae raised on PA, PR, and R diets, respectively.

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Figure 5: The influence of the treatment on larvae symbiotic microbiota communities'

composition. Discriminant analysis of symbiotic microbiota community based on taxa relative

abundances in larvae from non-sterilized (red) and sterilized (blue) treatments. The analysis

includes A) All identified ZOTUs on a PA diet B) All identified ZOTUs on a PR diet C) All

identified ZOTUs on a R diet D) 10 dominant genera on a PA diet E) 10 dominant genera on a

PR diet F) 10 dominant genera on a R diet. In figures D-F, the length of the vector is correlated

with the strength of the impact that it produced for the samples to be separated in the vector
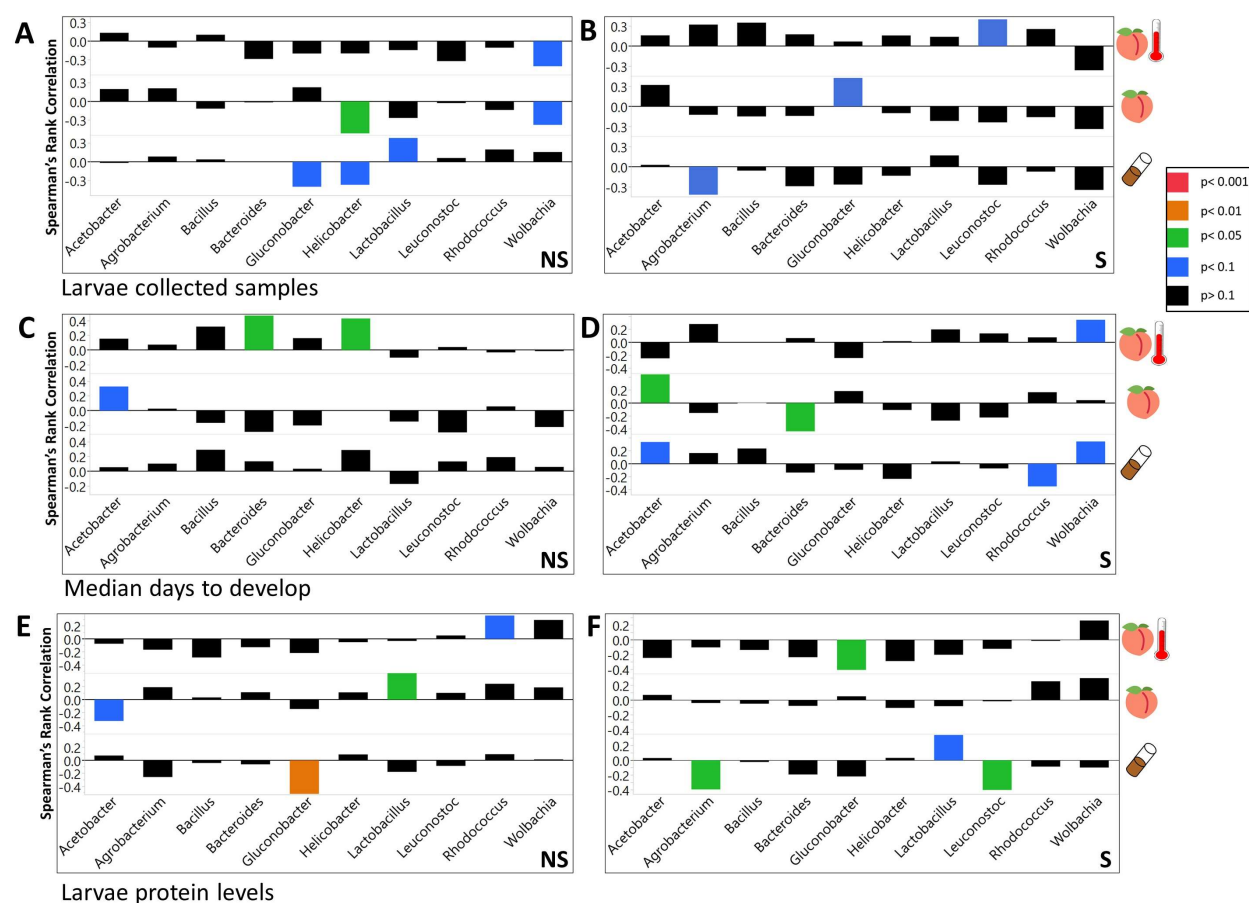
direction, on a canonical plot.
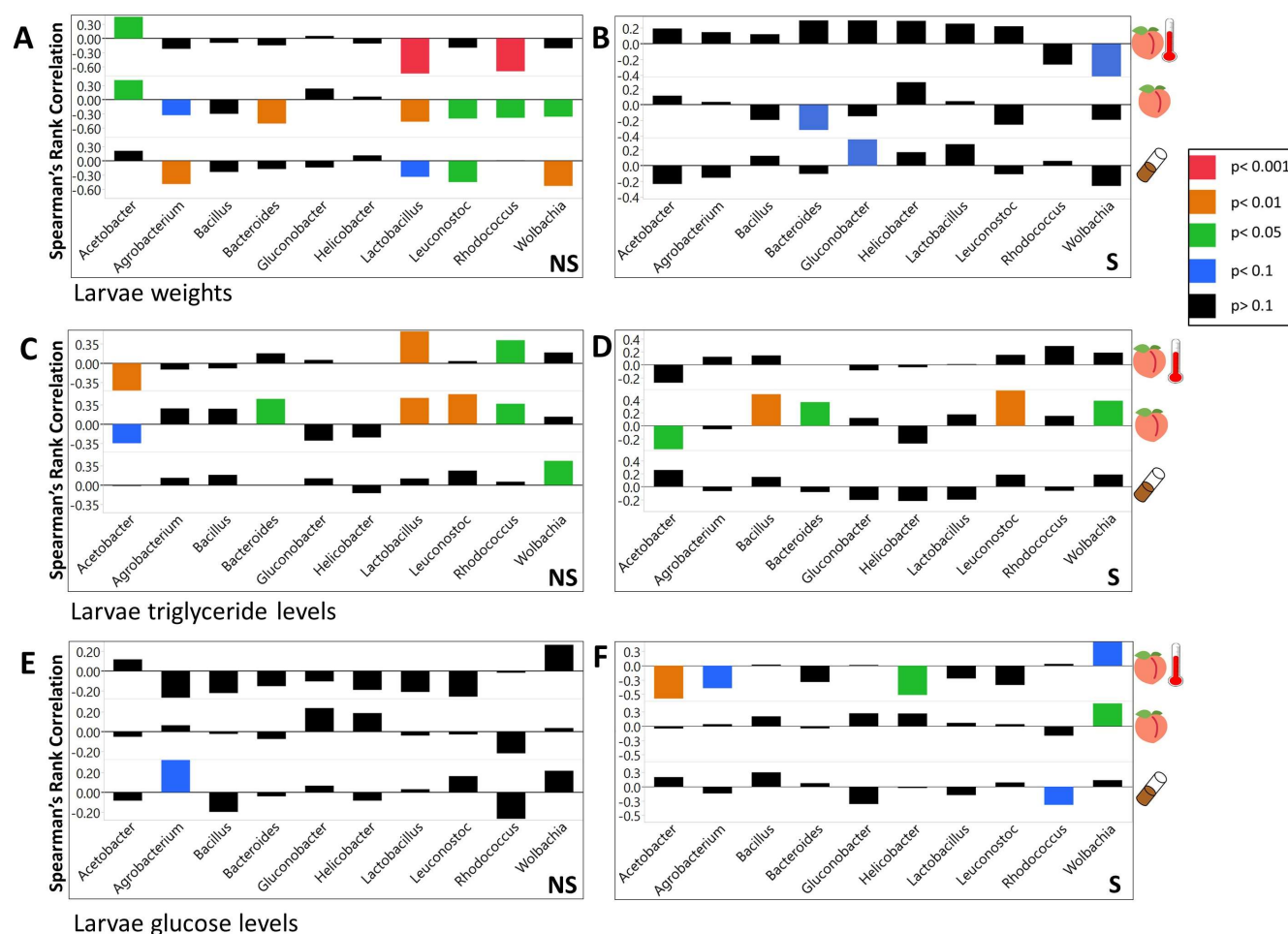
Figure 6: The influence of symbiotic microbial taxa on larvae metabolic phenotypes.

Spearman's rank correlation coefficients between the abundance of 10 dominant symbiotic microbiota genera and larvae phenotypes, on each diet. The color of the bars corresponds to the level of significance for each correlation. A) Weight of NS larvae B) Weight of S larvae C) Triglyceride levels of NS larvae D) Triglyceride levels of S larvae E) Glucose levels of NS larvae F) Glucose levels of S larvae

Figure 7: The influence of symbiotic microbial taxa on larvae life history traits and metabolic phenotypes. Spearman's rank correlation coefficients between the abundance of 10 dominant symbiotic microbiota genera and larvae phenotypes on each diet. The color of the bars corresponds to the level of significance for each correlation. A) Total number of collected NS larvae B) Total number of collected S larvae C) Median number of days to reach pre-pupation stage for NS larvae D) Median number of days to reach pre-pupation stage for S larvae E) Protein levels of NS larvae F) Protein levels of S larvae

Data Tables

Treatment	Survival	Development	Weight	Triglyceride	Protein	Glucose
NS	R> PR **	R< PR ***	R> RP ***	R< PR ***	R> PR **	R> PR ***
S	R> PR **	R< PR ***	R> PR ***	R< PR ***	R> PR ***	R> PR ***
NS	PR> PA ***	PR< PA ***	PR> PA ***	PR< PA ***	PR< PA	PR< PA **
S	PR> PA ***	PR< PA ***	PR> PA ***	PR< PA ***	PR< PA ***	PR< PA ***

Table 1.1: The influence of diet on larvae phenotypes. Comparison of larvae life history traits and metabolic phenotypes between larvae, from all 10 genetic lines, raised on regular lab diet (R), peach diet (PR), and autoclaved peach diet (PA). NS stands for non-sterilized larvae, S stands for sterilized larvae. Asterisks indicate the significance of comparisons $p< 0.001$ ***, $p< 0.01$ **, and $p< 0.5$ *

Genetic line	Diet	Survival	Development	Weight	Triglyceride	Protein	Glucose
All	PA	S< NS ***	S> NS ***	S< NS ***	S> NS	S> NS **	S> NS **
All	PR	S< NS	S> NS ***	S< NS ***	S> Ns	S< NS	S> NS
All	R	S< NS	S> NS	S< NS	S> NS	S> NS	S> NS

Table 1.2: The influence of parental microbiota on larvae phenotypes. Comparison of larvae life history traits and metabolic phenotypes between larvae, from all 10 genetic lines, raised on regular lab diet (R), peach diet (PR), and autoclaved peach diet (PA). NS stands for non-sterilized larvae, S stands for sterilized larvae. Asterisks indicate the significance of comparisons $p < 0.001$ ***, $p < 0.01$ **, and $p < 0.5$ *

Independent variable	Survival	Development	Weight	Triglyceride	Protein	Glucose
Diet	VE=28.4% ***	VE= 47.5% ***	VE= 31.4% ***	VE= 41.4% ***	VE= 4.24% ***	VE= 17.8% ***
Genetic line	VE=5.13% ***	VE=4.98% ***	VE=7.44% ***	VE=2.36% **	VE=5.71% ***	VE=3.15%
Treatment	VE=4.69% ***	VE=2.41% ***	VE=0.74% ***	VE= 0.36%	VE=0.40% *	VE= 2.61% ***
Diet*Genetic line	VE=3.13% ***	VE=0.96%	VE=2.88% ***	VE=5.03% ***	VE=5.09% ***	VE=5.52%
Diet*Treatment	VE=8.02% ***	VE=1.41% ***	VE=0.10%	VE=0.07%	VE=0.45%	VE= 3.55% ***
Genetic line*Treatment	VE=0.98% *	VE=2.82% ***	VE=1.06% ***	VE= 1.81% *	VE=3.53% ***	VE=0.72%
Diet*Treatment*Genetic line	VE= 2.37% ***	VE=0.82%	VE=2.75% ***	VE=4.81% ***	VE= 4.92% ***	VE= 2.37%

Table 1.3: The contribution of diet, genotype, treatment, and their interactive effects on formation of larvae life history traits and metabolic phenotypes. VE stands for variance explained, by each independent variable. Asterisks indicate the significance of comparisons p< 0.001 ***, p< 0.01 **, and p< 0.5 *

References

ADAIR, K. L., WILSON, M., BOST, A. & DOUGLAS, A. E. 2018. Microbial community assembly in wild populations of the fruit fly Drosophila melanogaster. *The ISME Journal*, 1.

AKTER, R., NESSA, A., HUSAIN, M., WAHED, F., KHATUN, N., YESMIN, M., NASREEN, S. & TAJKIA, T. J. M. M. J. M. 2017. Effect of Obesity on Fasting Blood Sugar. 26, 7-11.

ARMOUGOM, F., HENRY, M., VIALETTES, B., RACCAH, D. & RAOULT, D. J. P. O. 2009. Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. 4.

ASHBURNER, M. 1989. *Drosophila. A laboratory handbook*, Cold Spring Harbor Laboratory Press.

BEHAR, A., JURKEVITCH, E. & YUVAL, B. 2008. Bringing back the fruit into fruit fly–bacteria interactions. *Molecular Ecology,* 17, 1375-1386.

BERGER, Z., TTOFI, E. K., MICHEL, C. H., PASCO, M. Y., TENANT, S., RUBINSZTEIN, D. C. & O'KANE, C. J. J. H. M. G. 2005. Lithium rescues toxicity of aggregate-prone proteins in Drosophila by perturbing Wnt pathway. 14, 3003-3011.

BING, X., GERLACH, J., LOEB, G. & BUCHON, N. J. M. 2018. Nutrient-dependent impact of microbes on Drosophila suzukii development. 9, e02199-17.

BIRSE, R. T., CHOI, J., REARDON, K., RODRIGUEZ, J., GRAHAM, S., DIOP, S., OCORR, K., BODMER, R. & OLDHAM, S. 2010. High-fat-diet-induced obesity and heart dysfunction are regulated by the TOR pathway in Drosophila. *Cell metabolism,* 12, 533-544.

BOLGER, A. M., LOHSE, M. & USADEL, B. J. B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. 30, 2114-2120.

BORDENSTEIN, S. R. & THEIS, K. R. 2015. Host Biology in Light of the Microbiome: Ten Principles of Holobionts and Hologenomes. *Plos Biology,* 13.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry,* 72, 248-254.

BRUCE-KELLER, A. J., SALBAUM, J. M., LUO, M., BLANCHARD IV, E., TAYLOR, C. M., WELSH, D. A. & BERTHOUD, H.-R. J. B. P. 2015. Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. 77, 607-615.

BRUNO, D., BONELLI, M., DE FILIPPIS, F., DI LELIO, I., TETTAMANTI, G., CASARTELLI, M., ERCOLINI, D. & CACCIA, S. J. A. E. M. 2019. The intestinal microbiota of Hermetia illucens larvae is affected by diet and shows a diverse composition in the different midgut regions. 85, e01864-18.

CAPORASO, J. G., BITTINGER, K., BUSHMAN, F. D., DESANTIS, T. Z., ANDERSEN, G. L. & KNIGHT, R. J. B. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. 26, 266-267.

CAPORASO, J. G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F. D., COSTELLO, E. K., FIERER, N., PENA, A. G., GOODRICH, J. K. & GORDON, J. I. J. N. M. 2010b. QIIME allows analysis of high-throughput community sequencing data. 7, 335.

CARVALHO, G. B., KAPAHI, P. & BENZER, S. 2005. Compensatory ingestion upon dietary restriction in Drosophila melanogaster. *Nature methods,* 2, 813.

CHANDLER, J. A., LANG, J. M., BHATNAGAR, S., EISEN, J. A. & KOPP, A. 2011. Bacterial communities of diverse Drosophila species: ecological context of a host–microbe model system. *PLoS genetics,* 7, e1002272.

CHASTON, J. M., DOBSON, A. J., NEWELL, P. D. & DOUGLAS, A. E. 2016. Host genetic control of the microbiota mediates the Drosophila nutritional phenotype. *Applied and environmental microbiology,* 82, 671-679.

1

CHASTON, J. M., NEWELL, P. D. & DOUGLAS, A. E. J. M. 2014. Metagenome-wide association of microbial determinants of host phenotype in Drosophila melanogaster. 5, e01631-14.

CLARK, A. & KEITH, L. J. G. 1988. Variation among extracted lines of Drosophila melanogaster in triacylglycerol and carbohydrate storage. 119, 595-607.

CORBY-HARRIS, V., PONTAROLI, A. C., SHIMKETS, L. J., BENNETZEN, J. L., HABEL, K. E. & PROMISLOW, D. E. 2007. Geographical distribution and diversity of bacteria associated with natural populations of Drosophila melanogaster. *Applied and environmental microbiology,* 73, 3470-3479.

DE LUCA, M., YI, N., ALLISON, D. B., LEIPS, J. & RUDEN, D. M. J. O. R. 2005. Mapping quantitative trait loci affecting variation in Drosophila triacylglycerol storage. 13, 1596-1605.

DEW-BUDD, K., JARNIGAN, J. & REED, L. K. 2016. Genetic and sex-specific transgenerational effects of a high fat diet in Drosophila melanogaster. *PloS one,* 11, e0160857.

DOBSON, A. J., CHASTON, J. M., NEWELL, P. D., DONAHUE, L., HERMANN, S. L., SANNINO, D. R., WESTMILLER, S., WONG, A. C.-N., CLARK, A. G. & LAZZARO, B. P. 2015. Host genetic determinants of microbiota-dependent nutrition revealed by genome-wide analysis of Drosophila melanogaster. *Nature communications,* 6, 6312.

DOUGLAS, A. E. J. L. A. 2018. The Drosophila model for microbiome research. 47, 157-164.

EARLY, A. M., SHANMUGARAJAH, N., BUCHON, N. & CLARK, A. G. 2017. Drosophila Genotype Influences Commensal Bacterial Levels. *PloS one,* 12, e0170332.

EDGAR, R. C. J. B. 2010. Search and clustering orders of magnitude faster than BLAST. 26, 2460-2461.

FERGUSON, L. V., DHAKAL, P., LEBENZON, J. E., HEINRICHS, D. E., BUCKING, C. & SINCLAIR, B. J. J. F. E. 2018. Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity. 32, 2357-2368.

FLINT, H. J., DUNCAN, S. H. & LOUIS, P. 2017. The impact of nutrition on intestinal bacterial communities. *Current Opinion in Microbiology,* 38, 59-65.

FURET, J.-P., KONG, L.-C., TAP, J., POITOU, C., BASDEVANT, A., BOUILLOT, J.-L., MARIAT, D., CORTHIER, G., DORÉ, J. & HENEGAR, C. 2010. Differential adaptation of human gut microbiota to bariatric surgery–induced weight loss. *Diabetes,* 59, 3049-3057.

HAN, T. S. & LEAN, M. E. 2016. A clinical perspective of obesity, metabolic syndrome and cardiovascular disease. *JRSM cardiovascular disease,* 5, 2048004016633371.

HOFFMANN, A. A., HALLAS, R., SINCLAIR, C. & PARTRIDGE, L. J. E. 2001. Rapid loss of stress resistance in Drosophila melanogaster under adaptation to laboratory culture. 55, 436-438.

HUANG, J.-H. & DOUGLAS, A. E. 2015. Consumption of dietary sugar by gut bacteria determines Drosophila lipid content. *Biology letters,* 11, 20150469.

HUANG, W., MASSOURAS, A., INOUE, Y., PEIFFER, J., RÀMIA, M., TARONE, A. M., TURLAPATI, L., ZICHNER, T., ZHU, D. & LYMAN, R. F. 2014. Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines. *Genome research,* 24, 1193-1208.

IGNACIO, A., FERNANDES, M. R., RODRIGUES, V. A., GROPPO, F. C., CARDOSO, A. L., AVILA-CAMPOS, M., NAKANO, V. J. C. M. & INFECTION 2016. Correlation between body mass index and faecal microbiota from children. 22, 258. e1-258. e8.

JEHRKE, L., STEWART, F. A., DROSTE, A. & BELLER, M. J. S. R. 2018. The impact of genome variation and diet on the metabolic phenotype and microbiome composition of Drosophila melanogaster. 8, 1-15.

KING, E. G., MACDONALD, S. J. & LONG, A. D. 2012. Properties and power of the Drosophila Synthetic Population Resource for the routine dissection of complex traits. *Genetics,* 191, 935-949.

2

KLEPSATEL, P., PROCHÁZKA, E. & GÁLIKOVÁ, M. J. E. G. 2018. Crowding of Drosophila larvae affects lifespan and other life-history traits via reduced availability of dietary yeast. 110, 298-308.

KREZNAR, J. H., KELLER, M. P., TRAEGER, L. L., RABAGLIA, M. E., SCHUELER, K. L., STAPLETON, D. S., ZHAO, W., VIVAS, E. I., YANDELL, B. S. & BROMAN, A. T. J. C. R. 2017. Host genotype and gut microbiome modulate insulin secretion and diet-induced metabolic phenotypes. 18, 1739-1750.

KUMAR, R., EIPERS, P., LITTLE, R. B., CROWLEY, M., CROSSMAN, D. K., LEFKOWITZ, E. J. & MORROW, C. D. 2014. Getting started with microbiome analysis: sample acquisition to bioinformatics. *Current protocols in human genetics*, 18.8. 1-18.8. 29.

LEBOFFE, M. J. & PIERCE, B. E. 2012. *Microbiology: laboratory theory and application*, Morton Publishing Company.

LEITÃO-GONÇALVES, R., CARVALHO-SANTOS, Z., FRANCISCO, A. P., FIOREZE, G. T., ANJOS, M., BALTAZAR, C., ELIAS, A. P., ITSKOV, P. M., PIPER, M. D. & RIBEIRO, C. 2017. Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLoS biology,* 15, e2000862.

LEY, R. E., TURNBAUGH, P. J., KLEIN, S. & GORDON, J. I. J. N. 2006. Human gut microbes associated with obesity. 444, 1022-1023.

MACKAY, T. F., RICHARDS, S., STONE, E. A., BARBADILLA, A., AYROLES, J. F., ZHU, D., CASILLAS, S., HAN, Y., MAGWIRE, M. M. & CRIDLAND, J. M. 2012. The Drosophila melanogaster genetic reference panel. *Nature,* 482, 173.

MARTIN, T. L., BALSER, S. R., YOUNG, G. S. & LEWIS, S. D. J. J. O. T. A. A. F. L. A. S. 2016. Cost and effectiveness of commercially available nesting substrates for Deer Mice (Peromyscus maniculatus). 55, 412-418.

MARTINEZ-PORCHAS, M., VILLALPANDO-CANCHOLA, E., SUAREZ, L. E. O. & VARGAS-ALBORES, F. 2017. How conserved are the conserved 16S-rRNA regions? *PeerJ,* 5, e3036.

MARTYN, J. J., KANEKI, M. & YASUHARA, S. J. A. 2008. Obesity-induced insulin resistance and hyperglycemia: etiological factors and molecular mechanisms. 109, 137.

MATURIN, L., PEELER, J. J. B. A. M. U. F. & DRUG ADMINISTRATION, N. H. A. S. S. U. 2001. BAM aerobic plate count.

MAURICE, C. F., KNOWLES, S. C., LADAU, J., POLLARD, K. S., FENTON, A., PEDERSEN, A. B. & TURNBAUGH, P. J. J. T. I. J. 2015. Marked seasonal variation in the wild mouse gut microbiota. 9, 2423-2434.

MENDEZ, S., WATANABE, L., HILL, R., OWENS, M., MORACZEWSKI, J., ROWE, G. C., RIDDLE, N. C. & REED, L. K. 2016. The TreadWheel: a novel apparatus to measure genetic variation in response to gently induced exercise for Drosophila. *PloS one,* 11, e0164706.

MILLION, M., MARANINCHI, M., HENRY, M., ARMOUGOM, F., RICHET, H., CARRIERI, P., VALERO, R., RACCAH, D., VIALETTES, B. & RAOULT, D. J. I. J. O. O. 2012. Obesity-associated gut microbiota is enriched in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. 36, 817-825.

MOGHADAM, N. N., THORSHAUGE, P. M., KRISTENSEN, T. N., DE JONGE, N., BAHRNDORFF, S., KJELDAL, H. & NIELSEN, J. L. J. F. 2018. Strong responses of Drosophila melanogaster microbiota to developmental temperature. 12, 1-12.

MORAIS, P. B., MARTINS, M. B., KLACZKO, L. B., MENDONÇA-HAGLER, L. C. & HAGLER, A. N. 1995. Yeast succession in the Amazon fruit Parahancornia amapa as resource partitioning among Drosophila spp. *Applied and environmental microbiology,* 61, 4251-4257.

MURUGESAN, S., ULLOA-MARTÍNEZ, M., MARTÍNEZ-ROJANO, H., GALVÁN-RODRÍGUEZ, F., MIRANDA-BRITO, C., ROMANO, M., PIÑA-ESCOBEDO, A., PIZANO-ZÁRATE, M.,

HOYO-VADILLO, C., GARCÍA-MENA, J. J. E. J. O. C. M. & DISEASES, I. 2015. Study of the diversity and short-chain fatty acids production by the bacterial community in overweight and obese Mexican children. 34, 1337-1346.

NEWELL, P. D. & DOUGLAS, A. E. 2014a. Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in Drosophila melanogaster. *Applied and environmental microbiology,* 80, 788-796.

NEWELL, P. D. & DOUGLAS, A. E. J. A. E. M. 2014b. Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in Drosophila melanogaster. 80, 788-796.

PAIS, I. S., VALENTE, R. S., SPORNIAK, M. & TEIXEIRA, L. J. P. B. 2018. Drosophila melanogaster establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. 16, e2005710.

PARKS, B. W., NAM, E., ORG, E., KOSTEM, E., NORHEIM, F., HUI, S. T., PAN, C., CIVELEK, M., RAU, C. D. & BENNETT, B. J. J. C. M. 2013. Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. 17, 141-152.

PAWITAN, Y., MICHIELS, S., KOSCIELNY, S., GUSNANTO, A. & PLONER, A. J. B. 2005. False discovery rate, sensitivity and sample size for microarray studies. 21, 3017-3024.

PRICE, M. N., DEHAL, P. S., ARKIN, A. P. J. M. B. & EVOLUTION 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. 26, 1641-1650.

PUHL, R. M. & HEUER, C. A. 2010. Obesity stigma: important considerations for public health. *American journal of public health,* 100, 1019-1028.

QUAST, C., PRUESSE, E., YILMAZ, P., GERKEN, J., SCHWEER, T., YARZA, P., PEPLIES, J. & GLÖCKNER, F. O. J. N. A. R. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. 41, D590-D596.

READ, M. N. & HOLMES, A. J. 2017. towards an integrative Understanding of Diet–Host–Gut Microbiome interactions. *Frontiers in Immunology,* 8.

REED, L. K., LEE, K., ZHANG, Z., RASHID, L., POE, A., HSIEH, B., DEIGHTON, N., GLASSBROOK, N., BODMER, R. & GIBSON, G. 2014. Systems genomics of metabolic phenotypes in wild-type Drosophila melanogaster. *Genetics,* 197, 781-793.

REED, L. K., WILLIAMS, S., SPRINGSTON, M., BROWN, J., FREEMAN, K., DESROCHES, C. E., SOKOLOWSKI, M. B. & GIBSON, G. 2010. Genotype-by-diet interactions drive metabolic phenotype variation in Drosophila melanogaster. *Genetics,* 185, 1009-1019.

RIDAURA, V. K., FAITH, J. J., REY, F. E., CHENG, J., DUNCAN, A. E., KAU, A. L., GRIFFIN, N. W., LOMBARD, V., HENRISSAT, B. & BAIN, J. R. 2013. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science,* 341, 1241214.

RIDLEY, E. V., WONG, A. C., WESTMILLER, S. & DOUGLAS, A. E. 2012. Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster. *PloS one,* 7, e36765.

ROSENBERG, E., SHARON, G., ATAD, I. & ZILBER-ROSENBERG, I. 2010. The evolution of animals and plants via symbiosis with microorganisms. *Environmental microbiology reports,* 2, 500-506.

ROSENTHAL, N. & BROWN, S. J. N. C. B. 2007. The mouse ascending: perspectives for human-disease models. 9, 993-999.

RULIFSON, E. J., KIM, S. K. & NUSSE, R. J. S. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. 296, 1118-1120.

RUSSELL, T., KURTZ, R. J. S. & REVIEWERS-JULY 2012. A Comparison of Laboratory-Reared Stock and Captured Fruit Flies (Drosophila melanogaster) using Upward Movement, Phototaxic, and Starvation Assays Reveals Significant Behavioral Differences. 6.

RYU, J.-H., KIM, S.-H., LEE, H.-Y., BAI, J. Y., NAM, Y.-D., BAE, J.-W., LEE, D. G., SHIN, S. C., HA, E.-M. & LEE, W.-J. 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. *science,* 319, 777-782.

SANG, J. H. J. J. O. E. B. 1956. The quantitative nutritional requirements of Drosophila melanogaster. 33, 45-72.

SCHWIERTZ, A., TARAS, D., SCHÄFER, K., BEIJER, S., BOS, N. A., DONUS, C. & HARDT, P. D. J. O. 2010. Microbiota and SCFA in lean and overweight healthy subjects. 18, 190-195.

SEGANFREDO, F., BLUME, C., MOEHLECKE, M., GIONGO, A., CASAGRANDE, D., SPOLIDORO, J., PADOIN, A., SCHAAN, B. & MOTTIN, C. 2017. Weight-loss interventions and gut microbiota changes in overweight and obese patients: a systematic review. *Obesity Reviews.*

SGRO, C. M. & PARTRIDGE, L. J. T. A. N. 2000. Evolutionary responses of the life history of wild-caught Drosophila melanogaster to two standard methods of laboratory culture. 156, 341-353.

SHIN, S. C., KIM, S.-H., YOU, H., KIM, B., KIM, A. C., LEE, K.-A., YOON, J.-H., RYU, J.-H. & LEE, W.-J. 2011. Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science,* 334, 670-674.

SKORUPA, D. A., DERVISEFENDIC, A., ZWIENER, J. & PLETCHER, S. D. 2008. Dietary composition specifies consumption, obesity, and lifespan in Drosophila melanogaster. *Aging cell,* 7, 478-490.

STAUBACH, F., BAINES, J. F., KÜNZEL, S., BIK, E. M. & PETROV, D. A. J. P. O. 2013. Host species and environmental effects on bacterial communities associated with Drosophila in the laboratory and in the natural environment. 8.

STORELLI, G., DEFAYE, A., ERKOSAR, B., HOLS, P., ROYET, J. & LEULIER, F. J. C. M. 2011. Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. 14, 403-414.

TEFIT, M., GILLET, B., JONCOUR, P., HUGHES, S. & LEULIER, F. 2017. Stable association of a Drosophila-derived microbiota with its animal partner and the nutritional environment upon transfer between populations and generations. *bioRxiv*, 111492.

TILG, H. & MOSCHEN, A. R. 2016. Gut Microbiome, Obesity, and Metabolic Syndrome. *Metabolic Syndrome: A Comprehensive Textbook*, 447-459.

TONG, Q. & ZHANG, J.-T. J. F. I. M. 2019. Effects of captivity and season on the gut microbiota of the brown frog (Rana dybowskii). 10, 1912.

TURNBAUGH, P. J., LEY, R. E., MAHOWALD, M. A., MAGRINI, V., MARDIS, E. R. & GORDON, J. I. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *nature,* 444, 1027-131.

VACCHINI, V., GONELLA, E., CROTTI, E., PROSDOCIMI, E. M., MAZZETTO, F., CHOUAIA, B., CALLEGARI, M., MAPELLI, F., MANDRIOLI, M. & ALMA, A. 2017. Bacterial diversity shift determined by different diets in the gut of the spotted wing fly Drosophila suzukii is primarily reflected on acetic acid bacteria. *Environmental microbiology reports,* 9, 91-103.

WAHBA, I. M. & MAK, R. H. 2007. Obesity and obesity-initiated metabolic syndrome: mechanistic links to chronic kidney disease. *Clinical Journal of the American Society of Nephrology,* 2, 550-562.

WILLIAMS, S., DEW-BUDD, K., DAVIS, K., ANDERSON, J., BISHOP, R., FREEMAN, K., DAVIS, D., BRAY, K., PERKINS, L. & HUBICKEY, J. 2015. Metabolomic and gene expression profiles exhibit modular genetic and dietary structure linking metabolic syndrome phenotypes in Drosophila. *G3: Genes, Genomes, Genetics,* 5, 2817-2829.

5

WONG, A. C.-N., DOBSON, A. J. & DOUGLAS, A. E. 2014. Gut microbiota dictates the metabolic response of Drosophila to diet. *Journal of Experimental Biology,* 217**,** 1894-1901.

WONG, A. C.-N., LUO, Y., JING, X., FRANZENBURG, S., BOST, A. & DOUGLAS, A. E. J. A. E. M. 2015. The host as the driver of the microbiota in the gut and external environment of Drosophila melanogaster. 81**,** 6232-6240.

WONG, A. C., CHASTON, J. M. & DOUGLAS, A. E. 2013. The inconstant gut microbiota of Drosophila species revealed by 16S rRNA gene analysis. *The ISME journal,* 7**,** 1922.

WONG, C. N. A., NG, P. & DOUGLAS, A. E. 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environmental microbiology,* 13**,** 1889-1900.

WU, G. D., CHEN, J., HOFFMANN, C., BITTINGER, K., CHEN, Y.-Y., KEILBAUGH, S. A., BEWTRA, M., KNIGHTS, D., WALTERS, W. A. & KNIGHT, R. J. S. 2011. Linking long-term dietary patterns with gut microbial enterotypes. 334**,** 105-108.

ZHANG, C., ZHANG, M., WANG, S., HAN, R., CAO, Y., HUA, W., MAO, Y., ZHANG, X., PANG, X. & WEI, C. J. T. I. J. 2010. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. 4**,** 232-241.