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1 # NON-TARGETED METABOLOMICS REVEAL DIFFERENCES IN THE METABOLIC PROFILE
2 # OF THE FALL ARMYWORM STRAINS WHEN FEEDING DIFFERENT FOOD SOURCES

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17 ## ABSTRACT

18 *Spodoptera frugiperda,* the fall armyworm (FAW), is an important polyphagous agricultural pest feeding
19 on nearly 350 host plants. FAW is undergoing incipient speciation with two well-characterized host-adapted
20 strains, the "corn" (*CS*) and "rice" (*RS*) strains, which are morphologically identical but carry several genes
21 under positive selection for host adaptation. We used non-targeted metabolomics based on gas
22 chromatography/mass spectrometry to identify differences in metabolite profiles of the larval gut of *CS* and
23 *RS* feeding on different host plants. Larvae were fed on artificial diet, maize, rice, or cotton leaves from
24 eclosion to the sixth instar, when they had their midgut dissected for the analysis. This study revealed that
25 the midgut metabolome of FAW varied due to larval diet and differed between the FAW host-adapted
26 strains. Additionally, we identified several candidate metabolites that may be involved in the adaptation of

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27 *CS* and *RS* to their host plants. Our findings provide clues toward the gut metabolic activities of the FAW
28 strains.
29

30 **KEY WORDS:**
31 Metabolite, herbivore, insect-plant interaction, nutritional ecology, *Spodoptera frugiperda*
32

33 1. INTRODUCTION

34 *Spodoptera frugiperda,* the fall armyworm (FAW) (Lepidoptera, Noctuidae), feeds on approximately 350
35 different species from 76 families (Montezano et al., 2018). Despite this wide range of host plants, FAW is
36 best known as one of the most important agricultural pests of grasses (maize, millet, rice and sorghum) and
37 some cultivated dicots such as cotton (Barros et al., 2010). The FAW is native to the New World, but in the
38 last few years has invaded Africa and further spread to Asia and Oceania (Goergen et al., 2016; Johnson,
39 1987; Otim et al., 2018; Piggott et al., 2021; Suby et al., 2020), Therefore, FAW is currently considered of
40 a global concern due its polyphagy and capacity for rapid evolution of resistance to pesticides and Bt crops
41 (Huang, 2021; Jakka et al., 2016), representing an imminent threat to food security and a source of
42 significant economic losses.

43 The FAW is the only species of *Spodoptera* that usually feeds on grasses without having adapted, suitable
44 mandibles. Larvae that feed on grasses typically have specialized mandibles with chisel-like edges adapted
45 to the consumption of silica-rich leaves, which cause wear to larval mandibles(Djamin and Pathak, 1967;
46 Pogue, 2002; Smith, 2005). Mandibles of the *FAW* have serrate-like processes adapted to the consumption
47 of dicots or monocots that do not accumulate silica (Pogue, 2002). FAW is primitively polyphagous, but
48 because of the mandible-type it is thought to have started exploiting cultivated grasses as host plants only
49 recently (Kergoat et al., 2021, 2012).

50 Another interesting aspect of the FAW is the identification of two distinct strains known as the rice (*RS*)
51 and corn (*CS*) strains (Gouin et al., 2017; Pashley, 1986). There are indications that this divergence occurred
52 about 2 Myr ago (Kergoat et al., 2021, 2012).These strains differ in their performance and preference for

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53 host plants, and the correct classification of these two strains of the FAW is still controversial. Some authors
54 refer to them as "sibling species" (Drès and Mallet, 2002; Dumas et al., 2015), "host strains" (Pashley,
55 1986; Prowell et al., 2004), "host form" (Juárez et al., 2014) and "morphocryptic strains" (Sarr et al., 2021).

56 The lack of consensus is due to the fact these strains co-exist in sympatry and still hybridize, but also due
57 to inconsistencies in the associations with the named host plants. At the adult stage, both corn and rice
58 strains showed weak evidence of preference for their expected host plant in choice and non-choice
59 laboratory experiments (Orsucci et al. 2020). Despite the fact that the corn strain is often associated with
60 maize, sorghum, and cotton, with the rice strain associated with rice and pasture grasses, some reports show
61 the rice strain larvae developed better on corn and sorghum than corn strain larvae (Meagher et al. 2004).
62 Moreover, both strains performed poorly when feeding on rice (Silva-Brandão et al. 2017). Therefore,
63 further studies are still needed to understand how the process of host-plant adaptation is taking place in
64 FAW.

65 Every novel acquisition of host plant by herbivores constitutes a new niche adaptation program that opens
66 several evolutionary possibilities, but not without associated costs. In order to exploit a novel host, insects
67 have to become adapted to deal with new defensive secondary metabolites, such as phenolics and
68 terpenoids, and the nutritional quality of the new host plant (Singer, 2008). However, the mechanisms
69 behind the best performance of a given host-adapted strain on a given plant are poorly understood so far.
70 Different approaches can be used to address this question. One alternative is to access the insect
71 metabolome, the set of all low-molecular-weight metabolites that are produced during cell metabolism (Sun
72 and Hu, 2016). Ultimately, the metabolome is a product of genomic, transcriptomic, and/or proteomic
73 processes (Johnson and Gonzalez 2012). Non-targeted metabolomics provides a holistic view of the insect
74 metabolic profile. It makes no assumptions about which metabolites are important in distinguishing sample
75 types (Sévin et al., 2015). This approach provides a direct functional measurement of cellular activity and
76 physiological state, reflecting environmental changes such as new host plants as well as aspects related to
77 their genome, as different host-adapted strains. Therefore, the non-targeted study of metabolomes is a good
78 tool to highlight candidate metabolites involved in insect-plant interactions (Maag et al., 2015). Particularly,

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79 the assessment of the insect midgut may be useful, bearing in mind that it is a selectively permeable and
80 metabolically active tissue, in which most digestion and almost all nutrient absorption takes place (Dow,
81 1987). However, approaches focused on the assessment of the gut metabolomics of insect herbivores are
82 not common, and little is known on how host plants impact the metabolic profile of the herbivore gut.

83 The gut microbiome is also a key player in th
84 e metabolic processes of their hosts. Gut microbes can play important roles in several metabolic functions,
85 including vitamin production (Chen et al., 2016; Salem et al., 2014), amino acid synthesis (Ayayee et al.,
86 2016; Xia et al., 2017) and detoxification of secondary plant compounds and synthetic insecticides
87 (Almeida et al., 2017; Ceja-Navarro et al., 2015). Among the numerous factors that influence the gut
88 microbiota (R J Dillon and Dillon, 2004; Yun et al., 2014), diet has received considerable attention due to
89 its strong effect on the composition of the microbial community (Mason et al., 2020; Wongsiri and
90 Randolph, 1962; Yun et al., 2014). Diet provides the substrates to produce a plethora of small molecules
91 that can be converted by the gut microbiota, and which are not produced by the host (Krishnan et al., 2015;
92 Wang et al., 2020). Therefore, the gut microbiota may also facilitate adaptation to new host plants by
93 regulating or participating in the host's metabolic processes (Hammer and Bowers, 2015; Zhang et al.,
94 2020). Microbial contribution will depend on substrate availability and on microbial gene diversity and
95 activity (Wu et al., 2016). Thus, taxonomic or metagenomic information of the gut microbiota is limited in
96 predicting the metabolome of a microbial community, as it may under- or overestimate the functional
97 contribution of associated gut microbiota depending on the nutritional conditions the host is exposed to
98 (Wu et al., 2016).

99 The FAW is a good model to study adaptation of phytophagous insects to agricultural plants. Moreover,
100 the metabolic processes underlying host shifts or differentiation in this species are not well understood. In
101 terms of metabolome, we would expect different metabolic profiles to reflect new adaptations. We predict
102 differences in larval adaptation to host plants to be reflected in the metabolome of their gut. These
103 differences might highlight adaptations in response to plant chemistry, changes in metabolic pathways,
104 and/or new roles for microbial symbionts. The aim of the present research is to investigate if the gut

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105 metabolome of FAW is determined by the diet and/or by host genotype. Highlighting the metabolic
106 differences in the midgut of the FAW strains may represent the starting point for future research that aims
107 to clarify : 1) how different host plants affect insect nutritional metabolism and 2) how larvae of the two
108 host strains differ in their metabolism of different host plant chemistries.
109

110 ## 2. MATERIAL AND METHODS

111 ### 2.1 Insect rearing and strains identification

112 Colonies of FAW were initiated in the laboratory from field-collected populations. The *RS* was
113 originally obtained from rice fields in Santa Maria, RS, Brazil (29°68'68"S, 53°81'49"W) and the *CS* from
114 a maize field in Piracicaba, SP, Brazil (22°43'30"S, 47°38'56"O). Field-collected larvae were
115 individualized into plastic cups containing an artificial diet based on wheat germ, beans and brewer's yeast
116 (Burton and Perkins, 1972; Kasten Jr et al., 1978), brought to the laboratory and reared under controlled
117 conditions (25 ± 1°C; 70 ± 10% RH; 14 h photophase) until pupation. Pupae were transferred to clean
118 plastic cups lined with filter paper for adult emergence. The produced exuviae were used for DNA
119 extraction for strain identification as described below. After strain identification, all newly emerged adults
120 belonging to the same strain were transferred to PVC tubes lined with paper as a substrate for egg laying.
121 Egg masses were collected and transferred to artificial diet for later larval development.

122 Strain identification followed Levy et al. (2002) (Levy et al., 2002). DNA was extracted from individual
123 pupal exuviae using the genomic DNA preparation protocol from RNAlater™ preserved tissues with some
124 modifications. The exuviae were individually placed in 750 µL digestion buffer (60 mM Tris pH 8.0, 100
125 mM EDTA, 0.5% SDS) containing proteinase K at a final concentration of 500 µg/mL. Samples were
126 macerated using plastic pestles and mixed well by inversion. Samples were incubated overnight at 55°C.
127 Afterwards, 750 µL of phenol: chloroform (1:1) was added and samples were rapidly inverted for 2 min
128 before centrifugation at a tabletop centrifuge at maximum speed (10 min). The aqueous layer was
129 recovered and subjected to re-extraction twice before a final extraction with chloroform. The aqueous

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130 layer was collected and added to 0.1 volume of 3 M sodium acetate (pH 5.2) and an equal volume of 95%
131 ethanol. Samples were then mixed by inversion, incubated for 40 min at -80°C, and centrifuged (27,238 g
132 x 30 min x 4°C). The pellet obtained was washed twice with 1 mL of 85% ice-cold ethanol, centrifuged
133 for 10 min after each wash and dried at 60°C during 5-10 min in a SpeedVac. Finally, the pellet obtained
134 was resuspended in nuclease-free water. DNA concentration and quality were estimated by
135 spectrophotometry and agarose gel electrophoresis (Sambrook and Russell, 2001).

136 Polymerase chain reaction (PCR) amplification of the mitochondrial COI gene was conducted using the
137 primers set JM76 (5'-GAGCTGAATTAGGRACTCCAGG-3') and JM77 (5'-
138 ATCACCTCCWCCTGCAGGATC-3') to produce an amplicon of 569 base pairs (bp)(Levy et al. 2002).
139 The PCR mixture contained 100-150 ng of gDNA, 1.5 mM of $MgCl_2$, 1 x PCR buffer, 0.2 mM of each
140 dNTP, 0.32 µM of each primer and 0.5U of GoTaq® DNA Polymerase (Promega) in a total volume of 25
141 µL. The thermocycling conditions were one cycle at 94°C x 1 min followed by 33 cycles at 92°C x 45 s,
142 56°C x 45 s, 72°C x 1 min, with a final extension at 72°C x 3 min (1x). Amplicons were then subjected to
143 endonuclease restriction analysis using *Msp*I (HpaII) to produce two fragments (497pb and 72pb) for
144 amplicons of the *CS*, while no digestion is observed for *RS* amplicons. After the amplification, 10 µL of
145 the PCR reaction mixture was subjected to digestion with 10 U of *Msp*I following the manufacture
146 guidelines (product number ER0541®, Thermo Scientific). Samples were gently mixed, centrifuged for a
147 few seconds and incubated overnight at 37°C. Subsequently, digestion efficiency and the resulting
148 products were verified using a 1.5% agarose gel electrophoresis.

149 **2.2 Assay with natural diet and gut dissection**

150 Maize (*Zea mays,* family: Poaceae) var. "Conventional impact" and cotton (*Gossypium hirsutum,*
151 family: Malvaceae) var. IAC FC2 seeds were seeded in 500 mL plastic pots filled with soil conditioner,
152 while rice seeds (*Oriza sativa,* family: Poaceae) var. BRS Esmeralda were seeded in 1 L plastic pots. All
153 plants were maintained in a greenhouse.

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154 The leaves were cut and immersed in a container with distilled water for 30 minutes to maintain
155 turgidity. Newly emerged larvae of *RS* and *CS* strains were placed in 25 mL plastic cups containing a 3-
156 cm piece of the host plant leaf (cotton and maize - leaves from v3-v4 stages; rice - v11-13). The leaves
157 were replaced with fresh ones according to the needs of each instar and were replaced every other day or
158 earlier to avoid food shortages. Insects were kept under controlled laboratory conditions throughout the
159 experiments (25 ± 1°C; 60 ± 10%; 14-hour photophase).

160 The experimental groups were represented by larvae of rice (*RS*) and corn (*CS*) strains reared on the
161 following substrates: rice (RiRS; RiCS), corn (CoRS; CoCS), cotton (CtRS; CtCS), and artificial diet
162 (DiRS; DiCS). We used five replicates for each treatment, with each replicate corresponding to a pool of
163 midgut collected from five larvae.

164 The guts were collected from sixth instars larvae, one day after the molting. Larvae were surface-
165 sterilized in cooled 0.2% sodium hypochlorite in 70% ethanol and washed in cold sterile water. Surface-
166 sterilized larvae were dissected in sterile water under aseptic conditions. Gut tissues with the peritrophic
167 matrix and the enclosed intestinal content were flash-frozen in liquid nitrogen and stored at -80 until
168 metabolite extraction.

169 **2.3 Metabolite extraction**

170 Samples were subjected to metabolite extraction and analysis according to (Hoffman et al., 2010), with
171 some modifications. A pool of midguts from five larvae were macerated in liquid nitrogen, and 25 mg of
172 the macerate was homogenized in TissueLyser II (QIAGEN) at the highest speed for 1 min using 5 mm
173 tungsten beads in 500 μL of methanol-chloroform-water solution (3:1:1). Then the sample was sonicated
174 (60 $Hz.s^{-1}$ x 30 min) in an ice bath (4ºC) and centrifuged (16,000 g x 10 min x 4ºC). The supernatant was
175 collected and filtered through a Luer Lock 0.22 μM filter (Millex®, JBR6 103 03) directly into amber
176 glass vials.

177 Aliquots (50 μL) of each sample were freeze-dried in a Terrone model LS 3000 lyophilizer, and
178 subjected to derivatization with 30 μL methoxyamine-HCl (20 $mg.mL^{-1}$) in pyridine for 16 h at room
179 temperature. Trimethylsilylation was accomplished with the addition of 1% trimethylchlorosilane

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180 (TMCS) in 30 µL of *n*-methyl-*n*-(trimethylsilyl)-trifluoroacetamide (MSTFA) to the samples, followed by
181 incubation for 1 h at room temperature. After silylation, 30 µL of heptane was added to samples, which
182 were immediately analyzed in a random order in a 7890A Agilent Gas Chromatograph coupled to a
183 Pegasus HT TOF Mass Spectrometer (LECO, St. Joseph, MI, USA) (GC-TOF/MS) (Technologies).
184 Samples were injected together with mix of *n*-alkanes standards (C_{12} - C_{40}) for the correct calculation of
185 the retention times. Derivatized samples (1 µL) were injected in splitless mode using an automatic
186 sampler-CTC Combi Pal Xt Duo (CTC Analytics AG, Switzerland) coupled to the GC-MS system
187 equipped with two silica columns in line. The first was a DB 5 column (20 m long x 0.18 mm internal
188 diameter x 0.18 µm thick) (Agilent J & W Scientific, Folsom, CA, USA), and the second a Rxi-17 column
189 (0.84 m long x 0.1 mm internal diameter x 0.1 µm thick) (Restek Corporation, Bellefonte, PA, USA). The
190 injector was set at 280°C, the septum bleed rate was 20 $mL.min^{-1}$ and began after 250s from the start of
191 data acquisition (Budzinski et al., 2019), The gas flow was 1 $mL.min^{-1}$. The temperature of the first
192 column was maintained at 80°C for 2 min and increased at 15°$C.min^{-1}$ to 305°C, with a 10 min hold. The
193 temperature of the second column was maintained at 85°C for 2 min, and then raised to 310°C at
194 15°$C.min^{-1}$, with a 10 min hold. The column effluent was introduced into the ionization source of the
195 Pegasus HT TOF MS. The transfer line and ionization source temperatures were held at 280 and 250°C,
196 respectively. The ions were generated by an electron source (70-eV) at an ionization current of
197 2.0 mA, and 20 $spectra.s^{-1}$ were acquired in a mass range of 45-800 *m/z*, with the detector voltage set to
198 1500 V.

199 **2.4 GC-TOF/MS data processing**

200 The processing of GC-TOF/MS data was performed in two steps. Initially the generated chromatograms
201 were exported to the ChromaTOF program, version 4.32 Software (LECO, St. Joseph, MI, USA), in which
202 base line correction, deconvolution of the spectra, retention rate correction (RI), retention time correction
203 (RT), peak identification, and alignment and identification of metabolites were processed using the NIST
204 library, version 11. Only metabolites with three or more characteristic masses and a score of 700 or higher

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205 were considered valid. Isomers were manually checked and merged. Feature intensities were normalized
206 to total ion chromatogram (TIC).
207 MetaboAnalyst 5.0 was used to perform all the downstream analyses (Chong et al., 2019). Data was *log*-
208 transformed and scaled using Pareto. Hierarchical clustering was also performed with the *hclust* function
209 in package stat (R v3.5.1) using Ward as a clustering algorithm and Euclidean distances as measures.
210 Sample clustering was presented as a dendrogram. Principal Component Analysis (PCA) was performed in
211 order to separate and classify the sample groups. Type 1 two-way ANOVA was used to examine the effects
212 of strain and diet, and their interaction on metabolite abundance (Xia et al., 2011). False discovery rate was
213 applied to adjust the *p*-values (0.05). Heatmap was built based only on the significant features from
214 ANOVA. The distance measure used was the Euclidean, and Ward was used for clustering algorithm. In
215 order to identify the features that were potentially significant in discriminating the strains on each host
216 plant, pairwise analysis was performed using volcano plot, which combine fold change ($FC \geq |2.0|$) and *t*-
217 test analysis, that can control the false discovery rate ($p \leq 0.05$).
218
219 **3. RESULTS**
220 The host plants on which FAW larvae fed significantly affected the midgut metabolome. The midgut
221 metabolomes of *RS* and *CS* larvae also differed when feeding on the same diet. However, the food source
222 had a greater impact shaping the gut metabolome of FAW than the host strain (show stats on which this is
223 based, e.g., 2-way ANOVA of summed or overall effects). Metabolomic analyses led to the identification
224 of two major clusters of metabolites, allowing the clear separation of larvae fed on artificial diet when
225 compared to those fed on natural diets (Fig. 1A). The metabolomic profile obtained clearly separated FAW
226 larvae fed on monocots (corn and rice) from those feds on the dicot cotton. Additionally, the profile of
227 metabolites obtained for each FAW strain on each food source also led to their clear separation (Fig.1A).
228 The PCA analysis explained a small percentage of the variation among the samples. The first two principal
229 components explained 39.5% of the variation in the data and showed the clustering of the samples according
230 to food source, but the separation based on strains was not as evident as shown in the dendrogram.

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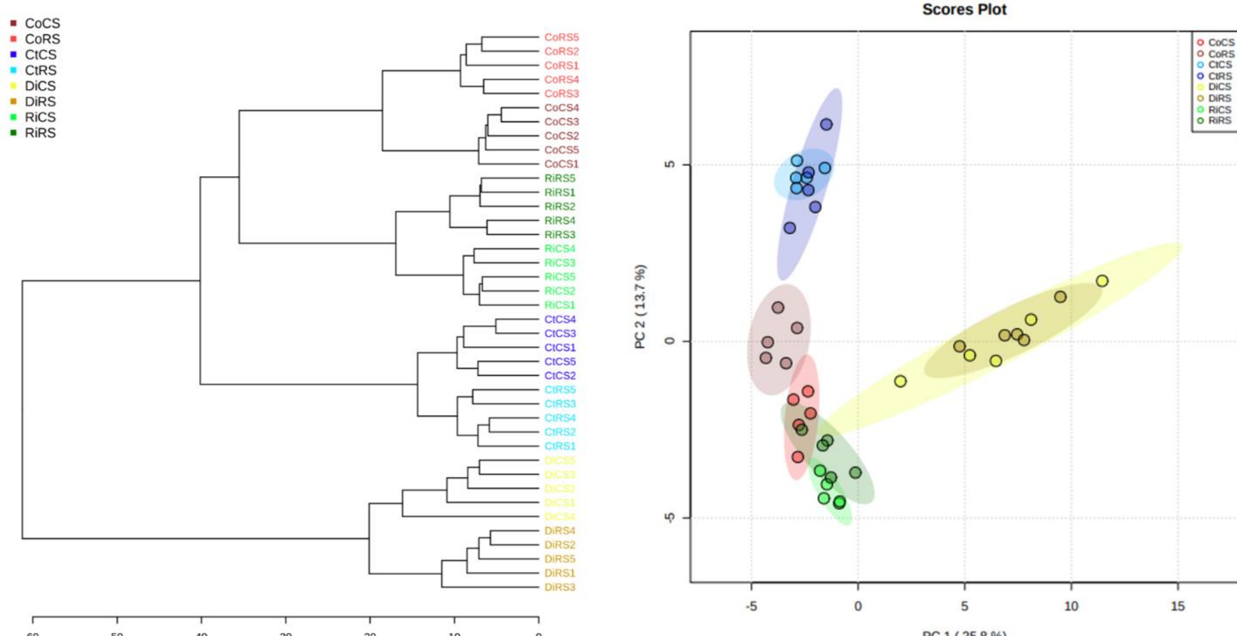

231

232 **Figure 1**: Clustering result shown as dendrogram with distance measure using Euclidean and clustering
233 algorithm using ward.D of the midgut metabolite profiles of *S. frugiperda* larvae (A). Principal Component
234 Analysis (PCA) Scores plot between the selected PCs. The explained variances are shown in brackets (B).
235 The experimental groups were rice strain on rice (RiRS), corn (CoRS), cotton (CtRS) and artificial diet
236 (DiRS) and corn strain on the same diets (RiCS, CoCS, CtCS and DiCS).

237

238 Among the 340 peaks identified in the gut samples of FAW larvae, 122 metabolites passed the filter
239 criteria for analysis, with the abundance of 107 them being affected by the diet, 13 by the host-adapted
240 strain, and 50 by the interaction of both factors (Table 1). The abundance of 12 metabolites was
241 simultaneously affected by diet, strain, and their interactions (Fig. 2). The compounds were predominantly
242 classified as amino acids, sugars, fatty acids, and organic acids.

243

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244
245 **Figure 2:** Venn diagram showing the important features of the midgut metabolome of *S. frugiperda* larvae
246 selected by two-way ANOVA whose levels were affected by strain, diet or interaction of strain and diet.
247
248 **Table 1.** Significant features identified by two-way ANOVA. FDR's correction was applied to adjust the
249 p-values.

METABOLITE	STRAIN		DIET		INTERACTION	
	F.VAL	ADJ.*p*	F.VAL	ADJ.*p*	F.VAL	ADJ.*p*
Homoserine	72.91	<0.01	72.91	<0.01	72.91	<0.01
Margaric acid	48.03	<0.01	48.03	<0.01	48.03	<0.01
2-ketoglucose	46.17	<0.01	19.54	<0.01	19.54	<0.01
Shikimic acid	42.12	<0.01	63.49	<0.01	24.54	<0.01
Protocatechuic acid	30.70	<0.01	112.80	<0.01	13.37	<0.01
Glycerol monostearate	22.08	<0.01	10.12	<0.01	24.41	<0.01
D-Erythronolactone	20.78	<0.01	7.28	<0.01	7.28	<0.01
Benzoic acid	18.96	<0.01	7.16	<0.01	4.89	0.02
Anthranilic acid	18.55	<0.01	12.22	<0.01	3.38	0.07
5-Hydroxynorvaline	15.64	<0.01	15.64	<0.01	15.64	<0.01
Glycerophosphoric acid	11.30	0.02	23.96	<0.01	20.50	<0.01
Galactaric acid	10.50	0.03	115.80	<0.01	6.30	0.01
2-Hydroxyisocaproic acid	10.33	0.03	10.33	<0.01	10.33	<0.01
Fumaric acid	8.47	0.05	94.81	<0.01	13.43	<0.01
Benzene, 4-chlorobutyl-	8.43	0.05	1.71	0.20	7.73	<0.01
Lanthionine	7.45	0.07	6.31	<0.01	1.42	0.41

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Phosphonoacetic Acid	7.15	0.08	26.53	<0.01	45.91	<0.01
Lyxose, linear form	7.04	0.08	3.21	0.04	3.83	0.05
Methyl 3,4-dimethoxyphenylhydroxyacetate	6.74	0.09	6.74	<0.01	6.74	0.01
Caffeic acid	6.58	0.09	23.93	<0.01	5.34	0.02
Phenylethanolamine	6.49	0.09	13.26	<0.01	6.12	0.01
Threonic acid	6.31	0.09	8.04	<0.01	1.42	0.41
Glutamine	6.07	0.10	22.59	<0.01	0.66	0.81
3-Deoxy-arabino-hexaric acid	5.64	0.11	5.64	<0.01	5.64	0.02
Proline	5.64	0.11	31.28	<0.01	1.69	0.33
4-Hydroxyhippuric acid	5.56	0.11	5.56	0.01	5.56	0.02
D-Xylose	5.49	0.11	5.49	0.01	5.49	0.02
2,4,4-Trimethyl-1-pentanol	5.34	0.11	5.34	0.01	5.34	0.02
d-Proline, N-methoxycarbonyl-, methyl ester	5.34	0.11	5.34	0.01	5.34	0.02
Bis2-ethylhexylamine	5.32	0.11	5.32	0.01	5.32	0.02
2-(4-(2-Hydroxy-3-(isopropylamino)-propoxy)phenyl)acetonitrile	5.22	0.11	5.22	0.01	5.22	0.02
Ethylamine, 2-((p-bromo-à-methyl-à-phenylbenzyl)oxy)-N,N-dimethyl-	5.14	0.11	5.14	0.01	5.14	0.02
2-O-Glycerol-alpha-d-galactopyranoside	5.10	0.11	5.10	0.01	5.10	0.02
4-Hydroxybenzoic acid	5.08	0.11	122.41	<0.01	1.96	0.26
D-Gluconic acid, delta-lactone	4.98	0.11	4.98	0.01	4.98	0.02
Aucubin	4.91	0.11	12.16	<0.01	0.22	1.00
Trifluoroacetamide	4.90	0.11	4.90	0.01	4.90	0.02
Hydracrylic acid	4.89	0.11	4.89	0.01	4.89	0.02
Timonacic	4.84	0.11	4.84	0.01	4.84	0.02
3-Octen-2-ol, E-	4.72	0.11	19.70	<0.01	9.51	<0.01
Lauric acid	4.63	0.11	4.63	0.01	4.63	0.02
Glyceryl-glycoside TMS ether	4.60	0.11	4.60	0.01	4.60	0.02
Oxalic acid	4.45	0.12	169.11	<0.01	3.32	0.07
D-Mannopyranose	4.24	0.13	4.24	0.02	4.24	0.03
alpha-D-glucose	4.11	0.14	10.21	<0.01	0.49	0.86
Xylitol	4.07	0.14	60.84	<0.01	4.43	0.03
Pentanedioic acid	3.98	0.14	59.34	<0.01	3.98	0.04
3,4-Dimethoxymandelic acid	3.67	0.16	3.67	0.03	3.67	0.05
D---Fructofuranose, pentakistrimethylsilyl ether	3.61	0.16	3.61	0.03	3.61	0.06
Alanine, N-methyl-N-ethoxycarbonyl-, dodecyl ester	3.51	0.17	18.39	<0.01	33.27	<0.01
Undecyl 2- [butoxycarbony(methyl)amino]propanoate	3.30	0.18	7.29	<0.01	1.29	0.46
Lactic acid	3.28	0.18	16.39	<0.01	18.71	<0.01
1-Aminocyclopentanecarboxylic acid	3.19	0.19	3.19	0.04	3.19	0.08
L-Aspartic acid	3.00	0.20	21.47	<0.01	0.22	1.00
Levulinic acid enol	2.96	0.20	104.76	<0.01	5.76	0.01
Inositol 1-phosphate	2.84	0.21	9.99	<0.01	3.08	0.09
Cadaverine	2.84	0.21	5.00	0.01	1.73	0.33
Glyceric acid	2.62	0.24	10.33	<0.01	1.96	0.26

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Urea	2.41	0.26	14.40	<0.01	0.49	0.86
L-5-Oxoproline	2.27	0.28	17.00	<0.01	0.39	0.92
Sorbic acid	2.24	0.28	155.37	<0.01	2.24	0.20
2-Isopropylaminoethanol	2.09	0.30	18.29	<0.01	13.34	<0.01
7,9-Di-tert-butyl-1-oxaspiro4,5deca-6,9-diene-2,8-dione	2.01	0.31	19.72	<0.01	3.81	0.05
L--Tartaric acid	1.94	0.31	55.28	<0.01	1.94	0.26
L-Threonine	1.93	0.31	9.18	<0.01	0.03	1.00
Tridecanoic acid	1.83	0.33	7.29	<0.01	1.52	0.39
b-Aminoisobutyric acid	1.80	0.33	9.42	<0.01	3.19	0.08
4-Methoxycarbonylphenol	1.50	0.40	145.70	<0.01	1.50	0.39
Pinitol	1.46	0.40	147.40	<0.01	1.46	0.40
Formamide	1.41	0.41	15.23	<0.01	0.36	0.93
Adrenaline	1.33	0.42	1.24	0.33	4.12	0.04
Citric acid	1.25	0.44	8.52	<0.01	0.45	0.89
L-Tryptophan	1.23	0.44	25.95	<0.01	9.77	<0.01
Methionine	1.21	0.44	10.77	<0.01	0.40	0.92
Ethyl .alpha.-D-glucopyranoside	1.12	0.46	7.08	<0.01	1.73	0.33
alpha-D-Talopyranose	1.05	0.49	9.28	<0.01	2.18	0.21
Cysteine	0.96	0.51	6.43	<0.01	2.90	0.11
Inositol	0.93	0.51	11.02	<0.01	4.47	0.03
Phenylalanine	0.79	0.55	4.86	0.01	0.04	1.00
D-Glucuronic acid	0.75	0.56	8.30	<0.01	1.14	0.53
Formylpiperidine	0.74	0.56	18.18	<0.01	5.28	0.02
Gluconic acid	0.67	0.58	15.41	<0.01	0.67	0.81
Putrescine	0.67	0.58	164.10	<0.01	0.79	0.72
L-Rhamnose	0.60	0.59	157.39	<0.01	0.60	0.83
3-Methylpiperazine-2,5-dione	0.52	0.63	247.03	<0.01	0.52	0.86
Linoleic acid	0.35	0.72	15.56	<0.01	0.57	0.85
Valine	0.34	0.73	5.54	0.01	0.61	0.83
Ritalinic acid	0.29	0.76	7.75	<0.01	0.29	0.98
Asparagine	0.25	0.78	11.89	<0.01	1.00	0.59
Glucopyranoside	0.23	0.78	28.63	<0.01	0.23	1.00
2,5-Dimethoxymandelic acid	0.22	0.78	3.12	0.05	0.13	1.00
2-3-Bromo-5,5,5-trichloro-2,2-dimethylpentyl-1,3-dioxolane	0.21	0.78	3.45	0.03	6.69	0.01
D--Galactopyranose, pentakistrimethylsilyl ether	0.15	0.83	11.46	<0.01	0.15	1.00
Salicylic acid	0.14	0.83	40.60	<0.01	0.14	1.00
Pipecolic acid	0.09	0.89	35.56	<0.01	0.09	1.00
Methyl glycoside	0.08	0.89	6.91	<0.01	8.00	<0.01
Aminomalonic acid	0.08	0.89	16.51	<0.01	0.08	1.00
Linolenic acid	0.07	0.89	13.44	<0.01	1.05	0.56
Ornithine	0.04	0.93	4.81	0.01	1.52	0.39
5-Methyluridine	0.04	0.93	135.55	<0.01	0.04	1.00
B-Alanine	0.03	0.95	3.81	0.02	2.80	0.11

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Tristrimethylsiloxyethylene	0.02	0.97	55.79	<0.01	0.02	1.00
Elaidic acid	0.01	0.98	93.77	<0.01	0.01	1.00
Serine	0.01	0.98	10.86	<0.01	0.05	1.00
1-Propene-1,2,3-tricarboxylic acid	<0.01	1.00	562.08	<0.01	<0.01	1.00
Malic acid	<0.01	1.00	11.09	<0.01	0.23	1.00
Alanylglycine	<0.01	1.00	127.33	<0.01	<0.01	1.00
L-Lysine	<0.01	1.00	30.24	<0.01	4.85	0.02
Palmitoleic acid	<0.01	1.00	23.00	<0.01	<0.01	1.00

In the heatmap, we also see two major clusters: one composed of metabolites mostly present in the gut of the larvae fed on the artificial diet (Fig. 3A) and another cluster composed of metabolites present in the larvae fed on the natural diets (Fig. 3B).

Within the first cluster (Fig. 3A), there are four other sub clusters: one where the metabolites are similarly abundant in both strains, two sub clusters where the differences between strains are most evident, being one sub cluster composed of metabolites more abundant in *RS* and the second sub cluster composed of metabolites more abundant in *CS;* Finally, there is a sub cluster where besides the metabolites were abuntant in larvae fed on artificial diet, the metabolites were also abundant in the midgut of caterpillars fed on rice and cotton (Fig. 3A).

Whereas, in the second large cluster (Fig. 3B), we highlighted five sub-clusters: one in which the gut metabolomic profile was associated with *CS* when feeding on rice, another in which the profile was similar among the strains feeding on rice, the next was characterized by similar abundance among all plants, and the last two sub-clusters were characterized by metabolites associated with cotton and corn plants, respectively (Fig. 3B).

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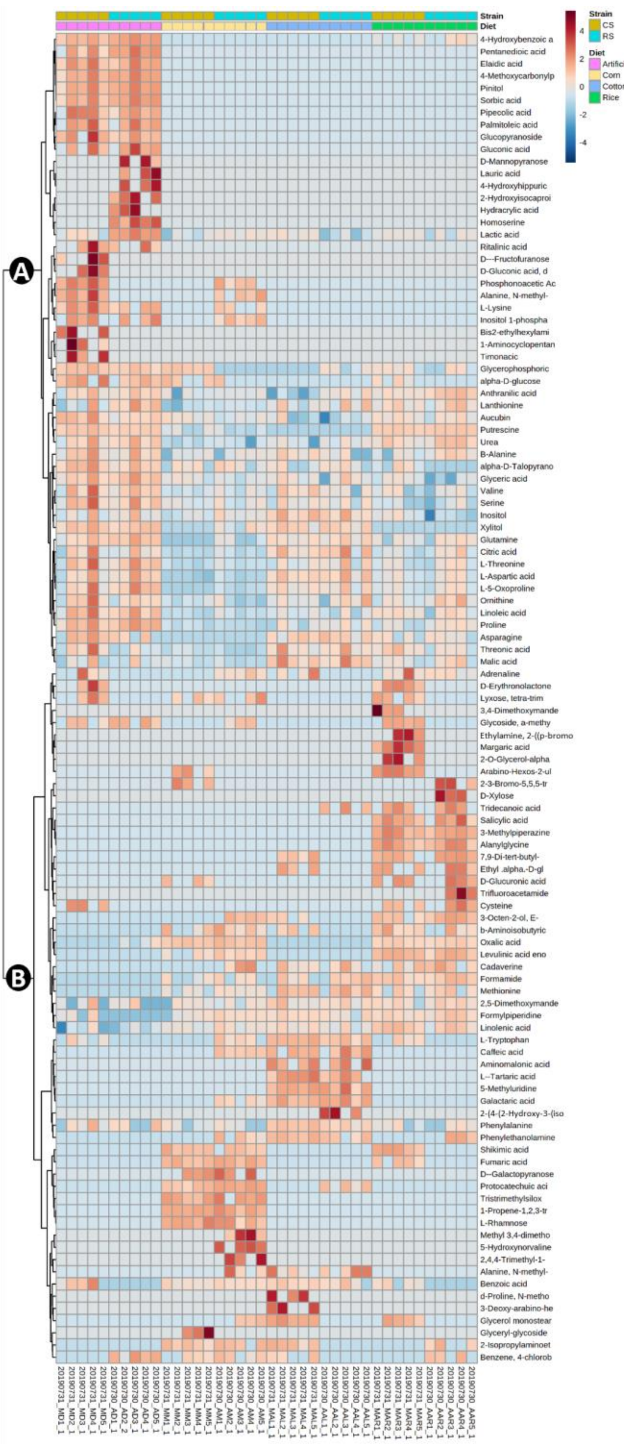

266
267 **Figure 3.** Heatmap of midgut metabolites from corn and rice strains of *Spodoptera frugiperda* larvae
268 after feeding on artificial diet, corn, cotton, or rice. The experimental groups were rice strain on rice (RiRS),
269 corn (CoRS), cotton (CtRS) and artificial diet (DiRS) and corn strain on the same diets (RiCS, CoCS, CtCS
270 and DiCS).
271

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272 Pairwise analyses of the *RS* and *CS* gut metabolomes of larvae fed on each food source led to the
273 identification of metabolites that differentiate FAW strains (Table 2). *RS* fed on corn had a higher
274 abundance of caffeic acid, inositol 1-phosphate, an unidentified biogenic amine, protocatechuic acid, 5-
275 hydroxynorvaline and 3-octen-2-ol, (E)- than the *CS* larvae. Only the abundance of glycerophosphoric acid
276 was higher in *CS* than in *RS* larvae (Table 2). In cotton, the abundances of glycerol monostearate and 2-
277 isopropylaminoethanol were higher in *CS* than in *RS* larvae. In rice-fed larvae, *CS* larvae had higher levels
278 of shikimic acid, 2-ketoglucose, D-erythronolactone and margaric acid, while *RS* had higher anthranilic
279 acid. The midgut of *RS* larvae fed on artificial diet were characterized by higher phosphonoacetic acid and
280 alanine, N-methyl-N-ethoxycarbonyl-, dodecyl ester than in *RS*, while homoserine, lactic acid, and 4-
281 chlorobutyl-benzene were more abundant in the midgut of *CS* larvae (Table 2).

282 **Table 2.** Significant features of midgut metabolome of *Spodoptera frugiperda* strains (*CS vs RS*) larvae
283 after feeding on different food sources identified by Volcano plot with fold change threshold 2 and t-tests.
284 False discovery rate correction was applied to adjust the p-values ($p \leq 0.05$) and the strain where the
285 compound were more abundant is identified.

	Metabolite	FC	log2(FC)	FDR	-log10(p)	*Sf* Strain
Corn	Caffeic acid	0.16	-2.64	0.00	5.80	*RS*
	Glycerophosphoric acid	8.18	3.03	0.00	4.22	*CS*
	Inositol 1-phosphate	0.04	-4.55	0.00	2.62	*RS*
	Unidentified biogenic amine	0.04	-4.51	0.03	1.55	*RS*
	Protocatechuic acid	0.11	-3.15	0.03	1.55	*RS*
	5-Hydroxynorvaline	0.20	-2.34	0.05	1.26	*RS*
	3-Octen-2-ol, (E)-	5.19	-2.38	0.05	1.26	*RS*
Cotton	Glycerol monostearate	6.35	2.67	0.00	4.78	*CS*
	2-Isopropylaminoethanol	22.24	4.48	0.00	3.05	*CS*
	Anthranilic acid	0.07	-3.78	0.05	1.30	*RS*
	Lactic acid	8.76	3.13	0.05	1.30	*CS*
Rice	Shikimic acid	9.28	3.21	0.00	3.84	*CS*
	2-ketoglucose	8.68	3.12	0.00	3.50	*CS*
	D-Erythronolactone	23.03	4.53	0.00	2.63	*CS*
	Margaric acid	20.90	4.39	0.00	2.63	*CS*
	Anthranilic acid	0.29	-1.79	0.03	1.59	*RS*
	Glycerol monostearate	8.05	3.01	0.05	1.28	*CS*
	Fumaric acid	7.48	2.90	0.05	1.28	*CS*
	Benzoic acid	6.71	2.75	0.05	1.28	*CS*
	Methyl Glycoside	6.09	2.61	0.05	1.28	*CS*
	Lyxose, Linear form	11.18	3.48	0.05	1.26	*CS*

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Artificial Diet	Homoserine	15.42	3.95	0.00	2.67	*CS*
	Lactic acid	73.43	6.20	0.01	1.95	*CS*
	Phosphonoacetic Acid	0.03	-4.94	0.01	1.95	*RS*
	Benzene, 4-chlorobutyl-	680.08	9.41	0.01	1.90	*CS*
	Alanine, N-methyl-N-ethoxycarbonyl-,dodecyl ester	0.06	-3.99	0.01	1.89	*RS*

286

287

288 **4. DISCUSSION**

289 The metabolic profile of the FAW larvae midgut is largely influenced by the food source used, and the
290 two strains differ in every food source analyzed. Our data demonstrates the *RS* and *CS* interact differently
291 with the substrate on which they are feeding, potentially due to differential metabolism of plant chemistry
292 (Silva-Brandão et al., 2017). Differences at the genomic level are reported for these strains (Dumas et al.,
293 2015), particularly with the large variation they have in the number of copies of genes and gene sequences
294 encoding for detoxification and digestive enzymes (Gouin et al., 2017). In addition, our results are also
295 consistent with the plethora of differences at the transcriptional level reported for the whole body of both
296 strains when feeding on the same host plants. These strains were demonstrated to have differences in the
297 expression levels of genes encoding for proteins with oxidoreductase activity, metal-ion binding, and
298 hydrolase activity, which are also related to the metabolism of xenobiotics (Orsucci et al., 2020; Silva-
299 Brandão et al., 2017).

300 Some compounds reported here in the FAW midgut have a defensive function in plants against insects,
301 such as shikimic acid. This compound has been shown to reduce intestinal proteolytic activity in insects by
302 acidifying the intestinal lumen. However, some specialist insects such as *Gilpinia hercyniae* (Hymenoptera)
303 are able to metabolize and neutralize the effect of this compound, through their gut bacteria (Schopf, 1986).
304 Therefore, the higher abundance of shikimic acid in the *CS* larval midgut comparing to *RS* feeding on rice
305 indicates the *CS* has a lower capacity to
306 process this metabolite. Additionally, the difference in shikimic acid metabolization may also be due to the
307 differential activity of the gut microbiota of the strains, similar to the adaptation found in *G. hercyniae*

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308 (Jensen, 1991). Likewise, margaric acid, or heptadecanoic acid, was shown to accumulate in *CS* when
309 feeding on rice. This compound was negatively correlated with oviposition, eclosion, and nymphal survival
310 of *Stephanitis pyrioides* (Hemiptera: Tingidae) on azalea (*Rhododendron sp*.), but positively correlated with
311 duration of development, indicating an arrestment of the developmental period (Wang et al., 1999).
312 Margaric acid is also found in rice plants (Jones et al. 2011) so it may play a role in the low performance
313 and survival of *CS* on rice plants (Silva-Brandão et al., 2017).

314 The reverse pattern was observed for strains feeding on corn, where the defensive metabolites, 5-
315 hydroxynorvaline, caffeic acid and protocatechuic acid, where measured in higher abundance in *RS* larval
316 midgut than in *CS*. The accumulation of 5-hydroxynorvaline in maize leaves has been demonstrated after
317 the feeding of *S. exigua* and the aphid, *Rhopalosiphum maidi* (Yan et al., 2015), suggesting that this
318 metabolite can provide protection against herbivores. When this compound was added to the artificial diet,
319 it reduced aphid growth and reproduction, but no significant effect was found on *S. exigua* larval growth.
320 However, 5-hydroxynorvaline also plays a defensive role by replacing amino acids in protein synthesis or
321 by inhibiting the biosynthetic pathways of many microorganisms(Guirard, 1958; Heremans and Jacobs,
322 1994; Huang et al., 2011; Kurtin et al., 1971; Washtien et al., 1977). Thus, it is possible 5-hydroxynorvaline
323 negatively impact the gut bacteria and impairs its contribution to the host.

324 The flavonoids caffeic and protocatechuic acids are referred as potential insecticides due to their toxic
325 effects (War et al., 2013). *Helicoverpa armigera* larvae fed on caffeic and protocatechuic acids displayed
326 reduced digestive and detoxification activity due to a reduction in serine protease, trypsin, and esterase
327 activity. The larvae also showed greater reduction in larval weight and higher mortality when compared to
328 the larvae fed on untreated control diet(War et al., 2013). Moreover, caffeic acid also increases the oxidative
329 stress in the gut of insect herbivores due to the elevation of protein oxidation, lipid peroxidation products
330 and release of free ions(Summers and Felton, 1994). The accumulation of these flavanoids in the *RS* larval
331 midgut could explain why *RS* does not perform as well as the *CS* when feeding on maize (Orsucci et al.,
332 2020; Silva-Brandão et al., 2017).

333

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334 The lower levels of defensive plant compounds in the midgut of the strains when they were feeding on
335 their preferred host plants (*RS* on and rice *CS* on maize) and their higher abundancies in the midgut when
336 larvae were feeding on the non-preferred host plants (Fig. S1), suggest either differential metabolization of
337 the food source as discussed above and/or differential elicitation of metabolic response in the host plant.
338 Additionally, FAW strains were demonstrated inducing different defense responses in maize and Bermuda
339 grass via specific differences in their saliva composition (Acevedo et al., 2018). The gut-associated
340 microbes in their oral secretions also play a role mediating the insect-plant interaction by regulating plant
341 defenses upon their secretion through insect oral secretions (Acevedo et al., 2017).

342 Our findings also suggest that the strains of FAW metabolize the artificial diet differently. The diet has
343 been widely used for several Lepidoptera, including FAW, demonstrating good performance (Gardner et
344 al., 1984; Perkins, 1979; Silva et al., 2018), However, most experiments were performed using the corn
345 strain. There is only one study as far as we know showing that *CS* larvae were significantly heavier than *RS*
346 larvae when they were fed the artificial diet (Silva-Brandão et al., 2017). Furthermore, artificial diets
347 generally provide unrealistic amounts of soluble carbohydrates, proteins, and fats. Perhaps the reason for a
348 greater accumulation of compounds in the larval gut when compared to other diets is due to the large amount
349 of these compounds in the food, which does not allow their complete metabolization.

350 The fact that only glycerol monostearate and 2-isopropylaminoethanol were differentially abundant
351 between *CS* and *RS* strains, being both more abundant in the midgut of *CS* larvae suggest that the strains
352 behave in a very similar way when feeding on this plant. Interesting, it is suggested that feeding on dicot is
353 a primitive condition of the FAW complex and feeding on grasses is a more recent event (Kergoat et al.,
354 2012). Additionally, studies also demonstrated that FAW presents low performance and low survival rate
355 when feeding on cotton (Ali et al., 1990; Barros et al., 2010)

356 In conclusion, our study documented the effects of host strains and dietary on the metabolome of the
357 FAW midgut. Our analyses found not only diet effects on the metabolome but indicate differential digestive
358 metabolism between FAW strains. and identified marker metabolites that may help us to better understand
359 the mechanisms involved in host adaptation. Our results shed light on our understanding of metabolic

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360 activities in the FAW, being a unit composed of its own metabolome and the metabolome of the associated
361 gut microbiota. Further analyses are essential to reveal the links between gut microbiota composition and
362 host metabolic phenotype, thus providing a holistic understanding of the functionality and adaptability of
363 strains to host plants.
364

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371

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378

379 ## 7. CONFLICTS OF INTEREST

380 Authors declare they have no conflicts of interest or competing interests.
381

382 ## 8. REFERENCES

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594 ## 9. SUPPLEMENTARY MATERIAL
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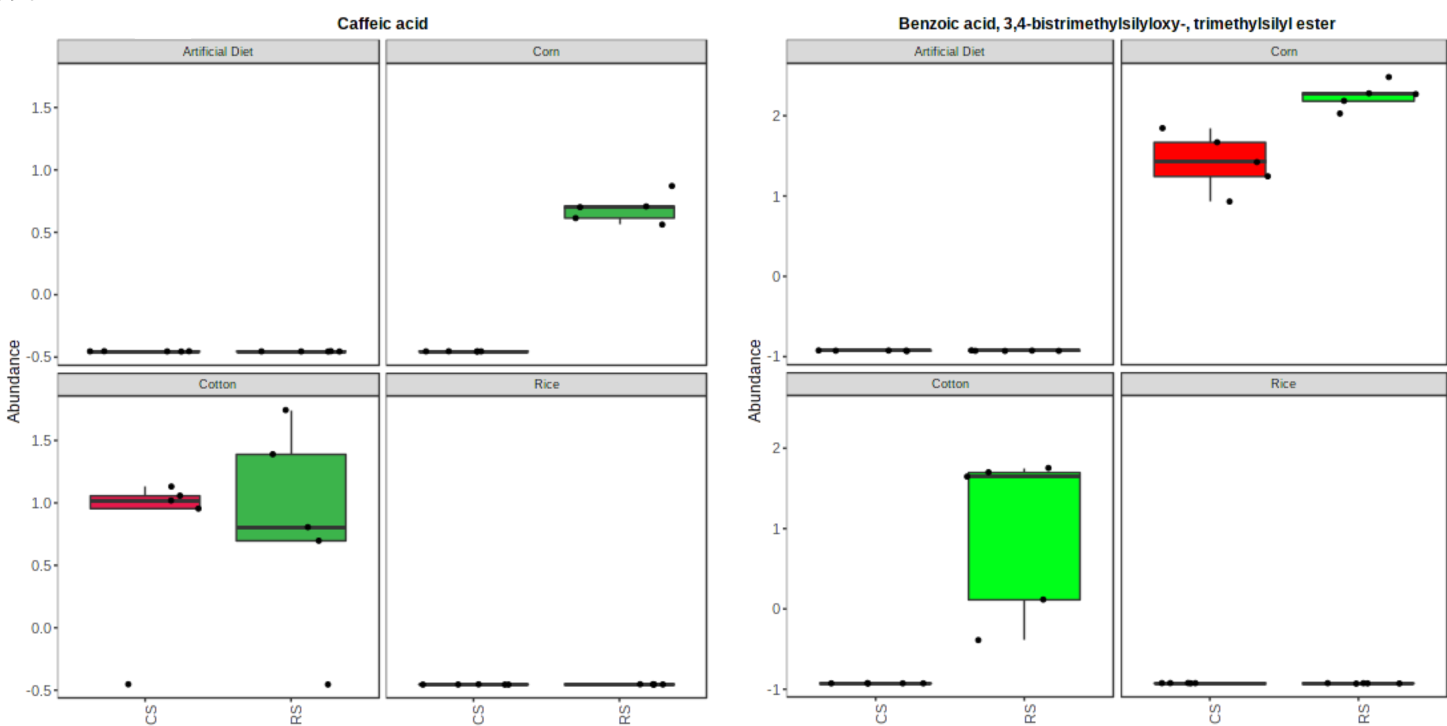
596 **A**



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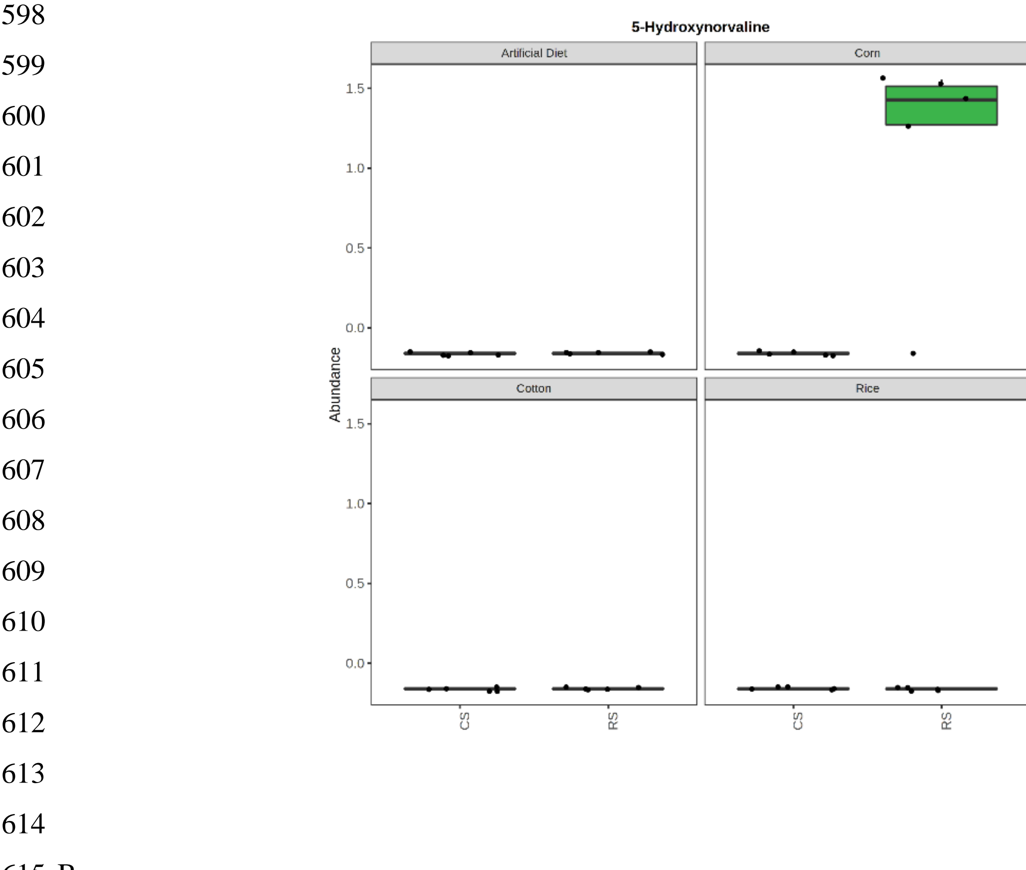

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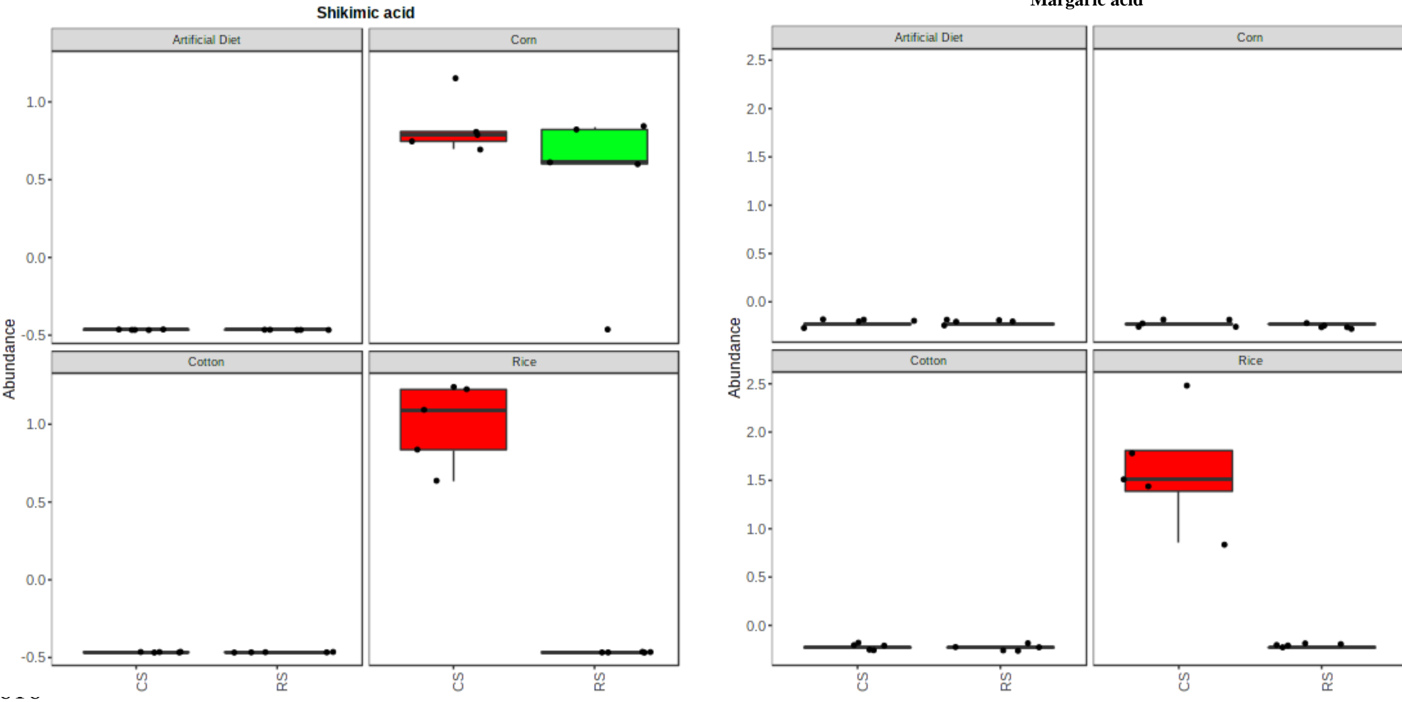

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617 **Figure S1.** Boxplot of significant features of midgut metabolome of *Spodoptera frugiperda* strains larvae
618 after feeding on maize (A) and rice (B) identified by Volcano plot with fold change threshold 2 and t-tests
619 threshold 0.05. False discovery rate correction was applied to adjust the p-values (0.05).
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