A low dose of an organophosphate insecticide causes dysbiosis and sex-dependent responses in the intestinal microbiota of the Japanese quail (*Coturnix japonica*)

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Organophosphate insecticides have been directly or indirectly implicated in avian populations declining worldwide. Birds in agricultural environments are commonly exposed to these insecticides, mainly through ingestion of invertebrates after insecticide application. Despite insecticide exposure in birds occurring mostly by ingestion, the impact of organophosphates on the avian digestive system has been poorly researched. In this work we used the Japanese quail (*Coturnix japonica*) as an avian model to study short-term microbial community responses to a single dose of trichlorfon at low concentration in three sample origins of the gastrointestinal tract (GIT): cecum, large intestine and faeces. Using next-generation sequencing of 16S rRNA gene amplicons as bacterial markers, the study showed that ingestion of insecticide caused significant changes in the GIT microbiome. Specifically, microbiota composition and diversity differed between treated and untreated quail. Insecticide-associated responses in the cecum showed differences between sexes which did not occur with the other sample types. In cecal microbiota only treated females showed significant shifts in a number of genera within the Lachnospiraceae and the Enterobacteriaceae families. The major responses in the large intestine were a significant reduction in the genus *Lactobacillus* and increases in abundance of a number of Proteobacteria genera. All microbial shifts in faeces occurred in phylotypes that were represented at low proportional abundances. In general, changes in microbiota possibly resulted from contrasting responses towards the insecticide, either positive (e.g. biodegrading bacteria) or negative (e.g. insecticide-susceptible bacteria). This study demonstrates the significant impact that organosphate insecticides have on the avian gut microbiota; showing that a single small dose of trichlorfon caused dysbiosis in the GIT of the Japanese quail. Further research is necessary to understand the implications on birds' health, especially in females.

1   **A low dose of an organophosphate insecticide causes**

2   **dysbiosis and sex-dependent responses in the intestinal**

3   **microbiota of the Japanese quail (*Coturnix japonica*)**

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Abstract

Organophosphate insecticides have been directly or indirectly implicated in avian populations declining worldwide. Birds in agricultural environments are commonly exposed to these insecticides, mainly through ingestion of invertebrates after insecticide application. Despite insecticide exposure in birds occurring mostly by ingestion, the impact of organophosphates on the avian digestive system has been poorly researched. In this work we used the Japanese quail (*Coturnix japonica*) as an avian model to study short-term microbial community responses to a single dose of trichlorfon at low concentration in three sample origins of the gastrointestinal tract (GIT): caecum, large intestine and faeces. Using next-generation sequencing of 16S rRNA gene amplicons as bacterial markers, the study showed that ingestion of insecticide caused significant changes in the GIT microbiome. Specifically, microbiota composition and diversity differed between treated and untreated quail. Insecticide-associated responses in the caecum showed differences between sexes which did not occur with the other sample types. In caecal microbiota only treated females showed significant shifts in a number of genera within the Lachnospiraceae and the Enterobacteriaceae families. The major responses in the large intestine were a significant reduction in the genus *Lactobacillus* and increases in abundance of a number of Proteobacteria genera. All microbial shifts in faeces occurred in phylotypes that were represented at low relative abundances. In general, changes in microbiota possibly resulted from contrasting responses towards the insecticide, either positive (e.g. biodegrading bacteria) or negative (e.g. insecticide-susceptible bacteria). This study demonstrates the significant impact that organophosphate insecticides have on the avian gut microbiota; showing that a single small dose of trichlorfon caused dysbiosis in the GIT of the Japanese quail. Further research is necessary to understand the implications on birds' health, especially in females.

Introduction

Organophosphates are the most widely applied insecticides (Bondarenko et al. 2004; Zamy et al. 2004), and they primarily act by inhibiting cholinesterase, a nervous system enzyme (Marrs 1993). Avian exposure to these contaminants occurs through dermal contact, maternal transfer, inhalation and ingestion (Smith et al. 2007). Among all of these routes, ingestion is the predominant pathway of avian exposure, which can occur through the consumption of prey items

53   such as insects that have been previously exposed and/or killed by insecticides (Golden &

54   Rattner 2003). Several studies have reported how organophosphate insecticides caused the mass

55   death of birds (e.g. Fleischli et al. 2004; Frank et al. 1991), some of these through ingestion of

56   contaminated prey (e.g. Goldstein et al. 1999; Goldstein et al. 1996). While these lethal

57   responses to insecticide ingestion highlight toxicity to birds, few studies have examined effects

58   of ingestion of sub-lethal organophosphate dosage on the gastrointestinal tract which may lead to

59   chronic effects on bird health.

60   Trichlorfon is an organophosphate insecticide, which has been used since the 1950s in a wide

61   number of systems, including tree orchards, vines, fruits, vegetables, field crops, pastures,

62   forests, and turf (de Oliveira et al. 2002). Trichlorfon is considered moderately toxic to birds

63   (e.g. LC50 values in bobwhite quail [*Colinus virginianus*]=720 ppm; in Japanese quail [*Coturnix*

64   *japonica*]=1901 ppm) (Hill et al. 1975). Most of the toxicological research on trichlorfon in

65   avian species has focused on its neurotoxicity; generally shown as severe decreases in

66   anticholinesterase activity (e.g. Hill 1982; Iko et al. 2003). The majority of the toxicological

67   assessments have used relatively high insecticide concentrations derived from general measures

68   of toxicity such as the lethal dose or lethal concentration values of the species tested (e.g. de

69   Oliveira et al. 2002; Slott & Ecobichon 1984). However, little work has assessed the

70   toxicological impacts of insecticides using insecticide concentrations representative of likely

71   exposure levels in the field; such as residues present at a given time after their application (e.g.

72   Joly et al. 2013). Residue levels in pesticide-exposed items can be measured to calculate how

73   much of the contaminant is ingested by birds to then understand the context of impact

74   assessment (Smith et al. 2007).

75   Although the digestive system is the first contact point for contaminants, few studies have aimed

76   to understand the toxicological impacts of pesticides on the gastrointestinal tract (GIT) (Joly et

77   al. 2013). The microbiota present throughout the GIT is essential in development and maturation

78   of the immune system (Kelly & Conway 2005; Umesaki et al. 1999). The GIT microbiota

79   contributes to digestive and fermentative processes, and prevents colonization by pathogens

80   (Kamada et al. 2013), ultimately contributing to the energy and nutrient supply, immunity, and

81   general well-being of the host (Stanley et al. 2014). The importance that host-microbiome

82   symbiotic relationships have in the success of animals is only recently being recognized by

83 evolutionary biologists and ecologists (McFall-Ngai et al. 2013). However, environmental

84 stresses can shift these relationships, thus affecting host performance and/or health (Myers

85 2004).

86 To date, most of the studies characterising the GIT microbiota of birds have been carried out in

87 chickens (e.g. Dumonceaux et al. 2006; Stanley et al. 2013). Only a few other studies have

88 characterised the microbiota of other bird species, including kakapo (*Strigops habroptilus*), emu

89 (*Dromaius novaehollandiae*), turkey (*Meleagris spp.*) (Waite & Taylor 2014), bobwhite quail

90 (Su et al. 2014), and Japanese quail (Wilkinson et al. 2016). The avian GIT is shorter than that of

91 mammals, thus decreasing food transit time (Golian & Maurice 1992), and it is colonized by a

92 unique microbiome adapted to the host (Stanley et al. 2014). Previous research has assessed the

93 impact of chronic low-dose organophosphate insecticide exposure on the GIT microflora of rats

94 and a human GIT simulator (Joly et al. 2013). However, to our knowledge, none has studied the

95 impact of organophosphate insecticides on GIT microbiota of any avian species.

96 In this study, trichlorfon was selected as a representative organophosphate insecticide, and the

97 Japanese quail was chosen as an indicative avian model, as suggested elsewhere (Foudoulakis et

98 al. 2013). The general aim of this study was to understand the immediate toxicological effects

99 that trichlorfon residues may produce on the intestinal microbiota of the Japanese quail.

100 Specifically, our aims were to: i) assess the potential impacts that a single oral dose of a low

101 concentration of trichlorfon (equivalent to that present in a bird when feeding upon arthropods in

102 an orchard 48 hours after spraying at the minimum commercially recommended dose) had on the

103 microbial GIT of the Japanese quail after 24 hours of exposure. The impact of trichlorfon were

104 assessed by means of shifts in the microbial community composition of the caecum, large

105 intestine and faeces, and ii) compare these shifts between sexes.

Materials and methods

Experimental birds

108 Fertilized eggs were obtained from Banyard Game Birds farm, Toowoomba, Queensland. The

109 line of quail has been bred for over 20 years without the use of antibiotics and growth promoters.

110 The Banyard Game Birds practice natural hatchery operations without fumigation or sterilization

111 of the eggs, thus ensuring essential transfer of maternal microbiota to the next generation

112   (Stanley et al. 2013). Hatchlings were housed together in a rearing pen until they reached three

113   weeks of age, when they were separated to male and female to avoid male fighting over the

114   females, and allocated into a number of cages, each containing four birds, until the start of the

115   experiment at eight weeks of age. The bird stock at the time of the trial was three times higher

116   than the number used in this experiment allowing us to select the birds with near identical initial

117   weights taking each of the birds allocated to the same treatment from the different cage thus

118   avoiding the cage effects on microbiota. At the start of the experiment birds from the rearing

119   pens were transferred to individual cages and allocated to treatment groups. Throughout the

120   rearing and experimental period the birds were housed in a temperature controlled room at 25 °C,

121   natural light, feed and water was supplied ad libitum. Feed supplied to the birds from hatch was a

122   commercial turkey starter (Ridley, Barastoc) (22% protein, 2.5% fat, 5% fibre, with added 0.3%

123   salt, 1% calcium, 8mg/kg copper and 0.3 mg/kg selenium). The starter feed was replaced with

124   Barastoc turkey grower feed (20% protein, 2% fat, 6% fibre, 0.3% salt, 0.95% calcium, 8 mg/kg

125   copper and 0.3 mg/kg selenium) at 4 weeks of age.

126   ***Experimental design***

127   Twenty healthy adult quails of eight weeks-old (10 male and 10 female) were used for this

128   experiment, (average weights; male=300 g and female=313.25 g). At the beginning of the

129   experiment, no significant differences in weight were found between treated and untreated quails

130   by sex. Five birds of each sex were assigned to each of two treatments, insecticide-treated and

131   untreated (control). Each of these 20 selected birds were then caged individually throughout the

132   duration of the experiment. Japanese quails are early maturing, short lived birds with the lifespan

133   of up to three years (Woodard & Abplanalp 1971). By six weeks of age all females were laying

134   eggs and all birds, both male and female were fully mature and considered adult.

135   Trichlorfon residue was estimated using a model specifically designed to calculate pesticide

136   residues on avian food items (i.e. T-REX model, developed by the US Environmental Protection

137   Agency [USEPA] [Sullivan & Wisk 2013]). The T-REX model estimated that 48 hours after a

138   single application of Lepidex 500 (containing 500 g/l trichlorfon) at the lowest commercially

139   recommended dose in tree crops (i.e. 100 ml/100 l), the concentration of trichlorfon residues

140   present on arthropods was 26.42 ppm. Following the toxicity test protocol of the USEPA, the

141   latter concentration was individually adjusted to each bird based on its estimated daily food

142   intake (average 133 g/day) and its bodyweight (average 306.6 g) (USEPA 1993). Final

143   trichlorfon concentrations (average 12 μg trichlorfon/g bird weight) were calculated by

144   multiplying the estimated concentration of the trichlorfon residues present on arthropods by the

145   estimated daily food intake of each bird in mg. These final trichlorfon concentrations were

146   adjusted to a final volume of 1ml using distilled water, and given to birds in the 'treated' group

147   by oral gavage in a single dose. Equal volumes of water were given by gavage to the birds in the

148   'untreated' group. Quails were continuously observed during the course of the experiment using

149   video cameras to monitor their behaviour. None of the birds died or showed abnormal behaviour.

150   Since the aim of the study was to characterize short-term GIT toxicological responses, birds were

151   euthanized 24 hours after the administration of trichlorfon by intramuscular overdose of

152   phenobarbitone sodium IP.

153   Samples from 3 origins (caecum, large intestine and faeces) were obtained from each quail.

154   Luminal specimens were obtained from caecum and large intestine by removing the contents and

155   placing into sterile containers. Faecal samples from each bird were collected within one hour

156   prior to necropsy. All samples were stored at -80°C for a maximum of one week until DNA was

157   extracted.

158   ***Animal ethics statement***

159   Animal ethics approval for the present project was obtained from the Animal Ethics Committees

160   of the Central Queensland University (permission number A14/03-309).

161   ***Bioinformatics analysis***

162   Total DNA was isolated using the Bioline ISOLATE Faecal DNA Kit (#BIO-52038) according

163   to the manufacturer's protocols. DNA was amplified across the V3-V4 16S rRNA gene region

164   using Q5 DNA polymerase (New England Biolabs). PCR conditions were; 98°C for 60 seconds

165   followed by 30 cycles of 98°C for 10 seconds, 49°C for 30 seconds, 72°C for 30 seconds,

166   followed by a final elongation at 72°C for 10 min. Sequencing was performed on an Illumina

167   MiSeq (2 x 300 bp), based on the method detailed by Fadrosh et al. (2014). The quality filtered

168   sequences were analysed in QIIME 1.8 software (Caporaso et al. 2010). Sequences were joined

169   in QIIME using the fastq-join method with zero percent error allowed across the overlapping

170   region. Sequences were demultiplexed retaining only sequences with Phred quality score higher

171 than 20. OTUs were picked using the Uclust algorithm (Edgar 2010) with 97% sequence

172 similarity threshold and inspected for chimeric sequences using Pintail (Ashelford et al. 2005).

173 The OTU table was filtered to remove nonbacterial OTUs and OTUs that were of less than

174 0.01% abundance. Samples with less than 2000 sequences were removed from the analysis

175 resulting in 51 samples with high quality joined sequences, 25 from control and 26 samples from

176 insecticide treated groups. The filtered table was normalised using QIIME's default cumulative

177 sum scaling (CSS) method (Paulson et al. 2013). Additional taxonomic assignment was

178 performed using blastn (Altschul et al. 1997) against the NCBI 16S Microbial database. The

179 complete dataset is available on MG-RAST database (http://metagenomics.anl.gov/) under

180 project ID 4693672.3.

181 ***Statistical analysis***

182 Permutational analysis of variance (PERMANOVA) (Anderson 2005) was used to test for

183 differences in microbiota composition at the OTU level. Specifically, three fixed factors were

184 tested: Treatment (treated and untreated quails), Sex (male, female), and Origin (caecum, large

185 intestine, and faeces). Normalised data was used to calculate matrices using Bray-Curtis

186 distances. Data was permuted 9999 times (Anderson & Braak 2003). Canonical Analysis of

187 Principal Coordinates (CAP) was used to visualize these differences in composition. Analysis of

188 Variance (ANOVA) tests were used to test for differences in community diversity, based on

189 Shannon diversity index values at the OTU level. ANOVA was also used to calculate significant

190 differences at higher microbiota taxonomic levels across factors. Additionally, a heat map was

191 created to depict positive and negative correlations between taxa based on Pearson's correlation

192 values. PERMANOVA and CAP were performed with PRIMER software (v.6.0) and

193 PERMANOVA+ software (v.1.0.6). Analyses of community diversity, taxonomic structure, and

194 heat maps were performed in Calypso (v.3.0) (http://bioinfo.qimr.edu.au/calypso/).

195 # Results

196 ***Microbiota composition***

197 Microbiota composition was significantly affected by Treatment (Pseudo-F=3.544, P=0.006) and

198 Origin (Pseudo-F=11.267, P≤0.001), but not by Sex (Pseudo-F=1.517, P=0.134) (Fig. 1). There

199 was a significant Treatment × Origin interaction (Pseudo-F=2.748, P=0.004). There were highly

200   significant differences in microbiota composition between treated and untreated quails in caecum

201   (t=1.652, P=0.001), faeces (t=1.759, P=0.001), and large intestine, although the level of

202   significance of the latter was lower than the other two origins (t=1.512, P=0.036). For untreated

203   quails, caecal microbiota differed significantly from those of faeces (t=2.883, P≤0.001) and large

204   intestine (t=3.406, P≤0.001). However the large intestine did not significantly differ in

205   microbiota composition from that of faeces (t=1.158, P=0.178) (Fig. 1). In treated birds,

206   differences in microbiota composition between each of the three origins were significant

207   (caecum, t=4.037, P≤0.001; large intestine, t=2.327, P≤0.001; faeces, t=2.030, P≤0.001) (Fig. 1).

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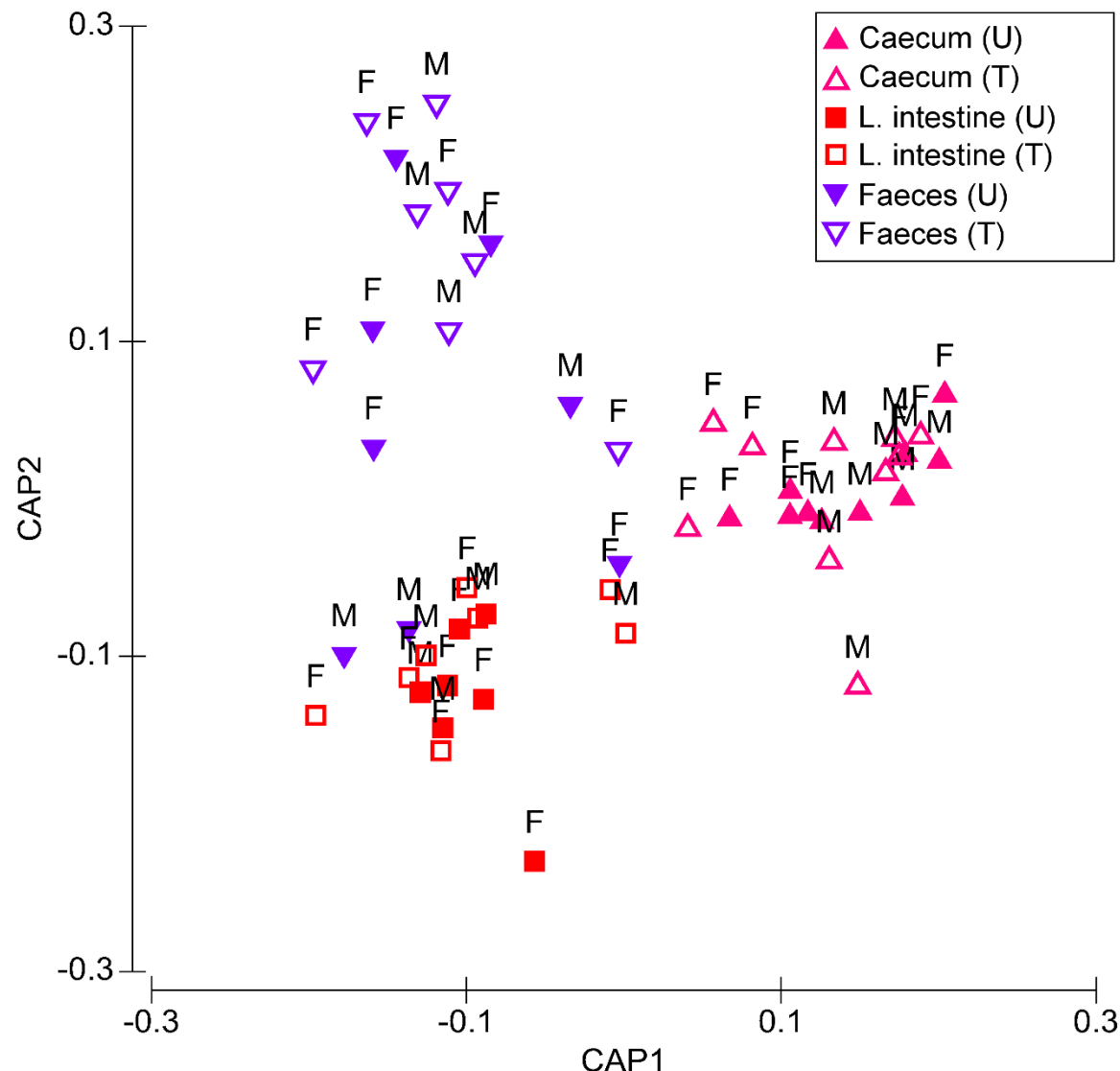
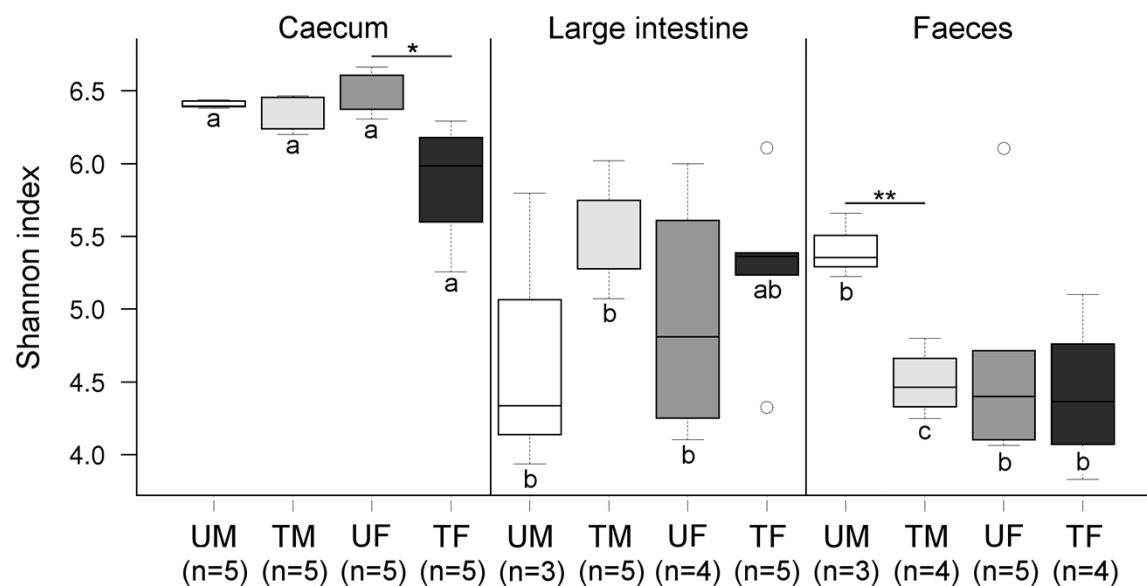


Fig. 1 Canonical analysis of principal coordinates (CAP) biplot showing a constrained by origin, non-dimensional ordination of microbial community across all factors. Letter U in the sample legend indicates untreated control while T indicates insecticide-treated birds. M stands for male and F for female birds.

Differences between sexes were recorded in the response of microbiota to insecticide. Particularly in the caecum, Treatment and Sex had a significant impact on the microbiota composition (respectively, Pseudo-F=2.728, P=0.002 and Pseudo-F=2.139, P=0.023). Pair-wise tests showed that treated females had a significantly different microbiota composition compared to non-treated females (t=1.615, P=0.036), while in males, these differences were not significant (t=1.212, P=0.122) (Fig. 1). Treatment was the only significant source of variation in both the large intestine (Pseudo-F=2.285, P=0.027) and faeces (Pseudo-F=3.093, P=0.002) (Fig. 1).

221 However, in the latter origin, pair-wise tests showed that Treatment had a significant effect on
222 the microbiota composition of males (Pseudo-F=1.607, P=0.028), but not on that of females
223 (Pseudo-F=1.224, P=0.085).

224 *Microbiota diversity*

225 Community diversity differed significantly across each of the three factors (P≤0.001) (Fig. 2).
226 Overall, the average caecal microbiota diversity, at an OTU level, was higher than the other two
227 origins. Microbiota diversity in the caecum differed significantly between treated and untreated
228 females (P=0.019), but no significant differences were shown for the male microbiotas (P=0.185)
229 (Fig. 2). Diversity in the large intestine did not differ significantly across Treatment or Sex (Fig.
230 2). In faeces, significant differences were found between treated and untreated males (P=0.003),
231 but not for the females (P=0.605) (Fig. 2).

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233 Fig. 2 Boxplots showing Shannon index values at the OTU level across factors. Within each origin, significant
234 differences are indicated ('*' P<0.05, '**' P≤0.01). Same colours indicate groups of quails of the same sex and
235 under the same treatment. Different letters indicate significant differences between these groups across origins.
236 Letter U in the sample legend indicates untreated control while T indicates insecticide-treated birds. M stands for
237 male and F for female birds. Number of samples are indicated.

238 *Caecum*

239   Overall, the caecum microbiota was represented by 9 phyla, 27 orders, 56 families, 87 genera,

240   and 1138 OTUs, based on 97% sequence similarity and using the Uclust algorithm. Across

241   factors, the main three phyla ordered by relative abundance levels were Firmicutes (constituting

242   approximately 81% of abundance across groups), Bacteroidetes (10%), and Proteobacteria (8%).

243   The main genera represented in the caecal microbiota were *Ruminococcus* (15%), *Bacteroides*

244   (10%), *Faecalibacterium* (8%), *Clostridium* (6%), and *Eubacterium* (4%).

245   Comparisons of the microbiota at the phylum level showed that in treated quails the abundance

246   of Proteobacteria was significantly increased (P=0.007) (Fig. 3A). This occurred due to changes

247   at the family level, by similar significant relative increases in abundance of Enterobacteriaceae

248   (5.4 to 9.7%, P=0.009). Four genera contained in this family increased significantly in abundance

249   within the treated females, *Klebsiella* (P=0.016), *Salmonella* (P=0.032), *Erwinia* (P=0.016), and

250   *Serratia* (P=0.021) (Fig. 3A). Nevertheless, each of the latter three genera represented relative

251   abundances below 1%. Significant increases also occurred in the phylum Bacteroidetes

252   (P=0.043), linked to equally significant increases in the genus *Bacteroides* (Fig. 3B). The relative

253   abundance of the phylum Firmicutes decreased significantly from 84% in untreated to 77% in

254   treated quails (P=0.005). These results were related to a significant decrease of the family

255   Lachnospiraceae (55 to 47%, P=0.023). Three genera present in this family significantly

256   decreased in treated females, *Eubacterium* (P=0.008), *Blautia* (P=0.032), and *Roseburia*

257   (P=0.025) (Fig. 3C). When combining male and female quails, *Coprococcus*, an additional genus

258   from the same family, decreased significantly (P=0.035) (Fig. 3C). No significant differences

259   were found at the genus level in males. The heat map showed how each of the genera increasing

260   significantly in female quails were positively correlated between each other and negatively

261   correlated with four other genera: *Coprococcus*, *Faecalibacterium*, *Ruminococcus*, and *Blautia*

262   (Fig. 3D).

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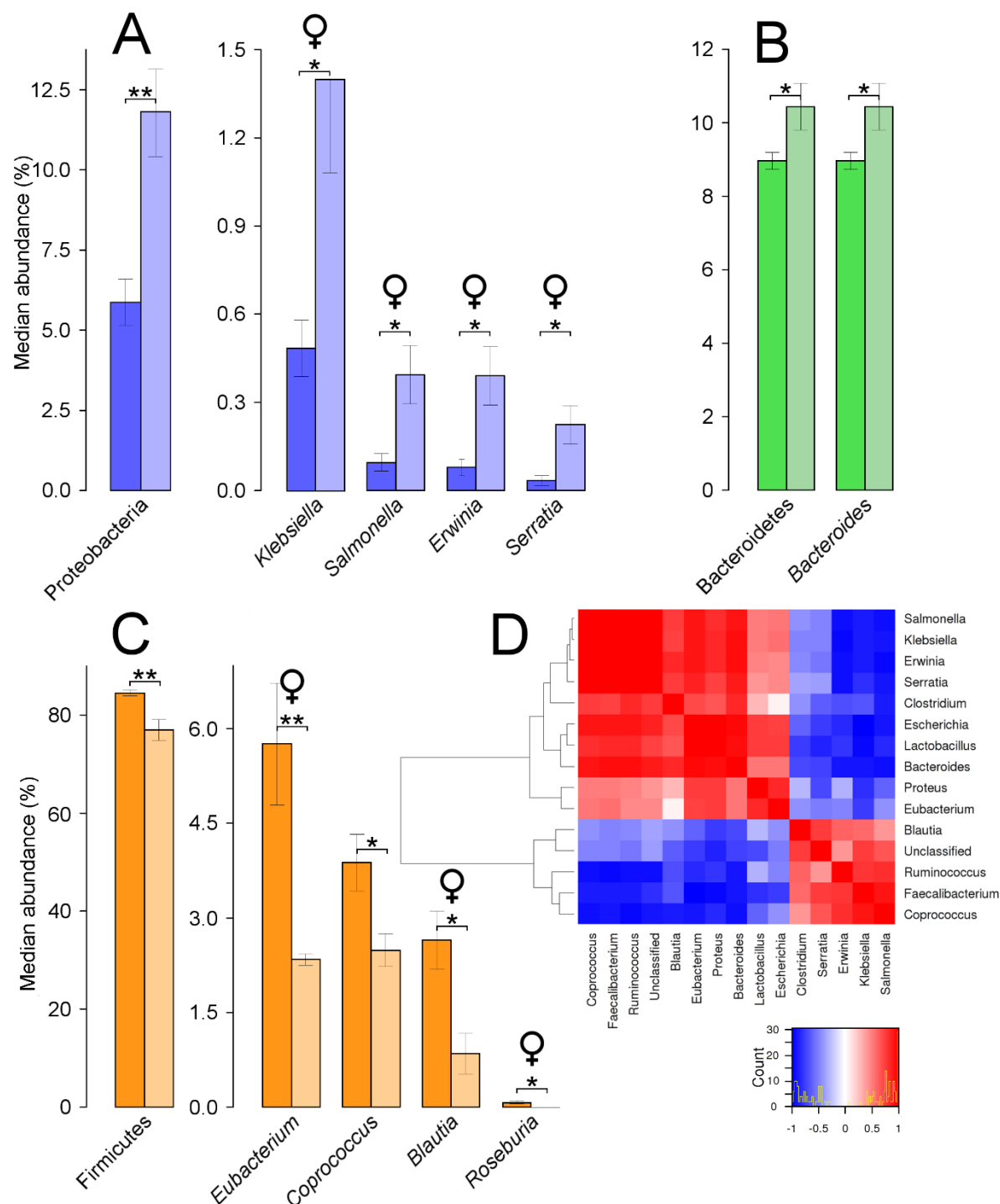
264   Fig. 3. Caecal microbial taxa showing significant shifts. These taxa were grouped in the phyla Proteobacteria (A),

265   Bacteroidetes (B), and Firmicutes (C); '*' indicates P≤0.05, and '**' P≤0.01. Light colours indicate treated birds.

266   Sex-dependent significant shifts are indicated with symbols. To facilitate comparisons across origins, taxa in the

267   same phylum is indicated by same colours in figures 3, 4, and 5. Panel D shows correlations between genera in

268   treated females (at levels of abundance >0.4%), based on Pearson's correlation. A histogram indicates the

269   distribution of counts based on the correlation values.

270   ***Large intestine***

271   The overall composition of the large intestine microbiota was represented by a total of 10 phyla,

272   33 orders, 71 families, 103 genera, and 1068 OTUs. Based on averaged values across all factors,

273   the main phyla were Firmicutes (65%), Proteobacteria (21%), Bacteroidetes (7%), and

274   Actinobacteria (5%). The five most abundant genera were *Lactobacillus* (25%), *Ruminococcus*

275   (6%), *Bacteroides* (6%), *Clostridium* (4%), and *Faecalibacterium* (3%).

276   Based on ANOVA rank tests, there were significant differences at the phylum level, as the

277   abundance of Firmicutes decreased from 76% in untreated to 55% in treated quails (P=0.014)

278   (Fig. 4A). These changes were linked to significant decreases at the genus level in *Lactobacillus*

279   (P=0.029) (Fig. 4A). The most abundant species under this genus was *L. agilis*, which was

280   assigned, using blast against 16S Microbial database, to a total of 36 OTUs as 'first choice'

281   identity (average=93.3%, min=86%, max=98%). This species' abundance dropped significantly

282   from 25% in untreated to 5% in treated quails (P=0.014) (Fig. 4A). The phylum

283   Verrucomicrobia, with a relative abundance of less than 1%, significantly increased in treated

284   quails (P=0.038), due to equally significant increases in the genus *Akkermansia* (Fig. 4B).

285   Although not-significant, the Proteobacteria phylum increased from 15% in untreated to 27% in

286   treated quails, partly due to significant increases at the genus level in *Klebsiella* (P=0.019), and

287   at lower proportions (i.e. <1%) in *Rhodospirillum* (P=0.045), *Alkanindiges* (P=0.019) and

288   *Lysobacter* (P=0.045). Also, significant increases in the genus *Agrobacterium* (P=0.044)

289   (comprised of an OTU 100% identical to *Agrobacterium fabrum*) were found only in females

290   (Fig. 4C). Lastly, the proportion of unclassified genera increased significantly in treated quails

291   (P=0.029) (Fig. 4D).

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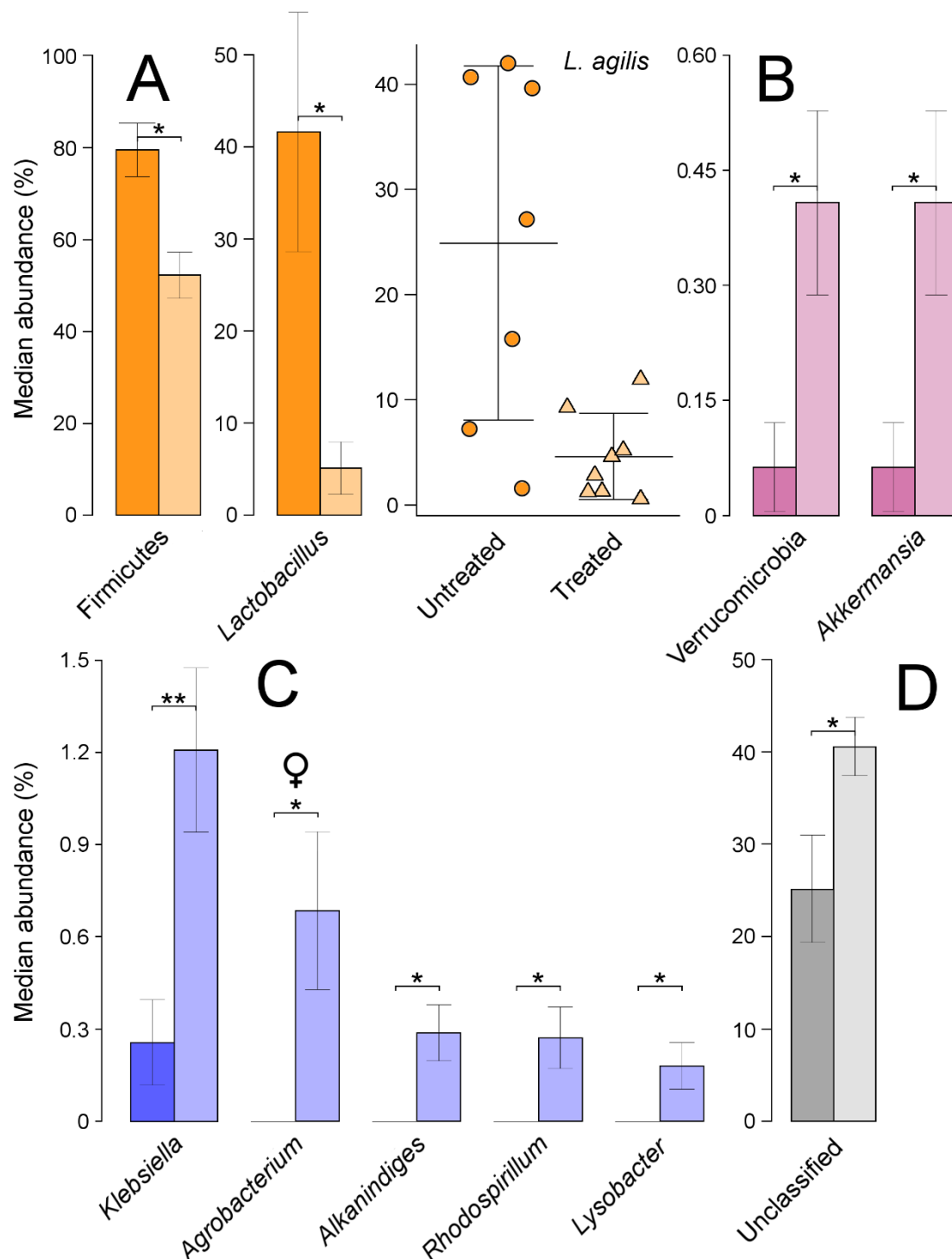


Fig. 4. Microbial taxa in the large intestine showing significant shifts. These taxa were grouped in the phyla

Firmicutes (A), Verrucomicrobia (B) and Proteobacteria (C). Unclassified taxa is shown in panel D; '*' indicates

P≤0.05, and '**' P≤0.01. Light colours indicate treated birds. Sex-dependent significant shifts are indicated with

296 symbols. To facilitate comparisons across origins, taxa in the same phylum is indicated by same colours in figures 3,

297 4, and 5.

298 Additionally, the phylum Actinobacteria increased non-significantly from approximately 3% in

299 untreated to 7% in treated quails, partly because of relatively low but significant increases in

300 *Janibacter* (0 to 0.3%, P=0.007), and only in females, in *Rothia* (0 to 0.5%, P=0.044).

301 Additionally, treated females experienced significant increases in the family Bacillaceae (0 to

302 0.7%, P=0.044).

303 ***Faeces***

304 Overall, after initial OTU filtering described previously, microbial taxa in quail faeces were

305 classified into 9 phyla, 31 orders, 67 families, 99 genera, and 980 OTUs. Across factors, the

306 main phyla were Firmicutes (approximately 57% of abundance), Proteobacteria (25%), and

307 Bacteroidetes (8%), and the main genera were *Lactobacillus* (10%), *Faecalibacterium* (6%), and

308 *Bacteroides*, *Clostridium*, and *Ruminococcus* (each 4%).

309 At the phylum level, only treated males showed significant increases in Verrucomicrobia

310 (P=0.05), which were linked to equally significant increases in the family Verrucomicrobiaceae,

311 and the genus *Akkermansia* (Fig. 5A). This genus was composed of a single OTU which was

312 assigned to the species, *A. muciniphila*, at 100% of identity across the amplified region.

313 Furthermore, the phylum Firmicutes decreased non-significantly when combining results from

314 both sexes, from 59% in untreated to 55% in treated quails. Two families from this phylum

315 showed significant increases, Turicibacteraceae (P=0.002), and Streptococcaceae (P=0.001), the

316 latter partly caused by a significant increase in the genus *Streptococcus* (P=0.001) (Fig. 5B).

317 Other genera from the same phylum showed significant shifts: significant decreases were found

318 in *Eubacterium* (P=0.032) in treated males, and significant increases were found in

319 *Jeotgalicoccus* (P=0.042) in treated females (Fig. 5B). Sphingomonadaceae, a family from the

320 phylum Proteobacteria, was significantly increased in treated quails (0.33 to 1.20% P=0.018),

321 which resulted from significant increases in the genus *Sphingomonas* (P=0.010) (Fig. 5C). Two

322 additional genera were significantly increased in abundances in treated quails, *Delftia* (P=0.010)

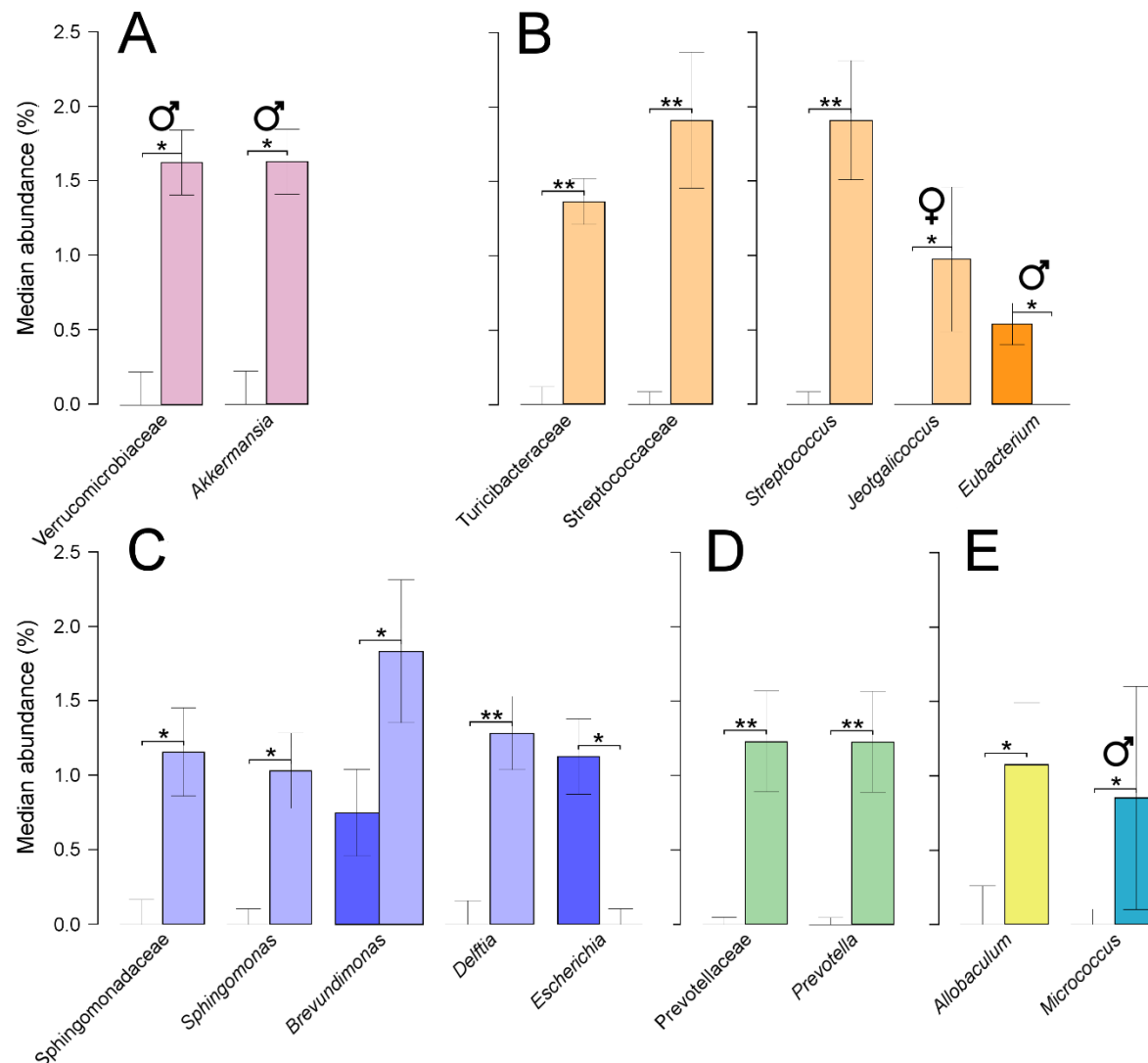
323 and *Brevundimonas* (P=0.031), while *Escherichia* showed a significant decrease (P=0.005) (Fig.

324 5C). Prevotellaceae, a family from the phylum Bacteroidetes, showed significant increases

325 (P=0.004), caused by an equally significant increase in the genus *Prevotella* (Fig. 5D).

326 Additionally, two genera, each from different phylum, varied significantly their abundances,

327 *Allobaculum* (Tenericutes) (P=0.027), and *Micrococcus* (Actinobacteria) (P=0.050), the latter

328 only in males (Fig. 5E).

329

330 Fig. 5. Faecal microbial taxa showing significant shifts. These taxa were grouped in the phyla Verrucomicrobia (A),

331 Firmicutes (B), Proteobacteria (C), Bacteroidetes (D), and shown together, Tenericutes and Actinobacteria (E). '*'

332 indicates P≤0.05, and '**' P≤0.01. Light colours indicate treated birds. Sex-dependent significant shifts are

333 indicated with symbols. To facilitate comparisons across origins, taxa in the same phylum is indicated by same

334 colours in figures 3, 4, and 5.

335 Additionally, several significant changes in abundance occurred in phylotypes that were present

336 in low abundance (below 2%). There was a significant increase in treated quails in the family

337 Bifidobacteriaceae (0.2 to 0.8%, P=0.029), linked to equally significant increases in the genus

338   *Bifidobacterium*. At similar low abundances, there were significant increases in the family

339   Pasteurellaceae (0 to 0.6%, P=0.05) linked to equally significant increases in the genus

340   *Haemophilus* (0 to 0.6%, P=0.03). There were also significant increases in the families

341   Clostridiaceae (0.1 to 1.1%, P=0.016), Propionibacteriaceae (0.4 to 1.2, P=0.022), and

342   'Clostridiales Family XI' (0 t 0.6%, P=0.013). Increased abundance of the latter two families

343   were linked to an equally significant increase in the genera *Propionibacterium* and

344   *Anaerococcus*. Additionally, there were significant increases in the genus *Oceanobacillus* (0 to

345   0.6%, P=0.013) and significant decreases in the genus *Eggerthella* (0.25 to 0%, P=0.013).

Discussion

347   This work explored the short-term impacts of an organophosphate insecticide on the intestinal

348   microbiota of the Japanese quail. Few studies have assessed the impact of insecticides on the

349   GIT (e.g. Joly et al. 2013), but to our knowledge none has been conducted on avian systems in

350   spite of results highlighting the central role that the GIT resident microflora plays in the bird's

351   health (Stanley et al. 2012; van Dongen et al. 2013). Our results revealed that a single oral dose

352   of trichlorfon at low-concentration induced significant shifts in bacterial community composition

353   and diversity across origins. Particularly, our results showed overall increases in Proteobacteria,

354   which have been recently regarded as an indicator of microbial dysbiosis in mammals (Shin et al.

355   2015). In humans, microbiota dysbiosis has been associated with immunological dysregulation

356   (Round & Mazmanian 2009) and diseases such as diabetes (Qin et al. 2012), irritable bowel

357   syndrome (Chassard et al. 2012), colorectal cancer (Sobhani et al. 2011), and polycystic ovarian

358   syndrome (Tremellen & Pearce 2012). Although the role of the GIT microbiota is not yet fully

359   understood in humans nor in birds, high microbial diversity is generally considered an indicator

360   of a healthy balance. In contrast, an imbalance generally promotes overgrowth of pathogenic or

361   non-beneficial microbiota, and diseases may occur (Karlsson et al. 2010). This study showed

362   consistency in microbial shifts at high taxonomic level in caecum and large intestine, but not on

363   faeces. However, at lower taxonomic levels, contrasting microbial responses were found between

364   all treatments and origins.

365   ***Caecum***

366   Caecal microbiota showed significantly higher diversity values at an OTU level and highly

367   significant differences in composition compared with the other two origins. These results agree

368   with findings from other studies of Japanese quail (Wilkinson et al. 2016). Significant shifts in

369   microbiota were observed at the phylum level. Firmicutes numbers decreased, while gram-

370   negative phyla such as Proteobacteria and Bacteroidetes increased in treated quails. Previous

371   studies have shown similar shifts in these three phyla, associated to applications of synthetic

372   pyrethroid insecticides in plant tissue (Zhang et al. 2009).

373   Interestingly, our results showed a significant effect of sex on the microbiota composition in the

374   caecum, which did not occur in the other two origins. Only in females, five genera belonging to

375   Proteobacteria and Bacteroidetes were increased significantly in abundances (i.e. *Salmonella*,

376   *Klebsiella*, *Erwinia*, *Serratia*, and *Bacteroides*). It is possible that these and other observed

377   increases in microbiota abundance resulted from their tolerance to the insecticide. Some bacteria

378   have the catabolic ability to dissolve insecticides and use them as a carbon source, an ability

379   termed as biodegradation (Akbar et al. 2014). For instance, the latter study showed that

380   Proteobacteria was the only dominant bacterial group selected by enrichment culturing using

381   chlorpyrifos (and organophosphate pesticide) as the carbon source. Other studies have found that

382   *Klebsiella* and *Serratia* are efficient organophosphate biodegraders (Cycoń et al. 2009; Pino &

383   Peñuela 2011; Sasikala et al. 2012). Furthermore, Joly et al. (2013) found significant increases in

384   *Bacteroidetes* in both the intestine of rats and in a human intestine simulator, after the continuous

385   application of low doses of an organophosphate insecticide. Burkholder et al. (2008) showed that

386   acute stressors resulted in shifts in GIT microbiota and increases in *Salmonella* abundances.

387   Thus, it is likely that some of the aforementioned taxa increased as they were not adversely

388   affected and hence had a relative advantage compared to other bacteria, as a consequence of their

389   biodegradation ability. Additionally, three genera from the Lachnospiraceae family decreased,

390   but *Blautia* and *Eubacterium* did so only in females. *Eubacterium* produce butyrate, which is

391   considered a gut health promoter through cell growth and colonic cell turnover (Gong et al.

392   2002). Another study reported increases in abundance of a number of caecal bacteria, including

393   *Blautia*, associated to an improvement of 10% in feed conversion ratio in broilers (Li et al.

394   2016). In general, the Lachnospiraceae family has been associated with improved growth

395   performance in chickens (i.e. lower food conversion ratio) (Stanley et al. 2012). Therefore, the

396   aforementioned decreases are potentially associated with negative effects on the health of quails.

397   Other studies have indicated that there are sex-dependent changes in environmental conditions

398   (e.g. pH and oxygen gradients, bile acid levels) throughout the GIT of female quails (Flint et al.

399   2012; Islam et al. 2011) and this may provide a clue as to why there are sex related differences in

400   the microbiota within the caecum. Our results indicate that in the caecum of treated females, a

401   number of bacteria, including all Enterobacteriaceae members, formed a microbial consortium

402   which negatively correlated with the abundance of Firmicutes. Although we cannot predict the

403   health risks that these microbiota shifts may invoke, a number of studies have shown the

404   detrimental impact that some of these bacterial groups have. Most of the genera that increased in

405   abundance belonged to Enterobacteriaceae. Based on findings from deceased birds, it has been

406   suggested that bacteria from this family predispose birds to diseases (Glünder 2002). Particularly

407   in the *Salmonella* genus, several reports in Europe and America have associated the death of

408   different wild bird species due to Salmonellosis (Craven et al. 2000). Other studies associated

409   very high abundances of Bacteroidetes in patients suffering irritable bowel syndrome (Ng et al.

410   2013). Moreover, increases in *Bacteroides* growth in the intestines of two different animals were

411   associated with decreases in probiotics such as lactobacilli and bifidobacteria (Joly et al. 2013).

412   Results from these studies suggest that the shifts we observed in our study, particularly in the

413   case of female quails, could predispose birds to the development of diseases. However, further

414   research is necessary to clarify the mechanisms and the consequences of these microbiota shifts.

415   ***Large intestine***

416   Significant Treatment × Origin interactions in microbiota composition were found in this study.

417   Significant changes in microbiota composition were found between the large intestine and faeces

418   in treated quails while no significant changes were found in the untreated quails. There were

419   large reductions of *Lactobacillus* (phylum Firmicutes) in the large intestine of treated birds. This

420   genus is generally present in normal mucosal microbiota in most animals (Kapczynski et al.

421   2000), and numerous studies have investigated their probiotic effects in avian systems. Early

422   research showed that high abundance of this genus can inhibit *Escherichia coli* growth in

423   chicken crop (Fuller 1977). More recently, Timmerman et al. (2006) showed that administering

424   probiotic *Lactobacillus* species to broilers promoted their growth and reduced mortality. In our

425   study, the most affected species of *Lactobacillus* was *L. agilis*. Lan et al. (2004) showed that this

426   species, along with other *Lactobacillus* strains, restored the balance and maintained the natural

427   stability of bacterial microbiota after heat stress-induced changes. In another study, probiotic

428   supplements composed of *L. agilis* and *L. salivarius* enhanced lactobacilli and bifidobacteria

429   abundance, and inhibited *Salmonella* growth in a simulated chicken caecum (Meimandipour et

430   al. 2010). However, there are number of studies questioning the beneficial effects of

431   *Lactobacillus* probiotics in birds (e.g. Goodling et al. 1987) and their effect on the bird health

432   cannot be taken as independent from the rest of the microbial community structure as it is often

433   done in the literature. In this study, differences observed in *Lactobacillus* abundance between

434   treated and untreated quails could point to the lack of adaptive responses of this biota to the new

435   chemical environment of the large intestine. Additionally, it is possible that the reductions

436   observed in *Lactobacillus* in treated quails could cause detrimental consequences for their health.

437   Similarly to the results found in caecum, the large intestine of treated quails had increases in the

438   abundance of Proteobacteria, which occurred as a result of significant increases in five genera

439   (i.e. *Alkanindiges*, *Lysobacter*, *Agrobacterium, Rhodospirillum*, and *Klebsiella*). Significant

440   increases in the latter genus were also found in the caecum. Furthermore, treated quails showed

441   increased abundances of Verrucomicrobia, a poorly characterized phylum (Hou et al. 2008), both

442   in the large intestine and in faeces. Increases in this phylum were attributed to a sole species,

443   *Akkermansia muciniphila*. This bacteria degrades intestinal mucin to obtain carbon and nitrogen

444   (Van Passel et al. 2011). In mice, it was suggested that intestinal bacteria producing excessive

445   mucin degradation could contribute to inflammatory bowel diseases, since of luminal antigens to

446   the intestinal immune system was facilitated (Ganesh et al. 2013). Increases in *A. muciniphila*

447   abundance have been negatively correlated with body weight in humans and rodents (Everard et

448   al. 2013). Moreover, studies have shown that overweighted people undergoing a fasting program

449   showed increased abundance of *Akkermansia* in their gut microbiota (Remely et al. 2015).

450   During our study, insecticide-treated quails were fasting (by reducing feed intake), most likely as

451   a consequence of trichlorfon ingestion. It is thus hypothesised that fasting in insecticide-treated

452   birds might have contributed to higher abundances of *A. muciniphila*, in comparison with

453   untreated birds which were feeding normally. Lastly, our results showed further insecticide

454   impact through an increase of unclassified bacteria to relative abundances above 40% in treated

455   quails. Although the identity of the latter group is unknown, it is possible that it could be

456   partially represented by phyla which showed positive responses to insecticide (e.g.

457   Proteobacteria and Bacteroidetes), as observed in other origins.

458 *Faeces*

459 Several microbiota taxa were significantly affected by trichlorfon in faeces, however, most of

460 these changes occurred at much lower abundance levels than in the other two origins. Compared

461 to the other origins, faecal microbiota showed consistent responses in taxa associated to the

462 phyla Verrucomicrobia and Proteobacteria as shown in this study. However, increases in taxa in

463 the Firmicutes phylum occurred concurrently with decreases in the same phylum in the other

464 origins, showing an inconsistency in the faecal microbiota responses. It is not unusual to observe

465 inconsistencies such as a lack of a unidirectional, dose-response correlation in microbial

466 toxicology tests (Petersen et al. 2004; Widenfalk et al. 2008). Furthermore, it is well accepted

467 that avian faecal microbiota oscillates based on the regular emptying of different GIT sections

468 (Sekelja et al. 2012). In a recent study, source-sink predictions of the seeding dynamics across

469 different GIT sections of Japanese quail showed that most of the faecal microbiota composition

470 had a multi-source predicted origin, with major origins contributing to it being caecum, and large

471 intestine (Wilkinson et al. 2016). Thus, it is possible that the results observed in the faecal

472 samples of treated quails originated from a combined reaction of different origins in the GIT to

473 the insecticide.

474 Conclusions

475 A single sub-lethal exposure to an organophosphate insecticide, at concentrations similar to

476 levels likely to be ingested following insecticide spraying in an orchard, caused significant

477 changes in avian gut microbiota within 24 hours. The effect size of this change, and the shifts in

478 microbial groupings, are consistent with microbiotas that previous studies have shown may

479 predispose birds to diseases. The changes differ between parts of the GIT, and interestingly also

480 differ between sexes, particularly in the caecum.

481 The study revealed that increases in abundance of certain microbial taxa occurred as a result of

482 direct or indirect positive interactions with trichlorfon administration, some possibly as a result

483 of their biodegradation ability. On the other hand, reductions observed in other groups likely

484 arose from their susceptibility to the insecticide, which may have produced antagonism and/or

485 competition with overgrowing biodegradative and/or insecticide-tolerant taxa.

486 This study raise several questions that should be addressed to understand the interactions
487 between GIT microbiota and avian hosts, the effects of pesticides on these interactions, and the
488 overall consequences for the bird's health. Further research is necessary to unravel the
489 mechanisms driving the interactions, and to understand how these are potentially associated with
490 damage, and/or the development of diseases in the GIT. Additionally, further study of short term
491 physiological responses and longer term health impacts are warranted based on the study results.

492 Considering that we carried out this study in a laboratory under defined and stable conditions,
493 our results can only partially mimic what is likely to occur under field conditions after
494 insecticides are sprayed. Nevertheless, our results suggest that changes in microbiota associated
495 to even small doses of insecticide could impose health risks on birds, particularly in females. The
496 implications of the significant response to a low dose of an organophosphate insecticide are that
497 acute and chronic health impacts are likely to occur in avian populations if similar GIT responses
498 occur under field conditions. These results are relevant for the conservation of avian
499 communities in agricultural areas where organophosphates are regularly sprayed.

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