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Role of wild birds and environmental contamination in the epidemiology of Salmonella infection in an outdoor pig farm

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32 ABSTRACT

33 Foodborne outbreaks caused by *Salmonella* are often attributed to the pork consumption. *Salmonella*
34 contamination of retail pork is directly linked to the *Salmonella* prevalence on farm. In UK,
35 approximately 40% of breeding pigs are kept outdoors. Aim of this study was to investigate the role
36 of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm. Three sampling visits were
37 carried out at monthly intervals to an outdoor farm consisting of two fields, one left empty of pigs for
38 more than 2 years (field A) while the second (field B) was occupied by pigs during the first visit only.
39 Faeces from wild bird droppings, environmental samples and pig faeces were tested for ~~the presence~~
40 ~~of~~ *Salmonella*. *Salmonella* spp. was isolated from environmental samples ~~even also~~ in field AB that
41 had not been occupied by pigs more than 2 years. Interestingly, the wild bird population accessing
42 the fields increased considerably once the pigs had left the farm and the proportion of *Salmonella*
43 positive wild bird droppings increased over time with 7.4%, 15.8% and 44.3% at the first, second and
44 third visit, respectively. The levels of *Salmonella* identified in some of the wild bird droppings were
45 unusually high (10^5 - 10^6 CFU/g) suggesting that *Salmonella* was actively replicating in the
46 gastrointestinal tract of these birds. Monophasic *Salmonella* Typhimurium DT193 was the
47 predominant serotype isolated in pigs as well as, in wild bird droppings and the environment,
48 ~~suggesting~~ supporting the hypotheses that the pigs were the original source of infection, as this serovar
49 is typically associated with pigs.

50

51 Highlights

- 52 • *Salmonella* was isolated from a field left empty by pigs for more than 2 years.
- 53 • Wild birds and pigs had the same *Salmonella* serotypes and phage types.
- 54 • The close relatedness of a selection of monophasic *S. Typhimurium* and *S. Rissen* isolated from
55 wild birds and pigs was confirmed using whole genome sequencing.
- 56 • Pigs are the likely to be the original source of *Salmonella* infection in wild birds. Pigs are the likely
57 source of *Salmonella* infection in wild birds.

58 • Wild birds are likely recycling and contributing to the persistence of *Salmonella*.

59 **Keywords:** *Salmonella*, Outdoor pig farm, Wild birds, Environment

60 **INTRODUCTION**

61

62 In the European Union, among the top-5 combinations related with the highest number of cases of
63 illness and hospitalisations in foodborne outbreaks in people, *Salmonella* is always included as
64 causative agent. Evidence foodborne outbreaks caused by *Salmonella* are were often attributable to
65 the consumption of contaminated eggs, pig meat, products thereof and other foods In the European
66 Union, among the top 5 combinations related with the highest number of cases of illness and
67 hospitalisations in outbreaks in people, *Salmonella* is always included as causative agent, in
68 combination with eggs, pig meat, products thereof and other foods (EFSA, 2016).

69 *Salmonella* infection can be introduced into a pig herd by many routes, for example through the
70 purchase of *Salmonella*-infected pigs, contaminated feed or other animals. Movements of pigs
71 between premises at different life stages represent a risk because during transport pigs are subjected
72 to stress. Stress makes pigs more susceptible to infection and increases the shedding rate of infected
73 pigs (Verbrugge et al., 2011). Furthermore, especially for outdoor and organic farms, wild fauna,
74 synanthropic and domestic animals living on the farm can constitute a source of introduction and
75 transmission of *Salmonella* through direct contact with pigs or indirectly through faecal
76 contamination of feed, water troughs or farm equipment (Zheng et al., 2007).

77 The herd prevalence of *Salmonella* infection in pig production holdings in the United Kingdom (UK)
78 was reported to be 44.4% in 2008 by EFSA (EFSA, 2009).

79 Andres and Davies (2015) suggested that there is a correlation between *Salmonella* prevalence on
80 farm and contamination of retail pork. Biosecurity measures applied at the farm ~~therefore~~ play an
81 important role in the reduction of contamination at retail, even if some of the risk of contamination
82 can be reduced at slaughter (Martelli et al., 2017). Biosecurity measures are also important to prevent
83 further spread within the pig industry, to other food animal sectors and potential zoonotic infections
84 due to contact with infected pigs and manure (Andres and Davies, 2015).

85 Several studies have been conducted to understand the role of wild birds in the cycle of *Salmonella*
86 infection in pigs (Andres-Barranco et al., 2014; Andres and Davies, 2015; Tizard, 2004; Zheng et al.,
87 2007). Various phage types of *S. Typhimurium* have been associated with wild birds in the UK. *S.*
88 *Typhimurium* definitive phage types (DT) 56, 40, 41, 195 were isolated from finches, waterfowl,
89 house sparrows, rooks, greenfinches, gulls. *S. Typhimurium* DT2 and DT99 are associated with
90 pigeons, and DT8 and DT30 with game birds (Pennycott et al., 2006). According to the data published
91 by APHA on the isolation of *Salmonella* from pig livestock in Great Britain between the 2011 and
92 2015, only 0.3% of *Salmonella* isolates from pigs were *Salmonella* serotypes and phage types
93 commonly associated with wild birds strains of *Salmonella* suggesting birds do not present a major
94 risk of infection for pigs (APHA, 2017). Andres et al., (2013), reported that wild birds could be a
95 reservoir of farm-resident strains and that birds can recycle the infection, but are less likely to be the
96 source of introduction. The presence of wild birds, rats and mice is of particular importance in outdoor
97 pig units where they can represent a risk factor for *Salmonella* seropositivity and where measures of
98 control are more challenging (Andres and Davies, 2015). In the UK, around 40% of the pig breeding
99 stock is kept outdoors, whilst most grower and finisher pigs are reared in indoor units (Houston,
100 2013), ~~while in the~~ In other European countries the number of pigs bred in organic or outdoor farms
101 has increased in recent years (European Commission, 2016).

102 The aims of the study were to investigate the role of wild birds in the epidemiology of *Salmonella* in
103 one outdoor pig farm and assess *Salmonella* prevalence in the environment when the farm was stocked
104 with pigs and after depopulation.

105

106 **MATERIALS AND METHODS**

107

108 **Sampling**

109 Between the 8th of September and the 15th November 2015, three sampling visits were carried out in
110 one outdoor pig farm at times determined by the depopulation schedule, during one
111 productionstoecking cycle.

112 The farm sampled inef this study was a fattening farm housing pigs from weaning to finishing. The
113 first visit was carried out when the pigs were still present, the second visit one month later and one
114 week after depopulation, and the third visit one month later. The farm consisted of two adjacent fields:
115 field A has been left empty of pigs for more than 2 years while the field B was occupied by weaners
116 and growers pigs during the first visit only.

117 All pigs were housed in pens adjacent to each other and located in a portion of the field B. The soil
118 of the fields was sandy and partially covered by weeds and wild shrubbery. The sizes of the fields
119 were 10.4 ha and 8.2 ha for field A and B respectively. Adjacent to the farm, there was a water course,
120 populated by a large number of aquatic wild birds.

121 In bothall two fields, swab samples of bird droppings, and environmental samples (soil, water puddles
122 and farm equipment) were collected from the areas unoccupied by pigs and all faecal samples
123 appeared to be fresh at the time of collection. The swab samples were either collected in sterile plastic
124 pots or directly placed into 225 ml of Buffered Peptone Water (BPW) using a hand held gauze.

125 The sample size was calculated to estimate *Salmonella* prevalence considering an expected
126 prevalence of 50%, an acceptable error of 56% and 95% confidence level.

127 During the first farm visit, intensive sampling (211 of 242 samples collected) was performed in field
128 B, which was occupied by pigs. In addition to what described above, pooled faeces samples were
129 taken from the weaners' and growers' pens at this visit.

130 A description of the samples taken at each visit is available in Tables 1 and Table 2.

131

132 **Bacteriological analyses**

133 Individual samples and pooled samples were suspended in Buffered Peptone Water (BPW)
134 enrichment media (1:10 w:v). All samples in BPW were incubated for 18 ± 2 hours at $37 \pm 1^\circ\text{C}$ and

135 after incubation, 100 µl of each sample was pipetted onto a semi-solid isolation medium; Modified
136 Semi-solid Rappaport Vassiliadis (MSRV) agar containing 0.01% novobiocin (MSRV; Difco
137 218681) and incubated at 41.5°C for 24 ± 3 hours. After incubation, Rambach agar was inoculated
138 from the MSRV by using a 1 µl loop from the edge of opaque growth on the MSRV (consistent with
139 *Salmonella* growth on MSRV). The Rambach agar plates were incubated at 37°C for 24h ± 3h. The
140 MSRV plates in which bacterial growth consistent with *Salmonella* was observed and ~~were~~ negative
141 for *Salmonella* on the Rambach agar plates were sub-cultured again onto Rambach agar after 48h
142 incubation. Suspect *Salmonella* colonies were identified by complete serotyping according to the
143 Kaufmann–White-Le Minor Scheme (Grimont and Weill, 2007). A random selection of the *S.*
144 Typhimurium and monophasic *S.* Typhimurium (mST) strains were also phage typed (Anderson et
145 al., 1977).

146 Quantitative analysis was performed on a random selection of positive individual faecal samples from
147 each age class (weaners and growers) and on a random selection of environmental samples collected
148 without enrichment media. Decimal dilutions and subsequent cultures of each dilution, as above, were
149 carried out to semi-quantitatively estimate the level of *Salmonella* per gram of sample (Wales et al.,
150 2006).

151

152 Sequencing and sequence data analyses

153 To further study the relatedness among wild bird and pig *Salmonella* isolates, a total of 6 isolates
154 were whole genome sequenced (WGS). These included 2 *S.* Rissen isolates, one isolate S05753-15
155 from a wild bird and one from pig (isolate S06138-15), and 4 mST isolates, 3 from wild birds
156 (isolates S05620-15, S05798-15 and S06144-15) and one from pigs (isolate S05634-15).
157 The assembled draft genome of the *S.* Rissen wild bird isolate S05753-15 was used as reference to
158 map the pig *S.* Rissen genome S06138-15 and extract the SNPs within the whole genome sequence.
159 The assembled draft genome S05620-15 was used as reference for the mST isolates. Genomic DNA
160 and was extracted from 6 *Salmonella* isolates using a commercial kit MagMAX™ CORE Nucleic

161 [Acid Purification Kit](#) together with a [KingFisher Duo Prime](#) magnetic particle processor (both from
162 [ThermoFisher Scientific](#), [Waltham, USA](#)) following manufacturer's instructions. The extracted
163 genomic DNA was fragmented, tagged for multiplexing with the [Nextera XT DNA Sample](#)
164 [Preparation Kit](#) ([Illumina, Inc. San Diego, USA](#)) and sequenced at the APHA on the [Illumina NextSeq](#)
165 [platform](#) ([Illumina, San Diego, USA](#)) to generate 150 base pair paired-end reads with minimum
166 coverage of 50 x. The quality of the short reads was evaluated with [FastQC](#)
167 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The short reads were quality trimmed
168 with [Trimmomatic](#) ([Bolger et al., 2014](#)) and mapped to the 2 de novo assembled draft genomes
169 [S05753-15](#) and [S05620-15](#) using [Snippy](#) ([Kwong et al., 2015](#)). The genomes were de novo assembled
170 using [SPAdes](#) ([Bankevich et al., 2012](#)) and assemblies corrected using [Shovill](#)
171 (<https://github.com/tseemann/shovill>). The ~~alignments~~alignments were further parsed to extract only
172 single nucleotide polymorphism (SNP) with the minimum number of 10 reads ~~covering the variant~~
173 ~~position and the minimum proportion of those reads which must differ from the reference being 0.9~~.
174 For phylogenetic analyses, a maximum-likelihood phylogenetic tree was constructed from the SNP
175 alignments after [Gubbins](#) was run to remove regions of recombination in the pseudofasta files from
176 [SNP calling](#) ([Croucher et al., 2014](#)). The recombination regions were removed using [SNP-sites](#) ([Page](#)
177 [et al., 2016](#)). The phylogenetic analysis was performed on the generated SNP alignment file to infer
178 core SNP phylogeny using the maximum likelihood method at 100 bootstraps by [RAxML](#) and
179 visualised using the [tree of life \(iTol\)](#) ([Letunic and Bork, 2016](#)). The SNP distance tables were
180 obtained using ~~a program called snp-dist~~ [\[snp-dist-\]\(https://github.com/tseemann/snp-dists\)](#). The raw
181 fastq files of the six isolates were also passed through the *Salmonella* pipeline which consists of
182 several programs including three serotyping programs, [MOST](#), [SeqSero](#) and [SISTR](#) to identify the
183 *Salmonella* serotypes based on WGS ([Tewolde et al., 2016](#); [Yoshida et al., 2016](#); [Zhang et al., 2015](#)).

184

185 **Statistical analyses**

186 *Salmonella* prevalence in environmental and wild bird droppings samples collected during the 3 farm
187 visits was investigated. Results from field A (empty at all visits) were compared to field B (occupied
188 by pigs at visit 1).

189 The change in *Salmonella* prevalence during the three visits was also studied in environmental and
190 wild bird droppings samples in each of the two fields ~~using Chi-squared test for trend~~. Chi-Square
191 test was used to compare all the data and the significance limit was set at $P < 0.05$. Confidence
192 intervals were calculated by binomial (Clopper-Pearson) “exact” method based on the β distribution.
193 Finally, Odds ratio (OR) was calculated for the risk of *Salmonella* contamination. All statistical
194 analyses were performed using the software SPSS 23.0 (IBM SPSS Statistics, NY, US).

195

196 RESULTS

197 During the three farm visits a total 661 samples were collected from the two areas investigated, field
198 A (empty at all visits) and field B (occupied by pigs at the first visit). Of these, ~~324~~182 were
199 environmental samples (254 ~~individual swabs collected without enrichment media~~ and 70 swabs
200 ~~collected in enrichment media~~), 182 were bird dropping (167 ~~swabs collected without enrichment~~
201 ~~media individual~~ and 15 ~~swabs collected in enrichment media swabs~~), 155 were swine feces- (120
202 ~~swabs collected without enrichment media individual faecal samples individual~~ and 35 ~~swabs pooled~~
203 ~~faeces collected in enrichment media~~).

204 The ~~individual non-enriched~~ environmental and wild bird dropping samples collected from field B
205 were more likely to be *Salmonella* positive ($P=0.001$) than those collected from field A (Table 5).
206 Overall, the odds of a sample being *Salmonella*-positive was 20.2 times higher in field B compared
207 to field A ($P=0.001$). For environmental samples and wild bird dropping, the OR values were 27.8
208 (95% CI: 12.5-62.2) and 12.6 (95% CI: 5.4-29.6), respectively ($P=0.001$) (Table 5).

209 At the first visit, 120 individual pig faeces samples were collected from field B. *Salmonella* was
210 isolated from 70.0% (42) and 91.7% (55) respectively of 60 samples from weaners’ pens and 60

211 samples from growers' pens (Table 3). *Salmonella* was also isolated from 58.3% (35 of 60) of
212 environmental samples and from 7.4% (2 of 27) of individual-wild bird faeces samples.

213 At the second visit, the 26.9% (36 of 134) of environmental samples and 15.8% (12 of 76) of wild
214 bird droppings were found to be *Salmonella* positive.

215 During the final third visit, *Salmonella* was isolated from 27.7% (36 of 130) of environmental samples
216 and from 44.3% (35 of 79) of wild bird samples.

217 ~~Overall, a significant difference ($P=0.001$) ($P < 0.05$) was observed in *Salmonella* prevalence over~~
218 ~~time in the field B.~~ Wild bird dropping samples collected in field B at the third visit were significantly
219 more contaminated with *Salmonella* than the samples collected in empty fields (field A) ($P=0.001$) (P
220 < 0.05).

221 Although *Salmonella* prevalence in environmental samples and wild bird samples collected from field
222 A did not vary significantly during the three visits (respectively: $P=0.82$ and $P=0.84$), significant
223 differences were observed in samples collected from field B over time. In particular, the proportion
224 of *Salmonella*-positive wild bird faeces increased significantly over time ($P=0.001$) ($P < 0.015$) while
225 no difference was observed for the environmental samples ($P=0.07$).

226

227 Enriched Swab samples

228 At the first visit *Salmonella* was isolated from 83.3% (15 of 18) and 100% (17 of 17) respectively of
229 the weaners' and growers' pens. Furthermore, *Salmonella* was isolated from all the 5 environmental
230 samples collected during the first farm visit and from the 23.1% (3 of 13) and 41.7% (5 of 12) of
231 environmental samples collected respectively during the second and third visit (Table 4).

232 From wild bird droppings, *Salmonella* was detected in 71.4% (5 of 7) and 100% (8 of 8) swabs
233 collected respectively during the second and third visit.

234

235 Semi-quantitative culture results

236 High levels of *Salmonella* were found in individual pig faecal samples (Table 3). In the growers'
237 faeces, 7 samples had a *Salmonella* concentration of 10^2 - 10^3 CFU/g, 3 samples of 10^3 - 10^4 CFU/g and
238 2 samples of 10^5 - 10^6 CFU/g. In the weaners' faeces the maximum *Salmonella* load was 10^3 - 10^4
239 CFU/g (3 samples) followed by 8 samples with 10^2 - 10^3 CFU/g while a lower *Salmonella* level was
240 found in the remaining 8 samples (10 - 10^2 CFU/g and 1 - 10 CFU/g).

241 In the environmental samples (Table 2) the levels of *Salmonella* were found to be low (1 - 10 CFU/g
242 or 10 - 10^2 CFU/g) for the majority of the samples. Only the environmental samples collected from the
243 field in which pigs had been housed had higher CFU/g (two samples had 10^2 - 10^3 CFU/g). Unusually
244 high *Salmonella* levels were found in some of the wild bird droppings: 10^5 - 10^6 CFU/g in geese
245 droppings collected during the second and third visit both of the two fields sampled (Table 2).

246

247 **Serovars and phage types**

248 In total 151 *Salmonella* strains were serotyped, most *Salmonella* isolates were *S. enterica* serovar
249 4,5,12:i:- (mST) (121, 80.1%), followed by *S. Rissen* (22, 14.6%), *S. Senftenberg* (3, 2%), *S.*
250 *Typhimurium* (2, 1.3%), *S. Panama* (2, 1.3%) and *S. Derby* (1, 0.7%) (Tables 1, 3 and 4).

251 Among individual faeces and swab samples mST was detected from 85.1% (40 of 47) of weaners'
252 and growers' samples, from 78.4% (29 of 37) of wild bird droppings samples (the majority of them
253 were from geese droppings) and 77.6% (52 of 67) of environmental samples (Tables 3 and 4).

254 *S. Typhimurium* was isolated from only two wild bird droppings samples collected during the first
255 and second visits.

256 Thirty four *S. Typhimurium* and mST isolate were phage typed. Different sample types, collected
257 during all the visit, such as bird droppings (12), environmental (18) and pig fecal samples (4) were
258 selected. Only two phage types were identified among the 34 *S. Typhimurium* and mST strains tested.

259 All were DT193 except for one isolate of *S. Typhimurium*, isolated from wild bird droppings, which
260 was phage type DT41.

261

262 Phylogenetic clustering of wild bird and pig *Salmonella* isolates

263 All 6 isolates sequenced in this study were highly related with only 1 SNP difference between the 2
264 *S. Rissen* isolates and a maximum observed difference of 9 SNPs between the 4 mST isolates. The
265 SNP difference of the monophasic *S. Typhimurium* isolates from wild birds was between 4 and 6.
266 Maximum likelihood core genome SNP phylogeny of *S. Rissen* and mST isolates and SNP
267 differences are presented in Figure 1 and Figure 2, respectively.

268

269 **DISCUSSION**

270

271 On farms rodents, birds, insects, are common inhabitants that can all be carrier vectors and can
272 mechanically transmit pathogens (Backhans et al., 2013). Bait traps or chemical pesticides can aid in
273 the management of rodent problems as well the removal of waste and feed spills can be helpful to
274 limit the attraction of birds and rodents (Andres and Davies, 2015).

275 The role of wild birds is a controversial matter in relation to potential hazards to livestock and to for
276 human health. Several studies support the hypothesis that wild birds play an important role in
277 *Salmonella* epidemiology in both humans and animals (Andres et al., 2013; Phalen et al., 2010; Vico
278 and Mainar Jaime, 2011). In contrast, other studies suggest that they do not represent a major public
279 health hazard, considering the low numbers of organisms shed and the short duration of *Salmonella*
280 carriage shedding (Hughes et al., 2008; Jensen et al., 2004; Marin et al., 2014). It is well recognized
281 that *Salmonella* is an ubiquitous agent that can colonize asymptotically the gut of birds and
282 consequently can be shed in their faeces (Andres et al., 2013). *Salmonella* prevalence studies are
283 usually associated with wild birds and focussed on host-adapted strains in some bird species, but
284 some studies report that birds near pig farms have higher probability of shedding *Salmonella* than
285 birds living far from pig premises (Andres et al., 2013).

286 This study was carried out on one outdoor pig farm occupied by *Salmonella* infected pigs at the first
287 visit only. Individual samples of pig faeces, environmental samples and wild bird droppings were
288 collected and a quantitative analysis of *Salmonella* was performed on positive samples.
289 Pools of faecal samples were also collected as they are regarded as more effective for isolating
290 *Salmonella* ~~salmonellae~~, than the sampling of a large number of individual samples (Cook et al.,
291 2005). A significantly higher prevalence ($P < 0.001$), as well ~~the~~ as the higher odds of *Salmonella*-
292 positive samples detected in samples collected from the field occupied by pigs, suggest that pigs are
293 the likely source of *Salmonella* in the pig farm environment. At the first visit >50% environmental
294 samples were found to be *Salmonella*-positive, and one and two months after the pigs had left the
295 farm, 27% of environmental samples were still *Salmonella*- positive. *Salmonella* was also found in
296 environmental samples in a field that had been empty for 2 years. It is likely that *Salmonella* can
297 survive outside the host for a significant length of time as reported by several authors (Funk and
298 Gebreyes, 2004; Jensen et al., 2004; Sandvang et al., 2000), and therefore the environment itself can
299 become a potential source of infection for subsequent batches of pigs and wildlife. However, it is also
300 possible that wild birds contributed to re-contaminate the soil, considering that *Salmonella* was
301 isolated from wild bird droppings (7.4% ~~(2 of 27)~~) of samples collected during the first farm visit, 15.8
302 % ~~(12 of 76)~~ and 44.3% ~~(35 of 79)~~ collected during the second and third farm visit, respectively). It
303 is apparent that once the pigs had left the farm, the proportion of *Salmonella*-positive wild bird faeces
304 increased significantly ($P < 0.01$). This could be linked to the fact that the wild bird population
305 accessing the fields increased considerably once the pigs left the farm (as observed by the sampling
306 team). The increase may be due to the presence of leftover pig feed and worm populations being
307 nearer the surface of the soil (Andres et al., 2013; Andres and Davies, 2015). Furthermore, the
308 increase in wild birds density over time, may have caused an increase in the transmission rate of this
309 infection among birds (Andres et al., 2013). It was not possible to collect samples from a field that
310 had never been occupied by pigs on this farm, as all fields had been occupied by pigs on a rotational

311 basis in the last decade. It was therefore not possible to assess the levels of contamination exclusively
312 related to wild bird droppings.

313 Livestock farms can act as areas where wild birds congregate for the availability of food and shelter
314 (Andres et al., 2013). At the same time farm environment with high levels of *Salmonella*
315 contamination, as well other pathogens may be an important potential source of infection and
316 potential biodiversity threat for those avian species of wild birds susceptible to the infection (Andres-
317 Barranco et al., 2014; Andres et al., 2013). It has been suggested that *Salmonellosis* has been
318 suggested as can can be be one of the causes of the decline of the house sparrow population (Pennycott
319 et al., 2006). *Salmonella enterica* serovar Typhimurium for passerine can result in a sever disease
320 with significant mortality (Tizard, 2004). ~~Sn this context, several~~ authors reported that the feeding
321 and migration behaviour as well as the seasonality may influence the prevalence of salmonellosis in
322 free-ranging birds (Andres-Barranco et al., 2014; Andres et al., 2013).

323 *Salmonella* prevalence was significantly higher in wild bird droppings collected from the field
324 occupied by pigs at the first visit (field B).

325 The majority of wild birds present in the farm were gulls or geese, both those bird species feeding on
326 the ground resulting in higher chances of getting infected than birds feeding from hanging feeders.
327 Moreover the significant positive relationship between the of detecting *Salmonella* in wild bird
328 droppings collected in field B. This that pigs represent an important risk factor as source of
329 *Salmonella* for wild birds.

330 Moreover in field B the odd of *Salmonella* positive samples was 12.6 times higher compared with
331 those samples collected from field A. These results together suggested that pigs represent an
332 important risk factor as source of *Salmonella* for wild birds.

333 In the proximity of the farm sampled in this study, there was also a river, which attracted a large
334 number of wild birds especially aquatic birds, such as Canada geese and seagulls.

335 Geese droppings had higher (10^5 - 10^6) *Salmonella* CFU/g, whilst compared with *Salmonella* levels
336 were lower in environmental samples (10 - 10^2 and 10^2 - 10^3 CFU/g). A study by Pennycott et al., (2006)

337 concluded that *Salmonella* strains in Great Britain originating from wild birds do not represent a
338 major primary source of infection, considering the low percentage of wild bird associated phage types
339 isolated from livestock. However, the high levels of *Salmonella* in geese faeces suggest that geese
340 can represent an important source of infection, able to maintain *Salmonella* in areas with-where geese
341 bird populations are present.

342 The *Salmonella* serotypes found in pigs, mST and *S. Rissen*, were the same as those found in wild
343 bird droppings. We confirmed the close relatedness of the mST and *S. Rissen* isolated from wild birds
344 and pigs using whole genome sequencing as ~~the~~ highly discriminative method for studying
345 population heterogeneity in bacteria. We found a single SNP difference between the *S. Rissen* isolates
346 and maximum of 9 SNPs among the mST isolates. In recent year WGS has been used successfully in
347 investigating a number of *Salmonella* related outbreak and trace back investigations and is becoming
348 a method of choice in linking different sources of infection (Ashton et al., 2015; Inns et al., 2017;
349 Inns et al., 2015) (Ashton et al., 2015, Inns et al., 2015, Inns et al., 2017). Within the *S. Typhimurium*
350 serovar a cluster of isolates that are grouped together in time and space and sharing 0-10 SNPs are
351 considered as common source of infection (Ford et al., 2018). *S. Senftenberg*, and *S. Typhimurium*
352 were found in wild bird droppings, and these are also serotypes typically commonly found in housed
353 breeding pigs in the UK (Hughes et al., 2008). *S. Typhimurium* is reported to be the most common
354 serotype identified in wild bird droppings (Andres et al., 2013; Horton et al., 2013; Hughes et al.,
355 2008; Lawson et al., 2011; Palmgren et al., 2006; Vico and Mainar Jaime, 2011). In contrast, the
356 majority of *Salmonella* isolates from wild bird droppings during this study were mST DT193. One of
357 the isolated *S. Typhimurium* serovars was phage type DT 41. *S. Typhimurium* DT 41 has been
358 reported previously in wild birds from the UK and is particularly associated with waterfowl (Barua
359 et al., 2013; Hughes et al., 2008; Pennycott et al., 2006).

360 Pigs have been recognized as the main reservoir of mST DT193 (Crayford et al., 2014), supporting
361 the hypothesis that pigs can act as a source of wild bird salmonellosis.

362 Interestingly, the 25 of 37 mST-positive samples, were from geese droppings and three of them
363 collected during the second and third visit presented an unusually high level of *Salmonella*-shedding
364 (~~10⁵-10⁶ CFU/g~~). Therefore, it is reasonable to postulate that mST infection in wild geese does not
365 cause clinical symptoms in birds. However, further studies are required to better understand the role
366 of geese and their role in the cycle of *Salmonella* infection in outdoor pig farms.

367 This study suggests a possible cyclical dissemination of *Salmonella* between pigs and wild birds, and
368 that wild birds are capable of contributing to the persistence of *Salmonella* between batches of pigs.
369 Adequate management practices to minimize the contact between pigs and wild birds (e.g. cover feed
370 and water sources, use of nets) should be implemented in outdoor pig units.

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375 **Conflict of Interests**

376 The authors declare that there is no conflict of interests regarding the publication of this paper.

377

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381 **References**

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Highlights

- *Salmonella* was isolated from a field left empty by pigs for more than 2 years.
- Wild birds and pigs had the same *Salmonella* serotypes and phage types.
- The close relatedness of a selection of monophasic *S. Typhimurium* and *S. Rissen* isolated from wild birds and pigs was confirmed using whole genome sequencing.
- Pigs are the likely to be the original source of *Salmonella* infection in wild birds.
- Wild birds are likely recycling and contributing to the persistence of *Salmonella*.

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3 **1 Role of wild birds and environmental contamination in the epidemiology of *Salmonella***
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5 **2**
6 **infection in an outdoor pig farm**
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8 **3**

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62 32 **ABSTRACT**
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64 33 Foodborne outbreaks caused by *Salmonella* are often attributed to the pork consumption. *Salmonella*
65 34 contamination of retail pork is directly linked to the *Salmonella* prevalence on farm. In UK,
66 35 approximately 40% of breeding pigs are kept outdoors. Aim of this study was to investigate the role
67 36 of wild birds in the epidemiology of *Salmonella* in one outdoor pig farm. Three sampling visits were
68 37 carried out at monthly intervals to an outdoor farm consisting of two fields, one left empty of pigs for
69 38 more than 2 years (field A) while the second (field B) was occupied by pigs during the first visit only.
70 39 Faeces from wild bird droppings, environmental samples and pig faeces were tested for *Salmonella*.
71 40 *Salmonella* spp. was isolated from environmental samples also in field A that had not been occupied
72 41 by pigs more than 2 years. Interestingly, the wild bird population accessing the fields increased
73 42 considerably once the pigs had left the farm and the proportion of *Salmonella* positive wild bird
74 43 droppings increased over time with 7.4%, 15.8% and 44.3% at the first, second and third visit,
75 44 respectively. The levels of *Salmonella* identified in some of the wild bird droppings were unusually
76 45 high (10^5 - 10^6 CFU/g) suggesting that *Salmonella* was actively replicating in the gastrointestinal tract
77 46 of these birds. Monophasic *Salmonella* Typhimurium DT193 was the predominant serotype isolated
78 47 in pigs as well as in wild bird droppings and the environment, suggesting that the pigs were the
79 48 original source of infection, as this serovar is typically associated with pigs.
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100 50 **Highlights**
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- 102 51 • *Salmonella* was isolated from a field left empty by pigs for more than 2 years.
103 52 • Wild birds and pigs had the same *Salmonella* serotypes and phage types.
104 53 • The close relatedness of a selection of monophasic *S. Typhimurium* and *S. Rissen* isolated from
105 54 wild birds and pigs was confirmed using whole genome sequencing.
106 55 • Pigs are the likely to be the original source of *Salmonella* infection in wild birds..
107 56 • Wild birds are likely recycling and contributing to the persistence of *Salmonella*.

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113 57 **Keywords:** *Salmonella*, Outdoor pig farm, Wild birds, Environment
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121 58 **INTRODUCTION**
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125 60 In the European Union, among the top-5 combinations related with the highest number of cases of
126 61 illness and hospitalisations in foodborne outbreaks in people, *Salmonella* is always included as
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128 62 causative agent. Foodborne outbreaks caused by *Salmonella* are often attributable to the consumption
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130 63 of contaminated eggs, pig meat, products thereof and other foods (EFSA, 2016).
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134 65 *Salmonella* infection can be introduced into a pig herd by many routes, for example through the
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136 66 purchase of *Salmonella*-infected pigs, contaminated feed or other animals. Movements of pigs
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138 67 between premises at different life stages represent a risk because during transport pigs are subjected
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140 68 to stress. Stress makes pigs more susceptible to infection and increases the shedding rate of infected
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142 69 pigs (Verbrugghe et al., 2011). Furthermore, especially for outdoor and organic farms, wild fauna,
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144 70 synanthropic and domestic animals living on the farm can constitute a source of introduction and
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146 71 transmission of *Salmonella* through direct contact with pigs or indirectly through faecal
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148 72 contamination of feed, water troughs or farm equipment (Zheng et al., 2007).
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151 73 The herd prevalence of *Salmonella* infection in pig production holdings in the United Kingdom (UK)
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153 74 was reported to be 44.4% in 2008 by EFSA (EFSA, 2009).
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155 75 Andres and Davies (2015) suggested that there is a correlation between *Salmonella* prevalence on
156
157 76 farm and contamination of retail pork. Biosecurity measures applied at the farm play an important
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159 77 role in the reduction of contamination at retail, even if some of the risk of contamination can be
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161 78 reduced at slaughter (Martelli et al., 2017). Biosecurity measures are also important to prevent further
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163 79 spread within the pig industry, to other food animal sectors and potential zoonotic infections due to
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165 80 contact with infected pigs and manure (Andres and Davies, 2015).
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168 81 Several studies have been conducted to understand the role of wild birds in the cycle of *Salmonella*
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170 82 infection in pigs (Andres-Barranco et al., 2014; Andres and Davies, 2015; Tizard, 2004; Zheng et al.,
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172 83 2007). Various phage types of *S. Typhimurium* have been associated with wild birds in the UK. *S.*
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174 84 *Typhimurium* definitive phage types (DT) 56, 40, 41, 195 were isolated from finches, waterfowl,
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180 84 house sparrows, rooks, greenfinches, gulls. *S. Typhimurium* DT2 and DT99 are associated with
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182 85 pigeons, and DT8 and DT30 with game birds (Pennycott et al., 2006). According to the data published
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184 86 by APHA on the isolation of *Salmonella* from pig livestock in Great Britain between the 2011 and
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186 87 2015, only 0.3% of *Salmonella* isolates from pigs were *Salmonella* serotypes and phage types
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188 88 commonly associated with wild birds suggesting birds do not present a major risk of infection for
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190 89 pigs (APHA, 2017). Andres et al., (2013), reported that wild birds could be a reservoir of farm-
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192 90 resident strains and that birds can recycle the infection, but are less likely to be the source of
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194 91 introduction. The presence of wild birds, rats and mice is of particular importance in outdoor pig units
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196 92 where they can represent a risk factor for *Salmonella* seropositivity and where measures of control
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198 93 are more challenging (Andres and Davies, 2015). In the UK, around 40% of the pig breeding stock is
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200 94 kept outdoors, whilst most grower and finisher pigs are reared in indoor units (Houston, 2013). In
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202 95 other European countries the number of pigs bred in organic or outdoor farms has increased in recent
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204 96 years (European Commission, 2016).
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206 97 The aims of the study were to investigate the role of wild birds in the epidemiology of *Salmonella* in
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208 98 one outdoor pig farm and assess *Salmonella* prevalence in the environment when the farm was stocked
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210 99 with pigs and after depopulation.
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216 101 **MATERIALS AND METHODS**

217 102 218 219 103 **Sampling**

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221 104 Between the 8th of September and the 15th November 2015, three sampling visits were carried out in
222
223 105 one outdoor pig farm at times determined by the depopulation schedule, during one production cycle.
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225 106 The farm sampled in this study was a fattening farm housing pigs from weaning to finishing. The first
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227 107 visit was carried out when the pigs were still present, the second visit one month later and one week
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229 108 after depopulation, and the third visit one month later. The farm consisted of two adjacent fields: field
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239 109 A has been left empty of pigs for more than 2 years while the field B was occupied by weaners and
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241 110 growers pigs during the first visit only.
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243 111 All pigs were housed in pens adjacent to each other and located in a portion of the field B. The soil
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245 112 of the fields was sandy and partially covered by weeds and wild shrubbery. The sizes of the fields
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247 113 were 10.4 ha and 8.2 ha for field A and B respectively. Adjacent to the farm, there was a water course,
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249 114 populated by a large number of aquatic wild birds.
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251 115 In both fields, swab samples of bird droppings, and environmental samples (soil, water puddles and
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253 116 farm equipment) were collected from the areas unoccupied by pigs and all faecal samples appeared
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255 117 to be fresh at the time of collection. The swab samples were either collected in sterile plastic pots or
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257 118 directly placed into 225 ml of Buffered Peptone Water (BPW) using a hand held gauze.
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259 119 The sample size was calculated to estimate *Salmonella* prevalence considering an expected
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261 120 prevalence of 50%, an acceptable error of 5% and 95% confidence level.
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263 121 During the first farm visit, intensive sampling (211 of 242 samples collected) was performed in field
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265 122 B, which was occupied by pigs. In addition to what described above, pooled faeces samples were
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267 123 taken from the weaners' and growers' pens at this visit.
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269 124 A description of the samples taken at each visit is available in Tables 1 and Table 2.
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275 126 **Bacteriological analyses**

277 127 Individual samples and pooled samples were suspended in Buffered Peptone Water (BPW)
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279 128 enrichment media (1:10 w:v). All samples in BPW were incubated for 18 ± 2 hours at $37 \pm 1^\circ\text{C}$ and
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281 129 after incubation, 100 μl of each sample was pipetted onto a semi-solid isolation medium; Modified
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283 130 Semi-solid Rappaport Vassiliadis (MSRV) agar containing 0.01% novobiocin (MSRV; Difco
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285 131 218681) and incubated at 41.5°C for 24 ± 3 hours. After incubation, Rambach agar was inoculated
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287 132 from the MSRV by using a 1 μl loop from the edge of opaque growth on the MSRV (consistent with
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289 133 *Salmonella* growth on MSRV). The Rambach agar plates were incubated at 37°C for $24\text{h} \pm 3\text{h}$. The
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291 134 MSRV plates in which bacterial growth consistent with *Salmonella* was observed and negative for
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298 135 *Salmonella* on the Rambach agar plates were sub-cultured again onto Rambach agar after 48h
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300 136 incubation. Suspect *Salmonella* colonies were identified by complete serotyping according to the
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302 Kaufmann–White-Le Minor Scheme (Grimont and Weill, 2007). A random selection of the *S.*
303 137
304 Typhimurium and monophasic *S.* Typhimurium (mST) strains were also phage typed (Anderson et
305 138
306 al., 1977).
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309 140 Quantitative analysis was performed on a random selection of positive individual faecal samples from
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311 141 each age class (weaners and growers) and on a random selection of environmental samples collected
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313 142 without enrichment media. Decimal dilutions and subsequent cultures of each dilution, as above, were
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315 143 carried out to semi-quantitatively estimate the level of *Salmonella* per gram of sample (Wales et al.,
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317 144 2006).
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321 322 146 **Sequencing and sequence data analyses**

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324 147 To further study the relatedness among wild bird and pig *Salmonella* isolates, a total of 6 isolates
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326 148 were whole genome sequenced (WGS). These included 2 *S.* Rissen isolates, one isolate S05753-15
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328 149 from a wild bird and one from pig (isolate S06138-15), and 4 mST isolates, 3 from wild birds
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330 150 (isolates S05620-15, S05798-15 and S06144-15) and one from pigs (isolate S05634-15).
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332 151 The assembled draft genome of the *S.* Rissen wild bird isolate S05753-15 was used as reference to
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334 152 map the pig *S.* Rissen genome S06138-15 and extract the SNPs within the whole genome sequence.
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336 153 The assembled draft genome S05620-15 was used as reference for the mST isolates. Genomic DNA
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338 and was extracted from 6 *Salmonella* isolates using a commercial kit MagMAX™ CORE Nucleic
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340 Acid Purification Kit together with a KingFisher Duo Prime magnetic particle processor (both from
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342 ThermoFisher Scientific, Waltham, USA) following manufacturer's instructions. The extracted
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344 genomic DNA was fragmented, tagged for multiplexing with the Nextera XT DNA Sample
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346 Preparation Kit (Illumina, Inc. San Diego, USA) and sequenced at the APHA on the Illumina NextSeq
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348 platform (Illumina, San Diego, USA) to generate 150 base pair paired-end reads with minimum
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350 coverage of 50 x. The quality of the short reads was evaluated with FastQC
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357 161 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The short reads were quality trimmed
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359 162 with Trimmomatic (Bolger et al., 2014) and mapped to the 2 de novo assembled draft genomes
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361 163 S05753-15 and S05620-15 using Snippy (Kwong et al., 2015). The genomes were de novo assembled
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364 164 using SPAdes (Bankevich et al., 2012) and assemblies corrected using Shovill
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366 165 (<https://github.com/tseemann/shovill>). The alignments were further parsed to extract only single
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368 166 nucleotide polymorphism (SNP) with the minimum number of 10 reads. For phylogenetic analyses,
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370 167 a maximum-likelihood phylogenetic tree was constructed from the SNP alignments after Gubbins
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372 168 was run to remove regions of recombination in the pseudofasta files from SNP calling (Croucher et
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374 169 al., 2014). The recombination regions were removed using SNP-sites (Page et al., 2016). The
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376 170 phylogenetic analysis was performed on the generated SNP alignment file to infer core SNP
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379 171 phylogeny using the maximum likelihood method at 100 bootstraps by RAxML and visualised using
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381 172 the tree of life (iTol) (Letunic and Bork, 2016). The SNP distance tables were obtained using snp-dist
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383 173 (<https://github.com/tseemann/snp-dists>). The raw fastq files of the six isolates were also passed
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385 174 through the *Salmonella* pipeline which consists of several programs including three serotyping
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387 175 programs, MOST, SeqSero and SISTR to identify the *Salmonella* serotypes based on WGS (Tewolde
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389 176 et al., 2016; Yoshida et al., 2016; Zhang et al., 2015).

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393 **Statistical analyses**

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395 179 *Salmonella* prevalence in environmental and wild bird droppings samples collected during the 3 farm
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398 180 visits was investigated. Results from field A (empty at all visits) were compared to field B (occupied
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400 181 by pigs at visit 1).

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402 182 The change in *Salmonella* prevalence during the three visits was also studied in environmental and
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404 183 wild bird droppings samples in each of the two fields using Chi-squared test. Chi-Square test was
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406 184 used to compare all the data and the significance limit was set at $P < 0.05$. Confidence intervals were
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408 185 calculated by binomial (Clopper-Pearson) “exact” method based on the β distribution. Finally, Odds
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ratio (OR) was calculated for the risk of *Salmonella* contamination. All statistical analyses were performed using the software SPSS 23.0 (IBM SPSS Statistics, NY, US).

RESULTS

During the three farm visits a total 661 samples were collected from the two areas investigated, field A (empty at all visits) and field B (occupied by pigs at the first visit). Of these, 324 were environmental samples (254 swabs collected without enrichment media and 70 swabs collected in enrichment media), 182 were bird dropping (167 swabs collected without enrichment media and 15 swabs collected in enrichment media), 155 were swine feces (120 individual faecal samples and 35 pooled faeces collected in enrichment media).

The non-enriched environmental and wild bird dropping samples collected from field B were more likely to be *Salmonella* positive ($P=0.001$) than those collected from field A (Table 5). Overall, the odds of a sample being *Salmonella*-positive was 20.2 times higher in field B compared to field A ($P=0.001$). For environmental samples and wild bird dropping, the OR values were 27.8 (95% CI: 12.5-62.2) and 12.6 (95% CI: 5.4-29.6), respectively ($P=0.001$) (Table 5).

At the first visit, 120 individual pig faeces samples were collected from field B. *Salmonella* was isolated from 70.0% (42) and 91.7% (55) respectively of 60 samples from weaners' pens and 60 samples from growers' pens (Table 3). *Salmonella* was also isolated from 58.3% (35 of 60) of environmental samples and from 7.4% (2 of 27) of wild bird faeces samples.

At the second visit, the 26.9% (36 of 134) of environmental samples and 15.8% (12 of 76) of wild bird droppings were found to be *Salmonella* positive.

During the final third visit, *Salmonella* was isolated from 27.7% (36 of 130) of environmental samples and from 44.3% (35 of 79) of wild bird samples.

Wild bird dropping samples collected in field B at the third visit were significantly more contaminated with *Salmonella* than the samples collected in empty fields (field A) ($P=0.001$).

473
474
475 211 Although *Salmonella* prevalence in environmental samples and wild bird samples collected from field
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477 212 A did not vary significantly during the three visits (respectively: P=0.82 and P=0.84), significant
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479 differences were observed in samples collected from field B over time. In particular, the proportion
480 213
481 of *Salmonella*-positive wild bird faeces increased significantly over time (P=0.001) while no
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483 difference was observed for the environmental samples (P=0.07).
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488 217 **Enriched swab samples**

489
490 218 At the first visit *Salmonella* was isolated from 83.3% (15 of 18) and 100% (17 of 17) respectively of
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492 219 the weaners' and growers' pens. Furthermore, *Salmonella* was isolated from all the 5 environmental
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494 220 samples collected during the first farm visit and from the 23.1% (3 of 13) and 41.7% (5 of 12) of
495
496 environmental samples collected respectively during the second and third visit (Table 4).
497 221
498 From wild bird droppings, *Salmonella* was detected in 71.4% (5 of 7) and 100% (8 of 8) swabs
499 222
500 collected respectively during the second and third visit.
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505 225 **Semi-quantitative culture results**

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507 226 High levels of *Salmonella* were found in individual pig faecal samples (Table 3). In the growers'
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509 227 faeces, 7 samples had a *Salmonella* concentration of 10^2 - 10^3 CFU/g, 3 samples of 10^3 - 10^4 CFU/g and
510
511 228 2 samples of 10^5 - 10^6 CFU/g. In the weaners' faeces the maximum *Salmonella* load was 10^3 - 10^4
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513 CFU/g (3 samples) followed by 8 samples with 10^2 - 10^3 CFU/g while a lower *Salmonella* level was
514 229
515 found in the remaining 8 samples (10 - 10^2 CFU/g and 1-10 CFU/g).
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518 231 In the environmental samples (Table 2) the levels of *Salmonella* were found to be low (1-10 CFU/g
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520 232 or 10 - 10^2 CFU/g) for the majority of the samples. Only the environmental samples collected from the
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522 233 field in which pigs had been housed had higher CFU/g (two samples had 10^2 - 10^3 CFU/g). Unusually
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524 234 high *Salmonella* levels were found in some of the wild bird droppings: 10^5 - 10^6 CFU/g in geese
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526 235 droppings collected during the second and third visit both of the two fields sampled (Table 2).
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237 **Serovars and phage types**

238 In total 151 *Salmonella* strains were serotyped, most *Salmonella* isolates were *S. enterica* serovar
239 4,5,12:i:- (mST) (121, 80.1%), followed by *S. Rissen* (22, 14.6%), *S. Senftenberg* (3, 2%), *S.*
240 *Typhimurium* (2, 1.3%), *S. Panama* (2, 1.3%) and *S. Derby* (1, 0.7%) (Tables 1, 3 and 4).

241 Among individual faeces and swab samples mST was detected from 85.1% (40 of 47) of weaners'
242 and growers' samples, from 78.4% (29 of 37) of wild bird droppings samples (the majority of them
243 were from geese droppings) and 77.6% (52 of 67) of environmental samples (Tables 3 and 4).

244 *S. Typhimurium* was isolated from only two wild bird droppings samples collected during the first
245 and second visits.

246 Thirty four *S. Typhimurium* and mST isolate were phage typed. Different sample types, collected
247 during all the visit, such as bird droppings (12), environmental (18) and pig fecal samples (4) were
248 selected. Only two phage types were identified among the 34 *S. Typhimurium* and mST strains tested.

249 All were DT193 except for one isolate of *S. Typhimurium*, isolated from wild bird droppings, which
250 was phage type DT41.

252 **Phylogenetic clustering of wild bird and pig *Salmonella* isolates**

253 All 6 isolates sequenced in this study were highly related with only 1 SNP difference between the 2
254 *S. Rissen* isolates and a maximum observed difference of 9 SNPs between the 4 mST isolates. The
255 SNP difference of the monophasic *S. Typhimurium* isolates from wild birds was between 4 and 6.

256 Maximum likelihood core genome SNP phylogeny of *S. Rissen* and mST isolates and SNP
257 differences are presented in Figure 1 and Figure 2, respectively.

259 **DISCUSSION**

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261 On farms rodents, birds, insects, are common inhabitants that can all be carrier vectors and can
262 mechanically transmit pathogens (Backhans et al., 2013). Bait traps or chemical pesticides can aid in

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593 263 the management of rodent problems as well the removal of waste and feed spills can be helpful to
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595 264 limit the attraction of birds and rodents (Andres and Davies, 2015).
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598 265 The role of wild birds is a controversial matter in relation to potential hazards to livestock and to for
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600 266 human health. Several studies support the hypothesis that wild birds play an important role in
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602 267 *Salmonella* epidemiology in both humans and animals (Andres et al., 2013; Phalen et al., 2010; Vico
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604 268 and Mainar Jaime, 2011). In contrast, other studies suggest that they do not represent a major public
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606 269 health hazard, considering the low numbers of organisms shed and the short duration of *Salmonella*
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608 270 carriage shedding (Hughes et al., 2008; Jensen et al., 2004; Marin et al., 2014). It is well recognized
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610 271 that *Salmonella* is an ubiquitous agent that can colonize asymptotically the gut of birds and
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612 272 consequently can be shed in their faeces (Andres et al., 2013). *Salmonella* prevalence studies are
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614 273 usually associated with wild birds and focussed on host-adapted strains in some bird species, but
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617 274 some studies report that birds near pig farms have higher probability of shedding *Salmonella* than
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619 275 birds living far from pig premises (Andres et al., 2013).
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621 276 This study was carried out on one outdoor pig farm occupied by *Salmonella* infected pigs at the first
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623 277 visit only. Individual samples of pig faeces, environmental samples and wild bird droppings were
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625 278 collected and a quantitative analysis of *Salmonella* was performed on positive samples.
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627 279 Pools of faecal samples were also collected as they are regarded as more effective for isolating
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629 280 *Salmonella*, than the sampling of a large number of individual samples (Cook et al., 2005). A
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631 281 significantly higher prevalence, as well as the higher odds of *Salmonella*-positive samples detected
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633 282 in samples collected from the field occupied by pigs, suggest that pigs are the likely source of
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636 283 *Salmonella* in the pig farm environment. At the first visit >50% environmental samples were found
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638 284 to be *Salmonella*-positive, and one and two months after the pigs had left the farm, 27% of
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640 285 environmental samples were still *Salmonella*- positive. *Salmonella* was also found in environmental
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642 286 samples in a field that had been empty for 2 years. It is likely that *Salmonella* can survive outside the
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644 287 host for a significant length of time as reported by several authors (Funk and Gebreyes, 2004; Jensen
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646 288 et al., 2004; Sandvang et al., 2000), and therefore the environment itself can become a potential source
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652 289 of infection for subsequent batches of pigs and wildlife. However, it is also possible that wild birds
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654 290 contributed to re-contaminate the soil, considering that *Salmonella* was isolated from wild bird
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656 291 droppings (7.4% of samples collected during the first farm visit, 15.8 % and 44.3% collected during
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658 292 the second and third farm visit, respectively). It is apparent that once the pigs had left the farm, the
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660 293 proportion of *Salmonella*-positive wild bird faeces increased significantly. This could be linked to the
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662 294 fact that the wild bird population accessing the fields increased considerably once the pigs left the
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664 295 farm (as observed by the sampling team). The increase may be due to the presence of leftover pig
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666 296 feed and worm populations being nearer the surface of the soil (Andres et al., 2013; Andres and
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668 297 Davies, 2015). Furthermore, the increase in wild birds density over time, may have caused an increase
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670 298 in the transmission rate of this infection among birds (Andres et al., 2013). It was not possible to
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672 299 collect samples from a field that had never been occupied by pigs on this farm, as all fields had been
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674 300 occupied by pigs on a rotational basis in the last decade. It was therefore not possible to assess the
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676 301 levels of contamination exclusively related to wild bird droppings.
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678 302 Livestock farms can act as areas where wild birds congregate for the availability of food and shelter
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680 303 (Andres et al., 2013). At the same time farm environment with high levels of *Salmonella*
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682 304 contamination, as well other pathogens may be an important potential source of infection and
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684 305 potential biodiversity threat for those avian species of wild birds susceptible to the infection (Andres-
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686 306 Barranco et al., 2014; Andres et al., 2013). It has been suggested that salmonellosis can be one of the
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688 307 causes of the decline of the house sparrow population (Pennycott et al., 2006). *Salmonella* enterica
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690 308 serovar Typhimurium for passerine can result in a sever disease with significant mortality (Tizard,
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692 309 2004). Several authors reported that the feeding and migration behaviour as well as the seasonality
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694 310 may influence the prevalence of salmonellosis in free-ranging birds (Andres-Barranco et al., 2014;
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696 311 Andres et al., 2013).
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698 312 *Salmonella* prevalence was significantly higher in wild bird droppings collected from the field
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700 313 occupied by pigs at the first visit (field B). Moreover in field B the odd of *Salmonella* positive samples
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711 314 was higher compared with those samples collected from field A. These results together suggested that
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713 315 pigs represent an important risk factor as source of *Salmonella* for wild birds.
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716 316 In the proximity of the farm sampled in this study, there was also a river, which attracted a large
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718 317 number of wild birds especially aquatic birds, such as Canada geese and seagulls.
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720 318 Geese droppings had higher *Salmonella* CFU/g, compared with *Salmonella* levels in environmental
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722 319 samples. A study by Pennycott et al., (2006) concluded that *Salmonella* strains in Great Britain
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724 320 originating from wild birds do not represent a major primary source of infection, considering the low
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726 321 percentage of wild bird associated phage types isolated from livestock. However, the high levels of
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728 322 *Salmonella* in geese faeces suggest that geese can represent an important source of infection, able to
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730 323 maintain *Salmonella* in areas where geese are present. The *Salmonella* serotypes found in pigs, mST
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732 324 and *S. Rissen*, were the same as those found in wild bird droppings. We confirmed the close
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734 325 relatedness of the mST and *S. Rissen* isolated from wild birds and pigs using whole genome
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736 326 sequencing as an highly discriminative method for studying population heterogeneity in bacteria. We
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738 327 found a single SNP difference between the *S. Risen* isolates and maximum of 9 SNPs among the mST
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740 328 isolates. In recent year WGS has been used successfully in investigating a number of *Salmonella*
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742 329 related outbreak and trace back investigations and is becoming a method of choice in linking different
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744 330 sources of infection (Ashton et al., 2015; Inns et al., 2017; Inns et al., 2015). Within the *S.*
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746 331 *Typhimurium* serovar a cluster of isolates that are grouped together in time and space and sharing 0-
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748 332 10 SNPs are considered as common source of infection (Ford et al., 2018). *S. Senftenberg*, and *S.*
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750 333 *Typhimurium* were found in wild bird droppings, and these are also serotypes typically commonly
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752 334 found in housed breeding pigs in the UK (Hughes et al., 2008). *S. Typhimurium* is reported to be the
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754 335 most common serotype identified in wild bird droppings (Andres et al., 2013; Horton et al., 2013;
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756 336 Hughes et al., 2008; Lawson et al., 2011; Palmgren et al., 2006; Vico and Mainar Jaime, 2011). In
757
758 337 contrast, the majority of *Salmonella* isolates from wild bird droppings during this study were mST
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760 338 DT193. One of the isolated *S. Typhimurium* serovars was phage type DT 41. *S. Typhimurium* DT 41
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770 339 has been reported previously in wild birds from the UK and is particularly associated with waterfowl
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772 340 (Barua et al., 2013; Hughes et al., 2008; Pennycott et al., 2006).
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774 341 Pigs have been recognized as the main reservoir of mST DT193 (Crayford et al., 2014), supporting
775
776 342 the hypothesis that pigs can act as a source of wild bird salmonellosis.
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778 343 Interestingly, the 25 of 37 mST-positive samples, were from geese droppings and three of them
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780 344 collected during the second and third visit presented an unusually high level of *Salmonella*-shedding.
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782 345 Therefore, it is reasonable to postulate that mST infection in wild geese does not cause clinical
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784 346 symptoms in birds. However, further studies are required to better understand the role of geese and
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786 347 their role in the cycle of *Salmonella* infection in outdoor pig farms.
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788 348 This study suggests a possible cyclical dissemination of *Salmonella* between pigs and wild birds, and
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790 349 that wild birds are capable of contributing to the persistence of *Salmonella* between batches of pigs.
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792 350 Adequate management practices to minimize the contact between pigs and wild birds (e.g. cover feed
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794 351 and water sources, use of nets) should be implemented in outdoor pig units.
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806 356 **Conflict of Interests**

807
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1 **Table 1.** *Salmonella* isolated from environmental and wild bird dropping samples (not collected in enrichment media) from the two fields sampled at
 2 the 3 sampling visits. Number of *Salmonella* positive samples/number tested, serotyping results are also reported. The number of positives for each
 3 category is shown in brackets.

Field	Samples	Farm visit	<i>Salmonella</i> positives/tested	Prevalence (%)	95% CI	Serotype	
A	Bird dropping	1	1/19	5.3	0.1-26.0	Typhimurium (1)	
		2	7/57	12.3	5.1-23.7	4,5,12:i:- (3), Senftenberg (3), Typhimurium (1)	
		3	3/46	6.5	1.4-17.9	4,5,12:i:- (1), Rissen (2)	
		Total	11/122	9.0	4.6-15.6		
Environment ^a	Environment ^a	1	2/12	16.7	2.1-48.4	4,5,12:i:- (2)	
		2	3/54	5.6	1.2-15.4	4,5,12:i:- (3)	
		3	3/63	4.8	1.0-13.3	4,5,12:i:- (3)	
		Total	8/129	6.2	2.7-11.9		
B	Bird dropping	1	1/8	12.5	0.3-52.7		
		2	0/12	0.0	0.0-26.5		
		3	24/25	96.0	79.7-99.9	4,5,12:i:- (23)	
		Total	25/45	55.6	40.0-70.4		
	Environment ^a	Environment ^a	1	28/43	65.1	49.1-79.0	4,5,12:i:- (16), Rissen (4), Panama (1)
			2	25/47	53.2	38.1-67.9	4,5,12:i:- (2), Rissen (3)
			3	28/35	80.0	63.1-91.6	4,5,12:i:- (23), Rissen (3), Derby (1)
Total	81/125	64.8	55.8-73.1				
Total			125/421	29.7	25.4-34.3		

4 ^a soil and water puddle samples

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Table 2. *Salmonella* isolated from environmental and wild bird dropping samples (not collected in enrichment media) from the two fields sampled at the 3 sampling visits. Enumeration and serotyping results are also reported. Number of isolates that were serotyped is shown in brackets.

Field	Samples	Farm visit	Count (CFU/g)	Serotype
A	Bird dropping	1	1-10	Typhimurium (1)
			1-10	Senftenberg (2), Typhimurium (1)
			10-10 ²	Senftenberg (1)
		2	10 ³ -10 ⁴	4,5,12:i- (1)
			10 ⁴ -10 ⁵	4,5,12:i- (1)
			10 ⁵ -10 ⁶	4,5,12:i- (1)
	Environmental*	3	1-10	Rissen (1)
			10-10 ²	Rissen (1)
			10 ³ -10 ⁴	4,5,12:i- (1)
		1	1-10	4,5,12:i- (2)
			2	4,5,12:i- (3)
			3	4,5,12:i- (1)
B	Bird dropping	3	1-10	4,5,12:i- (3)
			10-10 ²	4,5,12:i- (6)
			10 ² -10 ³	4,5,12:i- (8)
		1	10 ³ -10 ⁴	4,5,12:i- (4)
			10 ⁵ -10 ⁶	4,5,12:i- (2)
			1-10	4,5,12:i- (14), Rissen (3), Panama (1)
	Environmental*	2	10-10 ²	4,5,12:i- (2), Rissen (1)
			1-10	4,5,12:i- (2), Rissen (3)
			1-10	4,5,12:i- (17)
		3	10-10 ²	4,5,12:i- (5), Rissen (3)
			10 ² -10 ³	4,5,12:i- (1), Derby (1)

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* Environmental= soil and water puddle samples

12 **Table 3.** Prevalence of *Salmonella*-positive pig individual faecal samples from pigs at visit 1.

13 Number of *Salmonella* positive samples/number tested, enumeration and serotyping are also

14 reported. Number of isolates that were serotyped is shown in brackets.

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Samples	<i>Salmonella</i> positives/tested	Prevalence %	95% CI for prevalence	Count (CFU/g)	Serotype
Weaners	42/60	70.0	56.8-81.2	1-10	4,5,12:i:- (5) Rissen (1)
				10-10 ²	4,5,12:i:- (2)
				10 ² -10 ³	4,5,12:i:- (8)
				10 ³ -10 ⁴	4,5,12:i:- (3)
Growers	55/60	91.7	81.7-97.2	1-10	4,5,12:i:- (1)
				10-10 ²	4,5,12:i:- (6)
				10 ² -10 ³	4,5,12:i:- (7)
				10 ³ -10 ⁴	4,5,12:i:- (1), Rissen (2)
				10 ⁵ -10 ⁶	4,5,12:i:- (2)
total	97/120	80.8	72.6-87.4		

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Table 4. *Salmonella* isolated in swab samples collected in enrichment media from the two fields sampled at the 3 sampling visits. Number of *Salmonella* positive samples/number tested, and serotyping are also reported. Number of isolates that were serotyped is shown in brackets.

Field	Samples	Farm visit	no. positives/ no. tested	Serotype
A	Environmental	2	3/13	Rissen (3)
		3	5/12	
	Environmental	1	5/5	4,5,12:i:- (3)
		2	5/20	
		3	0/20	
B	Bird dropping	2	5/7	4,5,12:i:- (2)
		3	8/8	Rissen (1)
	Weaner	1	15/18	4,5,12:i:- (3), Panama (1)
	Grower	1	17/17	4,5,12:i:- (2), Rissen (3)

* Environmental= farm equipment samples

Table 5. Odds Ratio (OR) for environmental and wild bird dropping (not collected in enrichment media) from field A and B at the 3 sampling visits.

Sample	Field	Positive	Negative	Total	P	OR	95%CI for OR
Bird dropping	B	25	20	45	< 0.001	12.6	5.4-29.6
	A	11	111	122			
	Total	36	131	167			
Environment	B	81	44	125	< 0.001	27.8	12.5-62.2
	A	8	121	129			
	Total	89	165	254			
Total	B	106	64	170	< 0.001	20.2	11.5-35.4
	A	19	232	251			
	Total	125	296	421			

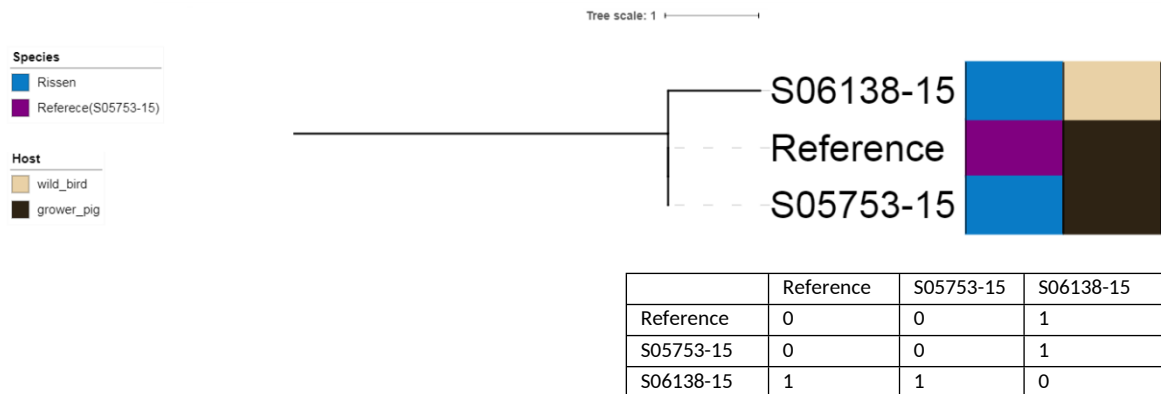


Figure 1. Maximum likelihood core genome SNP phylogeny of 2 *Salmonella* Rissen isolates S06138-15 and S05735-15 with assembled draft genome of S05735-15 used as reference. Figure created with Interactive Tree of Life (iTOL) (<https://itol.embl.de>). SNP, single-nucleotide polymorphism. Table: Number of SNPs in each isolate compared to the reference genome.

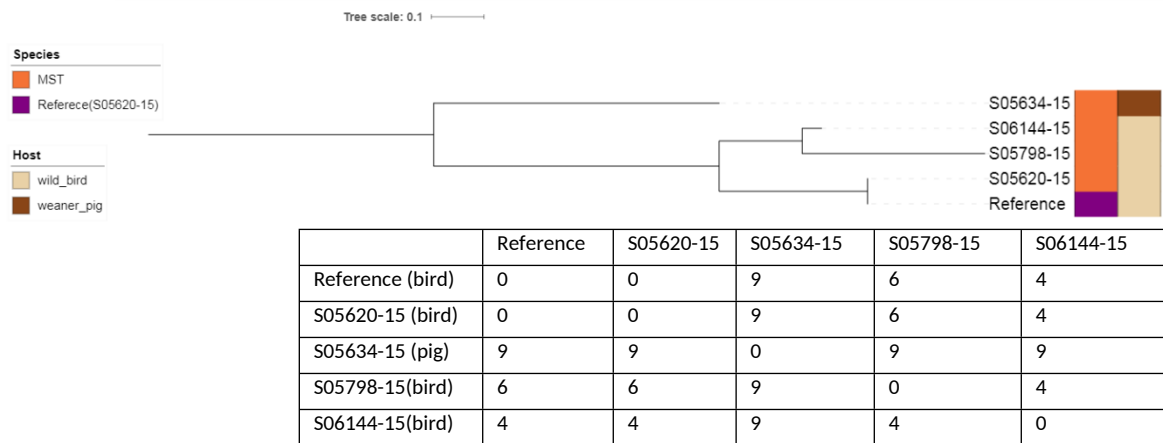


Figure 2. Maximum likelihood core genome SNP phylogeny of 4 monophasic *Salmonella* Typhimurium isolates S05620-15, S05634-15, S05798-15 and S06144-15 with assembled draft genome S05620-15 used as reference. Figure created with Interactive Tree of Life (iTOL) (<https://itol.embl.de>). SNP, single-nucleotide polymorphism. Table: Number of SNPs in each isolate compared to the reference genome.