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1 **Relationship between *Salmonella* infection, shedding and serology in fattening pigs in**  
2 **moderate prevalence areas**

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5 Running title: *Salmonella* infection, shedding and sero-prevalence in fattening pigs

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7 B. San Román,<sup>1</sup> V. Garrido,<sup>1</sup> S. Sánchez,<sup>1</sup> I. Martínez-Ballesteros,<sup>2</sup> J. Garaizar,<sup>2</sup> R.C. Mainar-Jaime,<sup>3</sup>

8 L. Migura-Garcia,<sup>4</sup> M.J. Grilló<sup>1,#</sup>

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11 <sup>1</sup>Instituto de Agrobiotecnología (CSIC-UPNA-Gobierno de Navarra), Avda. Pamplona, 123. 31192,  
12 Mutilva, Navarra, Spain.

13 <sup>2</sup>Dpto. de Inmunología, Microbiología y Parasitología, Facultad de farmacia, UPV/EHU, Calle  
14 Paseo de la Universidad, 7. 01006, Vitoria, Álava, Spain

15 <sup>3</sup>Facultad de Veterinaria, Universidad de Zaragoza, Spain.

16 <sup>4</sup>Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Centre de Recerca en Sanitat Animal,  
17 Campus de la Universitat Autònoma de Barcelona, Spain.

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20 # Corresponding author: María-Jesús Grilló

21 Phone: +34 948 168028; Fax: +34 948 232191; e-mail: [mj.grillo@csic.es](mailto:mj.grillo@csic.es)

22

23 **Abstract**

24 *Salmonella* is a major foodborne pathogen causing important zoonosis worldwide. Pigs  
25 asymptotically infected in mesenteric lymph nodes (MLN) can be intermittent shedders of  
26 the pathogen through feces, being considered a major source of human infections. European  
27 baseline studies of fattening-pig salmonellosis are based on *Salmonella* detection in MLN. This  
28 work studies the relationship between *Salmonella* infection in MLN and intestinal content (IC)  
29 shedding at slaughter, and the relationship between the presence of the pathogen and the  
30 serologic status at farm level. Mean *Salmonella* prevalence in the selected pigs (vertically-  
31 integrated production system of Navarra, Spain) was 7.2% in MLN, 8.4% in IC, and 9.6% in serum  
32 samples. In this low-moderate prevalence context, poor concordance was found between MLN  
33 infection and shedding at slaughter, and between bacteriology and serology. In fact, most of  
34 shedders were found uninfected in MLN (83%) or carrying different *Salmonella* strains in MLN  
35 and in IC (90%). The most prevalent *Salmonellae* were Typhimurium resistant to ACSSuT±Nx or  
36 ASSuT antibiotic families, more frequently found invading the MLN (70%) than in IC (33.9%).  
37 Multivariable analysis revealed that risk factors associated with the presence of *Salmonella* in  
38 MLN or in IC were different, mainly related either to good hygiene practices or to water and feed  
39 control, respectively. Overall, in this prevalence context, detection of *Salmonella* in MLN is an  
40 unreliable predictor of fecal shedding at abattoir, indicating that subclinical infections in  
41 fattening pigs MLN could have limited relevance in the IC shedding.

42

43 **Keywords:** *Salmonella*, fattening pigs, lymph-nodes infection, shedding, serology.

44

45 **Impact:**

- 46 • Poor concordance between *Salmonella* MLN infection and IC shedding, as well as  
47 between bacteriology and serology at farm level, was found by analysis of paired  
48 samples from 698 fattening pigs from a <10% *Salmonella* prevalence context.
- 49 • Multivariable analysis revealed that risk factors associated with the presence of  
50 *Salmonella* in MLN or in IC were different, being mainly related either to good hygiene  
51 practices or to water and feed control.
- 52 • *Salmonella* Typhimurium resistant to ACSSuT±Nx or ASSuT antibiotic families were more  
53 frequently found invading the MLN than in fecal IC samples.
- 54 • In low-moderate prevalence contexts, detection of *Salmonella* in MLN is an unreliable  
55 predictor of fecal shedding at abattoir, indicating that subclinical infections in fattening  
56 pigs MLN could have limited relevance in the IC shedding.

57

58 **Introduction**

59 Foodborne *Salmonella* infection is considered a major cause of human morbidity in  
60 industrialized areas such as USA (CDC, 2012) and EU (EFSA-ECDC, 2015). In USA, salmonellosis is  
61 the first cause of foodborne disease registering 1,027,561 of non-typhoid human cases in 2011,  
62 out of which 19,336 (1.9%) required hospitalization and 378 were fatal (CDC, 2012). Also, after  
63 campylobacteriosis, salmonellosis is the second most frequent zoonosis in EU, with 88,715  
64 confirmed cases in 2014 (EFSA-ECDC, 2015). Eggs and poultry products have been considered  
65 the most important source of human infections, responsible for 43.8% of the cases (Pires *et al.*,  
66 2011). Recent implementation of *Salmonella* control programs on fowl populations have  
67 resulted in a decreasing occurrence of *Salmonella* in eggs in the EU Member States (EFSA-ECDC,  
68 2015) and thus a clear decrease of human salmonellosis since 2007 (EFSA-ECDC, 2012).  
69 Currently, *Salmonella*-infected pigs are considered a major source of human infections (EFSA-  
70 ECDC, 2015, Pires *et al.*, 2011).

71 To preserve the consumer's health, the current EU authorities advocate for the control of  
72 *Salmonella* in pigs based on a "from farm to fork" strategy (DOUE, 2003). For this purpose, a EU  
73 baseline study was designed in order to estimate the prevalence of *Salmonella* in slaughtered  
74 pigs by analyzing the bacterium in mesenteric lymph nodes (MLN), which is considered the  
75 target organ of choice to demonstrate the *Salmonella* infection exists in asymptotically  
76 infected pigs (EFSA, 2008a) since (i) these tissues are quickly colonized by the pathogen after  
77 adhesion and invasion preferentially through the Peyer's patches and M cells of the gut wall;  
78 and (ii) a significant proportion of pigs become as chronic asymptomatic carriers in MLN and  
79 other tissues/organs, able to shed the pathogen through feces for long-lasting periods (Wood *et*  
80 *al.*, 1989; Evangelopoulou *et al.*, 2014; Evangelopoulou *et al.*, 2015). Alternatively, fecal samples  
81 have been used for *Salmonella* studies in live animals at farm level. However, the presence of  
82 *Salmonella* in feces could be attributed not only to an active infection of the intestine wall, MLN  
83 and/or other tissues and organs but also to a passive presence of the pathogen (EFSA, 2008a).

84 Also, serological studies are proposed as a cheaper and faster option for *Salmonella* surveillance  
85 by using the pig serum samples that are systematically collected in routine surveillance programs  
86 for other infectious diseases, such as Aujeszky's disease. This method is considered particularly  
87 useful to identify herds highly exposed to the pathogen, and to detect an increasing prevalence  
88 in very low (<3%) *Salmonella* prevalence countries/areas for interventions (Vico *et al.*, 2010).  
89 Large differences in fattening pigs *Salmonella* prevalence have been shown not only between  
90 EU Member States (EFSA, 2008a, EFSA, 2008b) but also between Spanish high and low pig-  
91 production regions (García-Feliz *et al.*, 2007). Our hypothesis is that, depending on the  
92 *Salmonella* prevalence in the country/region, the performance of the sample type for assessing  
93 the presence of the pathogen could vary widely. Accordingly, the aim of this study was to  
94 investigate the relationship between MLN infection and fecal shedding at abattoir in vertically  
95 integrated fattening pig from an area of low-moderate prevalence of *Salmonella* in these  
96 animals. Additionally, the concordance between bacteriology and serology was analyzed at farm  
97 level. For this, MLN and intestinal content (IC) paired samples were obtained at the slaughter  
98 line for bacteriology and subsequent thoroughly phenotypic and genotypic characterization of  
99 *Salmonella* isolates, and a representative number of sera from the same fattening pigs were  
100 obtained for ELISA analysis. Moreover, analysis of potential risk factors associated to *Salmonella*  
101 MLN infection and/or IC shedding were performed.

102

## 103 **Material and methods**

### 104 **Study design and sampling**

105 A total of 469,758 fattening pigs were registered in the region of Navarra (MAPAMA, 2012), most  
106 of them (78.6%) belonging to the 158 intensive farms vertically-integrated in 6 major pig  
107 companies (average of 2,900 pigs/farm). All the animals were slaughtered in 3 main abattoirs  
108 located within a 300-km radius. This was the sampling frame of this work.

109 The total number of farms and pigs to be sampled was calculated according to the expected  
110 herd and individual prevalence of *Salmonella*, i.e. around 50% farms containing at least one pig  
111 infected and less than 30% infected pigs per farm (EFSA, 2008a), and assuming a 10% error with  
112 a 95% confidence interval (95% CI). Thus, 30 farms (19% sampling fraction) and 25 pigs/farm  
113 were selected to avoid biases. In turn, farms were selected proportionally to the six major  
114 integrated-companies, the three main abattoirs implicated, the geographical location of farms,  
115 and the season of the year (18-months sampling). Twenty-five pigs per farm were selected  
116 randomly once in the slaughter line and systematically by selecting the first 25 sequential  
117 animals of each farm. Both MLN and intestinal content (IC) paired samples were collected from  
118 each pig. In 4 farms only 12 pigs/farm were collected due to logistic sampling limitations. Thus,  
119 a total of 1,396 samples (698 MLN and 698 IC) were finally obtained for bacteriological purposes.  
120 In addition, due to sampling limitations found in the abattoirs, the serological prevalence was  
121 determined at herd level in 19 out of the 30 farms, by sampling 12 pigs/farm (i.e. a total of 228  
122 out of the 698 pigs sampled for bacteriology). To avoid bias, random blood samples were taken  
123 in the slaughter line and the seroprevalence results were not used for the risk factors analysis.

#### 124 **Ethics committee approval**

125 Animal handling and slaughtering procedures were performed according to the current national  
126 legislation (Law 32/2007, for animal care on holdings, transportation, testing and slaughtering.

#### 127 ***Salmonella* spp. isolation and characterization**

128 The presence of *Salmonella* spp. in both MLN and IC samples was determined by the well-  
129 standardized ISO 6579:2002/Amd 1:2007 method (hereafter ISO 6579) (ISO, 2007), as  
130 recommended in the EU reference studies on pig salmonellosis (EFSA, 2008a) and previously  
131 detailed (Garrido, 2014). All the *Salmonella* isolates were confirmed and classified by serovars  
132 according to the Kaufmann-White scheme (Grimont & Weill, 2007) in the Reference National  
133 Centre for Animal Salmonellosis (MAPAMA, Madrid, Spain). The isolated *Salmonella* were  
134 thereafter analyzed by the Kirby-Bauer disk diffusion test (CLSI, 2006) against 12 antimicrobials

135 belonging to 8 different antimicrobial families (OIE, 2015), i.e. ampicillin and amoxicillin-  
136 clavulanic acid (A, Aminopenicillins); chloramphenicol (C, Phenicol); streptomycin and  
137 gentamycin (S, Aminoglycosides); sulphisoxazole, trimethoprim and trimethoprim-  
138 sulphometoxazole (Su, Sulfonamides); tetracycline (T, Tetracyclines); nalidixic acid (Nx, Natural  
139 Quinolones); ciprofloxacin (Fluoroquinolones); and cefotaxime (Third Generation  
140 Cephalosporins). Antimicrobial concentrations used were those recommended by the European  
141 legislation (DOUE, 2007). *Salmonella* susceptibility to each antimicrobial was determined by  
142 measuring the diameter of the inhibition halo induced around disk (BD, Madrid, Spain) in  
143 Mueller-Hinton (BD, Madrid, Spain) plates. Each strain was classified as resistant or susceptible,  
144 according to the Clinical and Laboratory Standards Institute recommendations (CLSI, 2006).  
145 Reference strains *E. coli* ATCC 25922, *S. Typhimurium* ATCC 14028 and *S. Typhimurium* ATCC  
146 DT104 were used as controls.

147 For further analysis of a possible relationship between *Salmonella* MLN infection and IC  
148 shedding, four additional colonies/sample were kept and characterized. Besides serotyping and  
149 antimicrobial resistance (AR) phenotypes, *S. Typhimurium* was submitted to phage typing in the  
150 National Centre of Microbiology (Instituto de Salud Carlos III, Madrid, Spain) by the 34 STM  
151 phage collection, following the standard procedures (Anderson *et al.*, 1977, Echeíta *et al.*, 2005).  
152 Also, strains showing the same phenotype were genotyped by MLVA, following the standard  
153 operating procedure proposed by the European Centre for Disease prevention and Control  
154 (ECDC, 2011). For this, a multiplex PCR was performed with the VNTR loci and the forward and  
155 reverse primers sequences described by Lindstedt *et al* (2004) in a GeneAmp Thermal  
156 Cycler2720 (Applied Biosystems). PCR products were subjected to capillary electrophoresis in a  
157 Genetic Analyzer ABI PRISM 3130XL (Applied Biosystems) and fragment sizes were determined  
158 with Peak Scanner v.1 (Applied Biosystems) using GS600 LIZ as size standard. An allele number  
159 was given to each fragment size according to the nomenclature proposed by Larsson *et al* (2009),  
160 representing the repeats copy number existing in the VNTR. MLVA profiles were expressed as a



161 string of five locus numbers (SSTR9-SSTR5-STTR6-STTR10-STTR3). Absent loci were named as  
162 “NA”, and all absent alleles were confirmed by single-plex PCR reactions (Larsson et al, 2009;  
163 Nadon et al, 2013). Cluster analysis was performed using the Dice similarity coefficient, and the  
164 unweighted pair group method with arithmetic mean (UPGMA) (<http://insilico.ehu.eus>;  
165 UPV/EHU). Shedding was considered associated to MLN infection when at least one *Salmonella*  
166 isolate showed identical phenotype simultaneously in both MLN and IC samples of a given pig.

#### 167 **Serological study**

168 Serum samples (n=228) were obtained after blood incubation (room temperature, 4 h) and  
169 centrifugation (Multifuge 3 L-R, SORVALL, Heraeus; 4°C, 10 min, 1,500 ×g) and kept frozen until  
170 its use. The Herd-Check® Swine *Salmonella* ELISA test (IDEXX™ Laboratories, Inc., Hoofddorp,  
171 Netherlands) was used following the manufacturer’s instructions. The 40% Optical Density cut-  
172 off was considered as the threshold to deem a positive result, according to the performance of  
173 this test reported by others (Methner et al., 2011, Nollet et al., 2005, Vico et al., 2010) and as  
174 used in some EU *Salmonella* surveillance programs (Merle *et al.*, 2011).

#### 175 **Questionnaire data and statistical analysis**

176 Questionnaires were designed in order to collect complementary information about the pig  
177 production from the abattoir, the major pig company, and the farm of origin, for each selected  
178 batch of pigs analyzed. Abattoir data (8 variables) were related to animal origin, travel time to  
179 slaughter and animal management previous to slaughtering, including the time spent by pigs in  
180 lairage before slaughter. The major pig company (8 variables) provided information on diet and  
181 antibiotics (if any) administration. Information from the farm (62 variables) dealt with data on  
182 basic infrastructures, biosecurity measures, animal health, feeding practices, antibiotic  
183 administration, and farmers’ information (Vico *et al.*, 2011). In order to provide more reliable  
184 information, the farmers were asked to fill out the questionnaires with the assistance of their  
185 veterinarians.

186 A farm was considered positive when *Salmonella* was isolated in at least one pig. Mean and 95%  
187 CI prevalence were calculated by considering MLN, IC and serum samples separately.  
188 Assessment of the agreement between infection in MLN and shedding was estimated by the  
189 Kappa statistic (*k*) and the strength of the concordance was interpreted according to the Landis  
190 & Koch criteria (Viera & Garrett, 2005). Agreement between bacteriology and serology was  
191 estimated exclusively at farm level, due to blood sampling limitations at abattoir.  
192 Questionnaire information was used to assess potential *Salmonella* risk factors for prevalence,  
193 or shedding. A univariable *Chi*-square test was carried out as a screening method, and significant  
194 ( $p \leq 0.05$ ) variables were further considered in a multivariable random-effect logistic regression  
195 model in which (i) the outcome variable was being “*culture positive*”; (ii) the explanatory  
196 variables included in the model as fixed effect were those from the questionnaire; and (iii) the  
197 random effect was the herd. The STATA software (StataCorp, L.P., College Station, TX, USA) was  
198 used for these statistical analyses.

199

## 200 **Results**

### 201 ***Salmonella* prevalence in MLN and IC, and herd-seroprevalence**

202 *Salmonella* spp. prevalence was similar in MLN (7.2%; 50/698) and in IC (8.4%; 59/698) samples  
203 (Table 1). However, only 14 pigs showed the pathogen simultaneously in MLN and feces.  
204 Therefore, the pathogen distribution in animals by farms was broader in IC than in MLN samples,  
205 being found in 70% and 46.7% of the farms analyzed, respectively (Table 1). In positive herds,  
206 the within-herd mean prevalence was 15.4% of pigs infected in MLN and 11.5% of shedders.  
207 However, most of the farms (93.3%) presented less than 20% of animals with *Salmonella* isolated  
208 in at least one sample (Table 1), showing 83.3% farms with *Salmonella* in less than 10% of pigs  
209 infected in MLN and 66.7% of farms with the presence of the pathogen IC samples from less  
210 than 10% of pigs (Figure 1).

211 ELISA results showed that 9.6% of pigs belonging to 52.6% of the farms were seropositive, with  
212 a 18.3% within-herd mean seroprevalence (Table 1). Similar to bacteriology, most of farms  
213 (78.9%) showed less than 20% of seropositive pigs, including 47.4% (9/19) farms with all pigs  
214 seronegative (Table 1). However, the percentage of farms with >20% of within-herd  
215 seroprevalence was higher ( $p<0.05$ ) than that detected by bacteriology either in MLN or in IC  
216 without agreement between bacteriological and serological prevalence at farm level (Figure 1).

#### 217 **Characterization of *Salmonella* strains**

218 From the 1,396 samples analyzed, *Salmonella* was found in 109 (7.8%) samples from 95 pigs, i.e.  
219 50 isolates from MLN and 59 from IC (Table 1). Eight different *Salmonella* serotypes were found  
220 in MLN, and 14 serotypes in IC samples (Table 2), being *Salmonella* Typhimurium the most  
221 common in both MLN (70%) and IC (33.9%) but more frequently ( $p<0.0001$ ) in the former. Other  
222 common serotypes were the monophasic 1,4,[5],12:i:- in both MLN (12%) and IC (11.8%); and  
223 Derby (16.9%), Anatum (13.5%), and Rissen (6.8%) in IC (Table 2).

224 A total of 74 (67.9%) *Salmonella* isolates (28 from MLN and 46 from IC samples) from 20 farms  
225 showed AR to at least one antimicrobial agent. Resistance to tetracycline (86.5%), streptomycin  
226 (82.4%), sulfisoxazole (77%) and ampicillin (64.9%) was common. Most (71.6%) of *Salmonella*  
227 strains showing some AR were resistant to 3 or more drugs, being ACSSuT±Nx (36.5%) and ASSuT  
228 (21.6%) the most prevalent multi-AR patterns in both MLN and IC samples (Table 2).

229 Furthermore, multi-AR strains were widely distributed, as they were present in 80% of the farms.  
230 In general, IC strains showed more variability than MLN strains in AR phenotypes (15 vs. 8 AR  
231 patterns, respectively; Table 2). Most of these AR patterns (11/15 in IC and 7/8 and in MLN)  
232 involved multiple antimicrobial agents belonging to 6 different families, but none included  
233 Fluoroquinolones (ciprofloxacin) or Third Generation Cephalosporin (cefotaxime). Noteworthy,  
234 AR to Natural Quinolones (nalidixic acid) was frequently associated to ACSSuT multi-AR pattern.  
235 At farm level, pansusceptible *Salmonella* isolates (35 out of 109 strains) were distributed in  
236 54.2% (13/24) of the farms where the pathogen was detected, but most (69.2%) of these farms

237 showed simultaneously pansusceptible and multi-AR strains. Regarding serotypes, around 50%  
238 of the strains showing AR were Typhimurium while less common serotypes such as Bardo,  
239 Enteritidis and Urbana, showed susceptibility to all the antibiotics tested (Table 2).

#### 240 **Relationship between *Salmonella* MLN infection, fecal shedding, and serology**

241 Although the overall prevalence of infection and shedding was similar, only mild agreement  
242 ( $k=0.19$ ) was observed between MLN and IC cultures (Table S1A). In fact, from the 95 pigs  
243 showing *Salmonella* spp. in at least one sample, only 14 (14.7%) pigs showed the pathogen  
244 simultaneously in both MLN and IC samples. The deeper characterization of these 28 isolates  
245 plus additional 4 colonies/sample (112 isolates) allowed to identify identical *Salmonella*  
246 phenotype in both MLN and IC samples from only 5/14 pigs, being Typhimurium (DT104B in 3  
247 pigs from the same farm and DT193 in 2 pigs) the serotype involved (Table S2). Other  
248 Typhimurium (2 pigs), Derby (2 pigs) and Anatum (1 pig) strains were discriminated exclusively  
249 by MLVA genotyping, showing different number of only 1 or 2 VNTR loci (Table S2). Overall, a  
250 relationship between MLN infection and fecal shedding could be established only in a 10% (5/50)  
251 of MLN infected pigs and 8.47% (5/59) of shedders. Noteworthy, 4 out of these 14 pigs (28.6%)  
252 showed simultaneous infections by different *Salmonella* types in MLN (Table S2, animals code  
253 5, 10, 11 and 12).

254 Regarding ELISA results, poor or slight concordance was observed at farm level between  
255 serology and MLN infection ( $k=0.05$ ), shedding ( $k=0.13$ ) or both simultaneously ( $k=0.24$ ) (Table  
256 S1B). In fact, 6 of the 9 farms where all the animals were serologically negative showed some  
257 pigs carrying *Salmonella* in both MLN and IC (4 farms, 5 pigs) or only in IC (2 farms).

#### 258 **Risk factors associated to *Salmonella* infection or shedding**

259 Twenty-three (76.7%) farms filled out the three questionnaires containing complementary  
260 information and, thus, they were eventually included in the statistical model. Considering the  
261 discrepancy observed between bacteriological results for both MLN and IC, the risk factor analysis  
262 was carried out separately for each type of sample. These 23 farms retained the large differences

263 in *Salmonella* MLN prevalence observed overall, since more than 50% of the infected pigs  
264 belonged to only 2 (8.7%) farms, while 14 (60.9%) farms were found free from *Salmonella*  
265 infection in pigs. Likewise, 45.7% of shedders belonged to 4 farms, while 7 (30.4%) farms showed  
266 all of the pigs analyzed free from *Salmonella* in IC.

267 A total of 56 variables (42 related to the farm and other 14 to both the company and the  
268 slaughterhouse) were initially associated with *Salmonella* spp. infection in MLN in the univariable  
269 analysis. However, 6 of them remained as risk factors in the final multivariable model, as shown  
270 in Table 3: (i) pigs with body weight at slaughter below 106 kg ("*final weight*"); (ii) pigs from farms  
271 with less than 1,800 animals ("*farm size*"); (iii) pigs slaughtered in autumn ("*season*"); (iv) pigs  
272 allocated to farms with only occasional or no rodent control programs ("*rodent control*"); (v) pigs  
273 from farms without a changing room and shower for workers ("*existence of changing room and*  
274 *shower*"); and (vi) pigs fed with fine-floured instead of pelleted feed ("*food type*").

275 In contrast, 20 variables (15 farm-related and 5 company-related) were associated with  
276 *Salmonella* fecal shedding in the screening univariable analysis but only 3 variables remained  
277 significant in the final model (Table 3): (i) "*food type*" (see above); (ii) "*food administration*" dry  
278 in contrast to feed mixed with water; and (iii) "*water analysis frequency*" performed only  
279 occasionally in contrast to at least once a year analysis. Thus, only the "*food type*" variable was a  
280 common risk factor identified for both MLN and IC positive samples (Table 3).

281

## 282 **Discussion**

283 The prevalence of *Salmonella* spp. infection in fattening pigs of our framework of Navarra (7.2%)  
284 was lower than that reported from similar studies carried out (i) at country level (29% in Spain)  
285 (EFSA, 2008a), (ii) in the major pig production areas of Spain (31.3% in Aragón) (Vico et al., 2011),  
286 and (iii) in the EU countries (10%) (EFSA, 2008a). Direct comparison to other pig *Salmonella*  
287 studies should be taken carefully since differences in sampling factors such as sample size (Funk  
288 et al., 2000), type of sample (EFSA, 2006, Mainar-Jaime et al., 2013) or the bacteriological

289 procedure used (Steinbach *et al.*, 2002) could lead to diagnostic accuracy variations. Differences  
290 between Navarra and Aragón were observed regarding not only the prevalence but also the  
291 variability of *Salmonella* serotypes and AR profiles found (Vico *et al.*, 2011), indicating  
292 differences in the epidemiological context and animal and herd management. Unlike major pig  
293 producing regions like Aragón (Gobierno-de-Aragón, 2012), Navarra has an important local gilt  
294 production that allows self-replacement, thus avoiding pig import and the subsequent cross-  
295 contamination (Lo Fo Wong *et al.*, 2004). Other subtler factors, likely associated with differences  
296 in the overall pig production system, may have also played a role in the observed differences  
297 between these neighboring regions, as shown by results from the multivariable analysis (Table  
298 3). Thus, the potential risk factors and the data were analyzed by using the same questionnaire  
299 and procedure as in the previous study in Aragón (Vico *et al.*, 2011). Only one variable, i.e. the  
300 absence of a continuous rodent control program in the farms, was found as a significant risk  
301 factor simultaneously in both regions, emphasizing the important role that rodents may play in  
302 the maintenance of the infection within the farm (Andrés-Barranco *et al.*, 2014). Other potential  
303 risk factors, such as the lack of changing rooms and showers for the staff, are considered a  
304 reflection of the farmer's level of awareness on farm hygienic practices. Moreover, pelleted feed  
305 has been associated with higher level of infection (Funk & Gebreyes, 2004), since it would modify  
306 the physical conditions of the gut, favoring the *Salmonella* survival. Herein, the presence of the  
307 pathogen not only in MLN (OR=5.73) but also in IC (OR=4.34) was favored by feed with fine flour.  
308 Factors modifying the intestinal microbiota have been proposed for controlling the infection by  
309 competitive exclusion of *Salmonella* (Andrés-Barranco *et al.*, 2015, Tanner *et al.*, 2014). In  
310 contrast to other studies, pigs with body weight below 106 Kg had a 39.6 higher risk of infection  
311 than heavier pigs under the same level of exposure, likely related to a poor nutritional and/or  
312 health condition.

313 Subclinical infections in MLN are considered as a main source of *Salmonella* that under certain  
314 circumstances of pig's stress can translocate to the digestive tract and shed by feces

315 (Evangelopoulou et al., 2014; Evangelopoulou et al., 2015) contributing to the contamination of  
316 other pigs, pig carcasses and meat (Callaway *et al.*, 2006, Larsen *et al.*, 2003, Argüello *et al.*,  
317 2012). In fact, while the slaughter process is designed to minimize external carcasses  
318 contamination, *Salmonella* invading MLN or other deeper tissues would seem to pose a high risk  
319 of direct contamination of meat, offal and their derived products. Alternatively, ingestion of the  
320 pathogen followed by its passive transit through the gut could be relatively frequent as well. In  
321 the low-medium prevalence context of this study, paired MLN and IC samples from 698 pigs  
322 were analyzed to estimate how frequent was the existence of simultaneous infections in both  
323 MLN and IC and, thus, the relevance of subclinical MLN infections in shedding at slaughter line,  
324 as a way of the pathogen introduction in the food chain. As result, only 10% (5/50) of pigs  
325 infected in MLN showed identical type of *Salmonella* in IC samples. This finding could be  
326 attributed either to a recent infection of the gut wall by *Salmonella* that reaches the MLN, or to  
327 a chronic infection of MLN ending up in *Salmonella* reactivation by stress and the subsequent  
328 shedding at the slaughter line (Monack et al., 2004). Differences between the isolation of  
329 *Salmonella* in MLN and IC samples could be attributed to a lower sensitivity of the bacteriological  
330 culture method from fecal samples, due to the presence of competitive flora and/or inhibitory  
331 substances in IC that could interfere in *Salmonella* isolation (EFSA, 2006, Mainar-Jaime et al.,  
332 2013). However, a high proportion (54/59) of pigs carrying the pathogen in IC appeared free  
333 from infection in MLN (45 pigs) or infected by different *Salmonella* strains (9 pigs), suggesting a  
334 recent ingestion of the pathogen that could have occurred during transport and/or lairage  
335 before slaughter, as demonstrated by others (Marg *et al.*, 2001). In our study, these parameters  
336 were not significant ( $p \geq 0.179$ ) in the univariate analysis. The time of transportation was less than  
337 1.5 hours in all cases and the time of lairage varied from 30 minutes to 7 hours. In most of the  
338 cases (20/30 herds) pigs waited less than 3 hours before slaughtering and only pigs from 3 herds  
339 waited 7 hours. Likewise, shedding could be attributed to a reactivation of a persistent  
340 *Salmonella* infection outside MLN, such as tonsils, gallbladder or intestinal wall (Evangelopoulou

341 et al., 2014; Evangelopoulou et al., 2015). Consequently, subclinical MLN infections seemed to  
342 play a limited role in pigs' shedding at slaughter, and subsequent introduction of the pathogen  
343 in the food chain.

344 The presence of a higher proportion of *S. Typhimurium* in MLN (70%) than in IC (33.9%) samples  
345 could indicate a higher invasiveness and/or persistence of this serotype in pigs MLN than those  
346 serotypes only found in the gut content, as reported in cattle (Gragg *et al.*, 2013). Additionally,  
347 the finding of simultaneous infection by *S. Typhimurium* strains with different phenotypes (i.e.  
348 antimicrobial susceptibility, phagetype and/or MLVA patterns) in 9 out of 14 pigs supported the  
349 relative high frequency of this phenomenon of co-infections, as previously reported (Garrido *et*  
350 *al.*, 2014). Coexistence of pansusceptible and AR *Salmonella* spp. in a same biological niche could  
351 favor the transference of mobile genetic elements carrying AR genes.

352 A large discrepancy was observed between bacteriology and serology at herd level. In spite of  
353 the low number of blood samples obtained, a significant proportion of farms showing all pigs  
354 seronegative had animals carrying the pathogen either in MLN (4 farms) and/or IC (6 farms),  
355 indicating that the one-time assessment of the presence of specific antibodies against  
356 *Salmonella* is a poor indicator of the actual status of infection in this epidemiological situation.  
357 This conclusion is supported by previous works indicating that: (i) *Salmonella* infection precedes  
358 by far (2-3 weeks) the sero-conversion, leading to seronegative but infected animals (Scherer *et*  
359 *al.*, 2008); (ii) the antibodies generated persist for more than 133 days post-infection, leading to  
360 seropositive but uninfected pigs (Scherer *et al.*, 2008); (iii) excretion can occur passively after  
361 the pathogen ingestion in absence of infection and seroconversion (Methner *et al.*, 2011, Nollet  
362 *et al.*, 2005); and (iv) other Gram-negative bacteria may cause false positive serological reactions  
363 (Vico *et al.*, 2010). Furthermore, some authors have suggested that discrepancies between  
364 serology and microbiology in pig salmonellosis could be attributed to serogroup differences  
365 between the antigens used in the ELISA test and the *Salmonella* serotypes prevalent in the  
366 region (Vico *et al.*, 2010, Steinbach *et al.*, 2002). This cannot explain our results since most of



367 *Salmonella* isolates (76.1%) belonged to serogroup B, the main target of the Herd-Check® Swine  
368 *Salmonella* ELISA test. Likely, false positive serological reactions caused by other  
369 *Enterobacteriaceae* may occur. In contrast to our results, in a 34.8% prevalence context, a strong  
370 association between herd serology and the prevalence of *Salmonella* bacteria measured at  
371 caecal-content but not at caecal-lymph nodes was established (Sorensen *et al.*, 2004).

372 In conclusion, the wide discrepancy between bacteriology in MLN and IC samples suggests a low  
373 impact of subclinical infections on *Salmonella* shedding at slaughter, in low-moderate  
374 prevalence contexts. Furthermore, the risk factors analysis strongly recommend a sustainable  
375 control based on good hygiene practices and rodent control. According to our results, a proper  
376 assessment of *Salmonella* in fattening pigs at abattoir should be done by analyzing both MLN  
377 and IC samples.

378

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387

### 388 **Conflict of interest**

389 None.

390

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537  
538

539 **Figure captions**

540

541 **Figure 1.** Distribution of *Salmonella* spp. prevalence at farm level (% of positive pigs/farm) in 698  
542 fattening pigs from the 30 farms analyzed. White bars: Mesenteric Lymph Nodes; black bars:  
543 Intestinal Content; grey bars: Blood Sera (ELISA).

544  
545

**Table 1.** Prevalence of *Salmonella* spp. in mesenteric lymph nodes, intestinal content and blood serum samples from vertically-integrated fattening pigs of Spain.

<i>Salmonella</i> spp. isolation	Mesenteric Lymph Nodes	Intestinal Content	Mesenteric Lymph Nodes and/or Intestinal Content	Serology
No. of positive pigs / total pigs analyzed (mean%; CI) <sup>a</sup>	50/698 (7.2%; 5.4-8.2)	59/698 (8.4%; 7.3-9.5)	95/698 (13.6%; 11.2-16.3)	22/228 (9.6%; 6.4-14.2)
No. of positive farms / total farms studied (mean%; CI) <sup>a</sup>	14/30 (46.7%; 33.9-66.1)	21/30 (70.0%; 53.8-86.1)	24/30 (80%; 70.0-96.6)	10/19 (52.6%; 31.7-72.6)
No. of positive pigs / pigs in positive farms (mean%; CI) <sup>a</sup>	50/324 (15.4%; 11.4-18.6)	59/512 (11.5%; 9.0-14.6)	95/574 (16.5%; 13.4-19.4)	22/120 (18.3%; 13.8-28.9)

546  
547

<sup>a</sup>at least 1 CFU of *Salmonella* spp. was isolated; <sup>b</sup>CI: 95% Confidence Interval.

548 **Table 2.** Phenotype of the *Salmonella* strains isolated from mesenteric lymph nodes or intestinal  
 549 content paired samples of 698 fattening pigs of Spain. Strains are grouped by antimicrobial resistance  
 550 pattern.

Antimicrobial resistance pattern <sup>a</sup> (No. of strains) <sup>a</sup>	Serotype (No. of strains) <sup>a</sup>	
	Mesenteric Lymph Nodes	Intestinal Content
ACSSuT(16)	Typhimurium(7)	Typhimurium(8) Rissen(1)
ACSSuTNx(11)	Typhimurium(5)	Typhimurium(6)
ASSuT(16)	Typhimurium(5) 1,4,[5],12:i:-(5)	Typhimurium(1) 1,4,[5],12:i:-(5)
ASSuTNx(1)	NA	Typhimurium(1)
ACSSu(1)	NA	Wien(1)
CSSuT(1)	NA	Derby(1)
ASSu(3)	1,4,[5],12:i:-(1)	1,4,[5],12:i:-(2)
SSuT(3)	NA	Derby(3)
STNx(1)	NA	Derby(1)
SSu(1)	Typhimurium(1)	NA
ST(4)	Anatum(1)	Anatum(3)
SuT(3)	Derby(1)	Agona(1) Derby(1)
Nx(1)	NA	Nottingham(1)
S(3)	Typhimurium(2)	<i>S. Salamae</i> (1)
Su(1)	NA	Anatum(1)
T(8)	NA	Typhimurium(1) Rissen(3) Derby(2) Anatum(2)
Susceptible(35)	Typhimurium(15) Bardo(2) Enteritidis(2) Other(3)	Typhimurium(3) Anatum(2) Derby(2) Urbana(2) Other(4)
<b>6 Antibiotic families</b>	<b>8 Serotypes (50)</b>	<b>14 Serotypes (59)</b>
<b>16 AR profiles (74)</b>		

551  
 552 <sup>a</sup> by typing one bacterial colony from each sample. A: ampicillin and/or amoxicillin-clavulanic acid;  
 553 C: chloramphenicol; S: streptomycin; Su: sulfisoxazole and/or trimethoprim-sulfometoxazole; T:  
 554 tetracycline; Nx: nalidixic acid. NA: No Applicable.

555 **Table 3.** Variables significantly associated with *Salmonella* prevalence in mesenteric lymph nodes or intestinal content of fattening  
 556 pigs, by a multivariable random-effect logistic regression analysis after clustering pigs by farm of origin.

Variable	Logistic Regression parameters for							
	Mesenteric Lymph Nodes				Intestinal Content			
	No. pigs	P value	OR <sup>b</sup>	(95% CI)	No. pigs	P value	OR <sup>b</sup>	(95% CI)
1. Final weight						NS		
	≥106 kg <sup>a</sup>	400		1	-		-	-
	<106 kg	175	0.000	39.6 (8-196)	-		-	-
2. Farm size						NS		
	≥1,800 pigs <sup>a</sup>	175		1	-		-	-
	<1,800 pigs	400	0.000	10.1 (3.8-26.6)	-		-	-
3. Season						NS		
	Winter <sup>a</sup>	150		1	-		-	-
	Spring	125	0.000	0.07 (0.03-0.16)	-		-	-
	Summer	175	0.028	0.23 (0.06-0.85)	-		-	-
	Autumn	125	0.046	7.41 (1.03-53.15)	-		-	-
4. Rodent Control						NS		
	Continuous <sup>a</sup>	425		1	-		-	-
	Sometimes/Never	150	0.000	20 (5.4-72.9)	-		-	-
5. Existence of changing room and shower						NS		
	Yes <sup>a</sup>	175		1	-		-	-
	No	375	0.005	11.92 (2.08-68.05)	-		-	-
6. Food type								
	Pelleted <sup>a</sup>	250		1	237		1	
	Meal	325	0.021	5.73 (1.3-25.2)	286	0.000	4.34 (1.92-10)	
7. Food administration								
	Mixed with water <sup>a</sup>	-		-	200		1	
	Dry	-	NS	-	298	0.001	4.2 (1.78-10)	
8. Water analysis frequency								
	≥1/year <sup>a</sup>	-		-	162		1	
	<1/year	-	NS	-	336	0.001	3.6 (1.69-7.96)	
Constant			0.09	3.1 (0.80-11.9)		0.000	0.15 (0.09-0.25)	

557  
 558 <sup>a</sup>Reference category assigned as OR=1 for statistical purposes; <sup>b</sup>Odds Ratio; NS: Not Significant.



559 **Table S1.** Contingency tables with the results of the *Salmonella* ISO 6579 on mesenteric lymph nodes  
 560 (MLN) and intestinal content (IC) paired samples (A); or with the *Salmonella* prevalences by serology and  
 561 microbiology (positive in MLN, IC or at least one of them) in 19 farms (B).  
 562

563 A)

No. of samples		MLN		Total
		Positive	Negative	
IC	Positive	14	45	59
	Negative	36	603	639
		50	648	698

564

565 B)

No. of farms		MLN		IC		MLN and/or IC		Totals
		Positive <sup>a</sup>	Negative	Positive <sup>a</sup>	Negative	Positive <sup>a</sup>	Negative	
Serology	Positive	5	5	8	2	9	1	10
	Negative	4	5	6	3	6	3	9
Totals		9	10	14	5	15	4	19
Kappa value vs. Serology (strength of concordance) <sup>b</sup>		k=0.05 (poor)		k=0.13 (slight)		k=0.24 (fair)		

566

567 <sup>a</sup> One farm was considered positive when at least one pig showed a positive result in the correspondent  
 568 analysis; <sup>b</sup> Strength of concordance determined by the Landis & Koch criteria (Viera & Garrett, 2005).

569  
570

**Table S2.** Phenotypic characterization of *Salmonella* strains isolated simultaneously in mesenteric lymph nodes (MLN) and intestinal content (IC) samples from fattening pigs.

Animal Code	Sample	Salmonella phenotype				Relationship MLN vs. IC <sup>a</sup>
		Serotype	AR pattern	Typhimurium phagetype	MLVA	
1	MLN	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	Yes
	IC	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	
2	MLN	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	Yes
	IC	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	
3	MLN	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	Yes
	IC	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	
5	MLN	Typhimurium	S/Susceptible	193	2-9-4-12-211	Yes
	IC	Typhimurium	S/Susceptible	193	2-9-4-12-211	
6	MLN	Typhimurium	S	193	2-9-4-12-211	Yes
	IC	Typhimurium Rissen	S ACSSuT	193 NA	2-9-4-12-211	
4	MLN	Typhimurium	ACSSuT	104B	3-13-15-24-311	No
	IC	Typhimurium	ACSSuT	104B	3-13-15-23-311	
10	MLN	Typhimurium	ACSSuT	104B/193/U302	3-13-16-24-311	No
	IC	Typhimurium	ACSSuT	104B	3-13-15-23-311	
7	MLN	Derby	SuT	ND	1-9-NA-19-111	No
	IC	Derby	SuT	ND	1-9-NA-NA-111	
8	MLN	Derby	Susceptible	ND	1-9-NA-NA-111	No
	IC	Derby	Susceptible	ND	1-9-NA-19-111	
9	MLN	Anatum	ST	ND	1-9-10-7-211	No
	IC	Anatum	ST	ND	1-9-NA-19-211	
11	MLN	Typhimurium	ACSSuT	104B	ND	No
	IC	Typhimurium	Susceptible	137/136	ND	
12	MLN	Typhimurium	S/Susceptible	193	ND	No
	IC	1,4,[5],12:i:-	ASSu	U311	ND	
13	MLN	Typhimurium	Susceptible	137	ND	No
	IC	Wien	ACSSu	ND	ND	
14	MLN	Typhimurium	ACSSuT	104B	ND	No
	IC	S. salamae	S/Susceptible	ND	ND	

<sup>a</sup>see Table 2; Possible relationship between infection in MLN and IC shedding; ND: Not Determined because not applicable; NA: No Amplification.

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