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

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

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Abstract

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

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

Impact:

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

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 asymptomatically
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 life 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).

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

Material and methods

Study design and sampling

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

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

Ethics committee approval

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

***Salmonella* spp. isolation and characterization**

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

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

For further analysis of a possible relationship between *Salmonella* MLN infection and IC shedding, four additional colonies/sample were kept and characterized. Besides serotyping and antimicrobial resistance (AR) phenotypes, *S*. Typhimurium was submitted to phagetyping in the National Centre of Microbiology (Instituto de Salud Carlos III, Madrid, Spain) by the 34 STM phage collection, following the standard procedures (Anderson *et al.*, 1977, Echeíta *et al.*, 2005). Also, strains showing the same phenotype were genotyped by MLVA, following the standard operating procedure proposed by the European Centre for Disease prevention and Control (ECDC, 2011). For this, a multiplex PCR was performed with the VNTR loci and the forward and reverse primers sequences described by Lindstedt et al (2004) in a GeneAmp Thermal Cycler2720 (Applied Biosystems). PCR products were subjected to capillary electrophoresis in a Genetic Analyzer ABI PRISM 3130XL (Applied Biosystems) and fragment sizes were determined with Peak Scanner v.1 (Applied Biosystems) using GS600 LIZ as size standard. An allele number was given to each fragment size according to the nomenclature proposed by Larsson et al (2009), 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 $\times g$) and kept frozen until
170 its use. The Herd-Check® Swine *Salmonella* ELISA test (IDEXX^{TM} 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.

A farm was considered positive when *Salmonella* was isolated in at least one pig. Mean and 95% CI prevalence were calculated by considering MLN, IC and serum samples separately. Assessment of the agreement between infection in MLN and shedding was estimated by the Kappa statistic (*k*) and the strength of the concordance was interpreted according to the Landis & Koch criteria (Viera & Garrett, 2005). Agreement between bacteriology and serology was estimated exclusively at farm level, due to blood sampling limitations at abattoir.

Questionnaire information was used to assess potential *Salmonella* risk factors for prevalence, or shedding. A univariable *Chi*-square test was carried out as a screening method, and significant ($p \leq 0.05$) variables were further considered in a multivariable random-effect logistic regression model in which (i) the outcome variable was being "*culture positive*"; (ii) the explanatory variables included in the model as fixed effect were those from the questionnaire; and (iii) the random effect was the herd. The STATA software (StataCorp, L.P., College Station, TX, USA) was used for these statistical analyses.

Results

Salmonella prevalence in MLN and IC, and herd-seroprevalence

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

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

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

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

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

Risk factors associated to *Salmonella* infection or shedding

Twenty-three (76.7%) farms filled out the three questionnaires containing complementary information and, thus, they were eventually included in the statistical model. Considering the discrepancy observed between bacteriological results for both MLN and IC, the risk factor analysis 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

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

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

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

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

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

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Conflict of interest

None.

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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).

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

***Salmonella* spp. isolation**	**Mesenteric Lymph Nodes**	**Intestinal Content**	**Mesenteric Lymph Nodes and/or Intestinal Content**	**Serology**
No. of positive[a] pigs/ total pigs analyzed (mean %; CI[b])	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)	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)	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)

[a]at least 1 CFU of *Salmonella* spp. was isolated; [b]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 (No. of strains)[a]	Serotype (No. of strains)[a]	
	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)	*S. salamae* (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)
6 antibiotic families **16 AR profiles (74)**	**8 serotypes (50)**	**14 serotypes (59)**

551

552 [a] 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[b]	(95% CI)	No. pigs	*P* value	OR[b]	(95% CI)
1. Final weight						NS		
³106 kg[a]	400		1		-		-	-
<106 kg	175	0.000	39.6	(8-196)	-		-	-
2. Farm size						NS		
³1,800 pigs[a]	175		1		-		-	-
<1,800 pigs	400	0.000	10.1	(3.8-26.6)	-		-	-
3. Season						NS		
Winter[a]	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[a]	425		1		-		-	-
Sometimes/Never	150	0.000	20	(5.4-72.9)	-		-	-
5. Existence of changing room and shower						NS		
Yes[a]	175		1		-		-	-
No	375	0.005	11.92	(2.08-68.05)	-		-	-
6. Food type								
Pelleted[a]	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[a]	-		-	-	200		1	
Dry	-	NS	-	-	298	0.001	4.2	(1.78-10)
8. Water analysis frequency								
³1/year[a]	-		-	-	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 [a] Reference category assigned as OR=1 for statistical purposes; [b] Odds Ratio; NS: Not Significant.

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

A)

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

B)

No. of farms		MLN		IC		MLN and/or IC		Totals
		Positive[a]	Negative	Positive[a]	Negative	Positive[a]	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)[b]		*k*=0.05 (poor)		*k*=0.13 (slight)		*k*=0.24 (fair)		

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

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[b]
		Serotype	AR pattern[a]	Typhimurium phagetype	MLVA	
1	MLN	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	Yes
	IC				4-15-10-7-310	
2	MLN	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	Yes
	IC				4-15-10-7-310	
3	MLN	Typhimurium	ACSSuTNx	104B	4-15-10-7-310	Yes
	IC				4-15-10-7-310	
5	MLN	Typhimurium	S/Susceptible	193	2-9-4-12-211	Yes
	IC				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				3-13-15-23-311	
10	MLN	Typhimurium	ACSSuT	104B/193/U302	3-13-16-24-311	No
	IC			104B	3-13-15-23-311	
7	MLN	Derby	SuT	ND	1-9-NA-19-111	No
	IC				1-9-NA-NA-111	
8	MLN	Derby	Susceptible	ND	1-9-NA-NA-111	No
	IC				1-9-NA-19-111	
9	MLN	Anatum	ST	ND	1-9-10-7-211	No
	IC				1-9-NA-19-211	
11	MLN	Typhimurium	ACSSuT/ Susceptible	104B 137/56	ND ND	No
	IC					
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	

[a]see Table 2; [b]Possible relationship between infection in MLN and IC shedding; ND: Not Determined because not applicable; NA: No Amplification.