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Zoonotic *Campylobacter* spp. and *Salmonella* spp. carried by wild boars in a metropolitan area: occurrence, antimicrobial susceptibility and public health relevance

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Abstract

Campylobacter spp. and *Salmonella* spp. are the most reported zoonotic agents in Europe. They can be transmitted from wildlife to humans, and wild boars (*Sus scrofa*) can harbour them. In the Metropolitan Area of Barcelona (MAB, NE Spain) wild boars are found in urbanized areas. To assess the potential public health risk of this increasing wild boar population, we collected stool samples from 130 wild boars from the MAB (June 2015 – February 2016), to determine the *Campylobacter* and *Salmonella* occurrence and the antimicrobial susceptibility of the isolates. We also investigated the genetic diversity and virulence potential of *Campylobacter*. *Campylobacter* prevalence in wild boars was 61%. Forty six percent of wild boars carried *Campylobacter lanienae*, 16% carried *Campylobacter coli*, and 1% carried *Campylobacter hyointestinalis*; 4% carried both *C. lanienae* and *C. coli*, and 1% carried both *C. lanienae* and *C. hyointestinalis*. This is the first report of *C. hyointestinalis* in wildlife in Spain. Using pulse-field gel electrophoresis and multilocus sequence typing, we observed a high genetic diversity of *Campylobacter* and identified new sequence types. Thirty-three percent of *C. coli* and 14% of *C. lanienae* isolates showed a high virulence potential. All of the *Campylobacter* isolates analysed were resistant to at least one antimicrobial agent. Multidrug resistance was only detected in *C. coli* (67%). *Salmonella enterica* subsp. *enterica* was detected in four wild boars (3%) and included a *S.* Enteritidis serovar (1/4 wild boars) and a multidrug-resistant (ASSuT) monophasic *S.* Typhimurium serovar (1/4 wild boars) which is associated with human infections and pig meat in Europe. The characteristics of some of the *Campylobacter* and *Salmonella* isolates recovered suggest an anthropogenic origin. Wild boars are a reservoir of *Campylobacter* and have the potential to spread antimicrobial resistant *Campylobacter* and *Salmonella* in urbanized areas in the MAB.

Keywords: antimicrobial resistance, MLST, PFGE, urban environment, virulence associated-genes.

1. Introduction

Campylobacteriosis and salmonellosis are the most frequent foodborne zoonoses in developed countries. In Europe, campylobacteriosis has been the most commonly reported zoonosis since 2005, followed by salmonellosis (EFSA and ECDC, 2021). Most human cases of campylobacteriosis are caused by *Campylobacter jejuni* (83.1%) and, to a lesser extent, by *Campylobacter coli* (10.8%) (EFSA and ECDC, 2021). Regarding salmonellosis, human cases are usually caused by *Salmonella enterica* subsp. *enterica* (Gaffuri and Holmes, 2012).

Consumption of contaminated food and water and the contact with infected domestic animals are the main routes of transmission to humans of both *Campylobacter* and *Salmonella* (Rukambile et al., 2019). Despite poultry is considered to be a major source of these zoonotic agents, other reservoirs may also be relevant (Greig et al., 2015; Sacks et al., 1986; Tomar et al., 2006). Hence, wildlife has been associated with the transmission of *Campylobacter* and *Salmonella* into the food chain, mainly through the contamination of water, farmland, farm produce, animal feed and pastures with faeces (Greig et al., 2015; Hilbert et al., 2012).

It is unknown whether *Campylobacter* can cause disease in free-ranging wild animals, although several species (e.g. *C. jejuni*, *C. coli, Campylobacter lari* and *Campylobacter lanienae*) have been isolated from wildlife (Horrocks et al. 2009; Moré et al. 2017; Navarro-González et al. 2014). As with *Campylobacter,* many wild birds and mammals carry *Salmonella* in their intestine without showing clinical signs (e.g. *Larus* spp. or red foxes *Vulpes vulpes,* among others), although mass mortalities of birds due to *Salmonella* outbreaks have seldom been reported (Giovannini et al., 2013; Velarde et al., 2012). Wildlife can acquire *Campylobacter* and *Salmonella* and their antimicrobial resistant (AMR) strains through environmental contamination, especially in areas affected by human activities (livestock farming, waste and sewage disposal,

etc), although the direction of the transmission between humans and wild animals is difficult to prove (Dolejska et al., 2016; Greig et al. 2015; Vittecoq et al. 2016).

Wild boars may harbour a wide variety of zoonotic pathogens that can infect livestock, companion animals and humans, including *Campylobacter* and *Salmonella* (Meng et al., 2009; Ruiz-Fons, 2017). Wild boar populations have been increasing and expanding their range across Europe during the last decades (Massei et al., 2015), which increases their potential to transmit diseases to humans (Ruiz-Fons, 2017). Wild boar is also receiving increasing attention due to its potential role as carriers, reservoirs and dispersers of AMR bacteria (Torres et al., 2020). Natural habitats impacted by human activities show increased prevalence of AMR bacteria carried by wildlife (Darwich et al. 2021; Dolejska et al., 2016; Vittecoq et al., 2016), and omnivorous species such as the wild boar are among the ones at a higher risk of acquiring and becoming carriers and spreaders of these bacteria (Vittecoq et al., 2016). The presence of AMR in zoonotic bacteria such as *Campylobacter* and *Salmonella* pose an additional hazard, compromising disease treatment both in animals and humans (Newell et al., 2010).

Moreover, wild boars inhabit a wide range of habitats (Abaigar et al., 1994; Meriggi and Sacchi, 2001; Virgós, 2002), including urbanized areas (Castillo-Contreras et al., 2018; Licoppe et al., 2013). In these areas, wild boars pose a greater risk of disease transmission for humans (Fernández-Aguilar et al., 2018; Jansen et al., 2007; Wang et al., 2019), in addition to other conflicts such as traffic accidents, damage to green spaces and even attacks on people and pets (Soulsbury and White, 2015).

The wild boar population from the Serra de Collserola Natural Park has also increased in recent years (González-Crespo et al., 2018), and wild boars search for food resources in urbanized areas, such as the city of Barcelona (Castillo-Contreras et

al., 2018). Barcelona is the largest municipality bordering the Serra de Collserola Natural Park, and both the park and the city are located within the Metropolitan Area of Barcelona (MAB, in northeast Spain). Wild boars have caused an increasing trend of conflicts in Barcelona from two incidents registered in 1998 (Llimona et al., 2007) to over 1,100 calls to the local emergency number in 2016 (Barcelona City Council, 2018). Wild boar presence has been reported in parks, gardens, private properties and the streets, where direct and indirect human-wild boar interactions occur (Barcelona City Council, 2018).

For these reasons, and given the potential role of wild boars as reservoir hosts or carriers of numerous zoonotic pathogens, including AMR bacteria, particularly in areas with a higher anthropogenic pressure, our objectives are: a) to assess the occurrence of zoonotic *Campylobacter* spp. and *Salmonella* spp. in wild boar faeces from the MAB; b) to determine the antimicrobial susceptibility of the isolates; c) to characterize the *Campylobacter* isolates in terms of genetic diversity and its potential association with anthropogenic sources, and the virulence potential, which is largely unknown in wild boars; d) to address the relationship between the carriage of *Campylobacter* by wild boars and the degree of urbanization of the study area, which has not previously been explored.

2. Material and methods

2.1. Study area

The MAB (in northeast Spain) is 636 km^2 in surface and has a population of approximately 3,200,000 people (Statistical Institute of Catalonia, 2015). We performed this study in three areas within the MAB: the city of Barcelona, the campus of the Universitat Autònoma de Barcelona (UAB), and the Serra de Collserola Natural Park (Collserola, hereafter) (Figure 1). Barcelona is a 101 km^2 city with 1,600,000 inhabitants (Statistical Institute of Catalonia, 2019), UAB is a 2.6 km^2 University

campus regularly used by 45,000 people (UAB, 2018), and Collserola is an 111 km^2 natural area that receives three million visitors each year (Parc de Collserola, 2020) (Figure 1). Barcelona and UAB are urbanized areas, but UAB contains garden, forestry and agricultural patches in a higher proportion of its surface than Barcelona (60% vs. 28%). On the contrary, Collserola is a natural area covered mainly by scrubland, forest and grassland, which also includes recreational spaces, built-up areas, and roads (see Castillo-Contreras et al. 2021a, for a detailed description of the three study areas). Therefore, landscape composition, human uses and pressure vary among these study areas, as also does the space use by wild boars, for instance with the exploitation of human-derived food resources in Barcelona but not in Collserola (Castillo-Contreras et al. 2021b).

2.2. Sampling

From June 2015 to February 2016, we aseptically obtained faecal swabs from the rectum of 130 wild boars. We placed the swabs in Amies transport medium containing charcoal (Deltalab, Barcelona Spain), and kept them refrigerated until arrival and processing at the laboratory, which occurred within the next 24h.

Wild boars were sampled in the abovementioned three areas within the MAB (Figure 1): Barcelona (n = 32), UAB (n = 25), and Collserola (n = 73). Wild boars were harvested by local hunters in Collserola, and tele-anaesthetised with a blowpipe by a veterinarian in Barcelona (contracts 15/0174, 16/0243 and 16/0243-00-PR/01 with *Ajuntament de Barcelona*-Barcelona City Council) and UAB (using cage traps prior to anaesthesia, authorization AC/190 from *Generalitat de Catalunya*-Government of Catalonia). Protocols of capture and anaesthesia can be found in Torres-Blas et al. (2020). Wild boar capture and hunting operations were done for population management purposes and/or when causing a potentially dangerous situation, not for research, and according to national and local legislation.

166 *2.3. Campylobacter and Salmonella isolation and identification*

167 We performed *Campylobacter* isolation and identification as described by Urdaneta et
168 al. (2015). We preserved up to four *Campylobacter* isolates from each wild boar host in
169 brain heart infusion broth with 20% glycerol at -75ºC until further analysis. For species
170 identification, we used a multiplex PCR targeting lipid A gene *lpx*A (Klena et al., 2004),
171 with forward primers lpxA-*C. coli* 5'-AGACAAATAAGAGAGAATCAG-3' and lpxA-*C.*
172 *jejuni* 5'-ACAACTTGGTGACGATGTTGTA-3', and a reverse primer lpxA-RKK2m 5'-
173 CAATCATGDGCDATATGASAATAHGCCAT-3' for both *C. coli* and *C. jejuni*. For *C.*
174 *lanienae*, we used a PCR using the primers CLAN76F (5'-
175 GTAAGAGCTTGCTCTTATGAG-3') and CLAN1021R (5'-
176 TCTTATCTCTAAGAGGTTCTTA-3'), as described by Logan et al. (2000). For the
177 isolates positive to the CLAN76F and CLAN1021R primers, we performed another
178 PCR for *C. hyointestinalis* with the primers JH0033 (5'-GGGGCAAATCCTATTGAGGT-
179 3') and JH0034 (5'-TCGCTATTTGCAGAGATCGTAG-3'), as described by Chaban et
180 al. (2009). We did this second PCR to confirm our identification of *C. lanienae* and *C.*
181 *hyointestinalis* isolates, given that results from a maximum likelihood tree (explained
182 later in the MLST section) suggested that a *C. hyointestinalis* isolate had been
183 misidentified as *C. lanienae*.

184

185 We performed *Salmonella* isolation and identification as previously described
186 (Antilles et al., 2015). Briefly, it was carried out by using buffered peptone water (Oxoid,
187 Basingstoke, UK) pre-enrichment, followed by a selective enrichment step in
188 Rappaport-Vassiliadis (Oxoid, Basingstoke, UK) and subculturing onto xylose lysine
189 tergitol 4 agar (Merck, Darmstadt, Germany). We subcultured up to four *Salmonell*a-
190 presumptive colonies from each wild boar host onto MacConkey agar plates and
191 lactose-negative colonies were confirmed as *Salmonella* sp. with the Mucap (Biolife,
192 Milano, Italy) and indole tests. We preserved the *Salmonella* isolates in brain heart
193 infusion broth with 20% glycerol at -75ºC until further analysis. *Salmonella* isolates

were serotyped at the Laboratori Agroalimentari (Cabrils, Spain) from the Catalan Government (Departament d'Acció Climàtica, Alimentació i Agenda Rural), according to White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007).

2.4. Molecular typing of Campylobacter and Salmonella isolates

In order to assess the genotypic diversity of *Campylobacter* strains, we first used two different typing methods, namely restriction fragment length polymorphism of the *fla*A gene (*fla*A-RFLP) for *C. coli* (n = 70 isolates, obtained from 21 wild boars), and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) for *C. lanienae* (n = 186 isolates, obtained from 60 wild boars). We initially attempted the typing of the *C. lanienae* isolates by *fla*A-RFLP, but most isolates were not typeable; thus we used ERIC-PCR instead. We considered as the same strain those isolates showing identical band patterns (either with *fla*A-RFLP or ERIC-PCR) and selected one of each from each positive individual, as well as non-typeable isolates (n = 82 *C. lanienae* and n = 31 *C. coli* isolates) for further typing by pulse-field gel electrophoresis (PFGE). A single *C. hyointestinalis* isolate initially misidentified as *C. lanienae* was also PFGE typed. Based on PFGE results, we selected 29 isolates (7 *C. lanienae*, 21 *C. coli* and one *C. hyointestinalis*) for further analysis by multilocus sequence typing (MLST). *Salmonella* isolates (n = 16) were typed by ERIC-PCR; we considered the strains being the same if the isolates had an identical band pattern by the ERIC-PCR.

<u>ERIC-PCR</u>

We performed ERIC-PCR of *C. lanienae* isolates as described by Antilles et al. (2015), using primers ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') and ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') (Versalovic et al., 1991). For *Salmonella* isolates we used the same ERIC-2 and ERIC-1R primer pairs but with a 50ºC annealing temperature, which is more adequate for Enterobacteriaceae (Versalovic et al., 1991).

We resolved the PCR products by electrophoresis in 2% agarose gel in 1x tris-acetate-electrophoresis (TAE) buffer at 60V for 3h.

*Fla*A-RFLP

We performed *fla*A-RFLP of *C. coli* isolates according to the CAMPYNET protocol (Harrington et al., 2003). For amplification of the *fla*A gene we used the forward A1 (5'-GGATTTCGTATTAACACAAATGGTGC-3') and reverse A2 (5'-CTGTAGTAATCTTAAAACATTTTG-3') primers (Nachamkin et al., 1993). The amplified PCR product (1.7 kb) was digested with the restriction enzyme *Dde*I (*Hyp*F3I, FastDigest®, Thermo Fisher Scientific, Waltham, MA, USA), and separated by electrophoresis in 2.5% agarose gel in 1x TAE buffer at 90V for 3h.

PFGE

We followed the standard operating protocol from PulseNet (www.pulsenetinternational.org/protocols/pfge/) for PFGE typing of the selected *Campylobacter* isolates (n = 82 *C. lanienae,* n = 31 *C. coli* and n = 1 *C. hyointestinalis*). We digested the genomic DNA with SmaI and KpnI restriction enzymes (Roche Applied Science, Indianapolis, IN, USA). Electrophoresis was performed in a CHEF-DR III System (Bio-Rad, Hercules, CA, USA).

We analysed PFGE band patterns with Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA), as previously described (Moré et al., 2017). We obtained a dendrogram for each restriction enzyme used, and another dendrogram combining SmaI and KpnI enzymes. We calculated similarity matrices by using the Dice coefficient with tolerance and optimization values of 1.0%. We then constructed dendrograms using an unweighted-pair group method with arithmetic mean (UPGMA). We considered PFGE band patterns with a similarity ≥ 90% to be the same pulsotype (PFGE type) and named them according to the restriction enzyme used.

MLST

Based on PFGE results, we selected 29 isolates (n = 7 *C. lanienae,* n = 21 *C. coli* and n = 1 *C. hyointestinalis*) for MLST typing, which was performed according to Miller et al. (2012) for *C. lanienae* and *C. hyointestinalis*. For *C. coli* isolates, we followed the procedure and primers reported by Miller et al. (2005) and, when no amplicon was obtained, we used the primers reported by Korczak et al. (2009). Primer sets are shown in supplementary files 1 and 2. Sanger sequencing of the PCR purified products was performed by Geneservice Source BioScience (Nottingham, United Kingdom).

We used the Fingerprinting II v3.0 software to edit and analyse the Sanger sequencing results and assigned alleles and sequence types (STs) based on the MLST scheme provided on the *Campylobacter* PubMLST database (http://pubmlst.org/campylobacter). We also searched the most frequent source (human stool, chicken or pig, among others) of the *Campylobacter* STs found in this study, already reported in the PubMLST database.

Also, to assess the phylogenetic relationship among *C. lanienae* and other *Campylobacter* isolates, we constructed two maximum likelihood trees based on the seven concatenated MLST loci, using the MEGA X software (Kumar et al., 2018). The first tree contained our isolates only, and the results obtained (the divergence of one *C. lanienae* isolate) led us to build a second maximum likelihood tree (not shown) to further address the phylogenetic relationship among our *C. lanienae* isolates and a selection of isolates from different *Campylobacter* species available in the PubMLST database. This second maximum likelihood tree contained our *C. lanienae* isolates and all the *C. hyointestinalis, C. fetus*, *C. insulaenigrae*, *C. helveticus* isolates, and a selection of *C. lanienae* isolates available in the PubMLST.

277 *2.5. Virulence-associated genes of Campylobacter*

278 In total, we tested 7 *C. lanienae,* 21 *C. coli* and one *C. hyointestinalis* isolates by PCR
279 for the presence of 14 genes encoding putative virulence factors. These included
280 genes related to motility (*fla*A and *fla*B), adhesion and colonization (*cad*F, *dna*J, *rac*R,
281 *pld*A, *vir*B11), invasion (*ceu*E, *cia*B), cytotoxin production (*cdt*A, *cdt*B, *cdt*C and *wla*N)
282 and the type VI secretion system (T6SS) (Bolton, 2015; Dasti et al., 2010). Primer sets
283 and corresponding annealing temperatures are indicated in supplementary file 3.

284

285 *2.6. Antimicrobial susceptibility testing of Campylobacter and Salmonella*

286 We tested the antimicrobial susceptibility of one *Campylobacter* isolate per pulsotype
287 and area (n = 6 *C. lanienae*, n = 17 *C. coli* isolates, and n = 1 *C. hyointestinalis*) and
288 one *Salmonella* isolate per ERIC-PCR profile (n = 4). We used a minimum inhibitory
289 concentration (MIC)-based broth microdilution (plate EUCAMP2 for *Campylobacter*,
290 Sensititre®, ThermoFisher Scientific, Spain; plate VetMIC GN-mo for *Salmonella*,
291 National Veterinary Institute, Uppsala, Sweden).

292

293 For *Campylobacter* isolates, antimicrobials tested included: erythromycin (1-128
294 mg/L), nalidixic acid (1-64 mg/L), ciprofloxacin (0.12-16 mg/L), tetracycline (0.5-64
295 mg/L), streptomycin (0.25-16 mg/L) and gentamicin (0.12-16 mg/L). We used *C. jejuni*
296 ATCC 33560 as control strain. Plates were incubated at 37ºC for 48h.

297

298 For *Salmonella* isolates, the antimicrobials tested were: ampicillin (1-128 mg/L),
299 cefotaxime (0.016-2 mg/L), ceftazidime (0.25-16 mg/L), ciprofloxacin (0.008-1 mg/L),
300 nalidixic acid (1-128 mg/L), gentamicin (0.12-16 mg/L), streptomycin (2-256 mg/L),
301 kanamycin (8-16 mg/L), tetracycline (1-128 mg/L), florfenicol (4-32 mg/L),
302 chloramphenicol (2-64 mg/L), colistin (0.5-4 mg/L), sulfamethoxazole (8-1024 mg/L)
303 and trimethoprim (0.12-16 mg/L). We used *E. coli* ATCC 25922 as control strain. Plates
304 were incubated at 37ºC for 24h.

An isolate was considered multidrug-resistant (MDR) when showing resistance to three or more unrelated antimicrobials. We designated each isolate as wild type (susceptible) or non-wild type (non-susceptible) based on the epidemiological cut-off values determined by EUCAST (https://mic.eucast.org/Eucast2/SearchController/search.jsp?action=init). These breakpoints were not available for *C. lanienae* or *C. hyointestinalis*, nor for three antimicrobial agents in *Salmonella* (kanamycin, colistin and sulphametoxazole). In these cases, we used the cut-off values from *C. coli* (for the *Campylobacter* species) and those from *Escherichia coli* (for *Salmonella*).

2.7. Statistical analyses

To compare the overall prevalence of *C. lanienae*, *C. coli, C. hyointestinalis* and the two types of co-infections (*C. lanienae* and *C. coli*, or *C. lanienae* and *C. hyointestinalis*), we used a 4-sample test for equality of proportions (Crawley, 2007), including post-hoc pairwise comparisons with the holm adjustment method.

In addition, we assessed the influence of the degree of urbanization (given that the three study areas have different landscape composition and uses by wild boar and people) on the different response variables explained below. For these analyses, Collserola was associated with low, UAB with medium, and Barcelona with high degree of urbanization.

To assess the influence of the degree of urbanization on the different *Campylobacter* prevalences (overall *Campylobacter*, *C. lanienae* and *C. coli*), we fitted logistic regression models including the isolation (positive/negative) of *Campylobacter* (any species), of *C. lanienae* and of *C. coli* as response variable, and the degree of urbanization (low, medium or high) as explanatory variable. We used the glm function

in R (stats package; R Core Team, 2020b) to fit these models (one null model and one model with the urbanization variable for each response variable), with binomial family and logit link function. We selected the model with the lowest Akaike's Information Criterion (AIC) as the best one, and gave consideration to the second model if the difference in their AIC values was lower than two points (Burnham & Anderson, 2002).

To assess the degree of urbanization on the number of virulence-associated genes detected and the number of antimicrobial agents to which the *Campylobacter* isolates showed resistance, we used the non-parametric Kruskal-Wallis test (Crawley, 2007).

We used the R software (Version 3.6.3; R Core Team, 2020a) for all statistical analyses. For 95% confidence interval (CI) calculation, we used the binconf function from the Hmisc package (Harrel Jr, 2018). In all comparisons, we considered a result as statistically significant at *P* values below 0.05.

3. Results

3.1. Campylobacter and Salmonella prevalence and the influence of the degree of urbanization

We isolated *Campylobacter* from 79 out of the 130 wild boars sampled (60.8% CI: 52.2 – 68.7, see Table 1). Overall, the prevalence was 46.2% for *C. lanienae* (95% CI: 37.8 – 54.7%), 16.2% (95% CI: 11.4 – 24.3%) for *C. coli*, and 0.8% (95% CI: 0 – 4.2%) for *C. hyointestinalis*. Prevalence and confidence intervals considering the wild boars co-infected with two different *Campylobacter* species are shown in Table 1. Wild boars carrying only *C. lanienae* were more frequent than those carrying only *C. coli* (X = 106.6, df = 3, $P < .001$), and co-infections (either with *C. coli* and *C. lanienae*, or with *C. lanienae* and *C. hyointestinalis*) were the least frequent (Table 1). *C. jejuni* was not detected. The single *C. hyointestinalis* isolated was initially identified by PCR as *C.*

361 *lanienae*, but it was reclassified as *C. hyointestinalis* when performing the MLST
362 genotyping analyses (see below).
363
364 Regarding the effect of the degree of urbanization on the carriage of
365 *Campylobacter* spp. by wild boars, the logistic regression models (see model selection
366 in supplementary file 4) showed that *C. lanienae* was more frequent in the wild boars
367 sampled in the less urbanized area than in the most urbanized one (estimate = 1.60*, Z*
368 = 3.14, $P < 0.05$; see Table 1), but there were no differences between these areas and
369 the one with medium degree of urbanization (medium *vs.* low urbanization: estimate =
370 0.71*, Z* = 1.49, $P > 0.05$; medium *vs.* high urbanization: estimate = 0.89*, Z* = 1.45, $P >$
371 0.05). On the contrary, the models did not show a relationship between *C. coli* or
372 *Campylobacter* (overall) and the degree of urbanization.
373
374 We isolated *Salmonella enterica* subsp. *enterica* from four out of the 130 wild
375 boars (3.1%, 95% CI: 1.2 – 7.6%), three from Collserola and one from Barcelona.
376 Through serotyping of these isolates, we identified one monophasic *Salmonella*
377 Typhimurium, one *Salmonella* Bardo, and one non-typeable *Salmonella* serovar in
378 three wild boars from Collserola, and one *Salmonella* Enteritidis in a wild boar from
379 Barcelona.
380
381 *3.2. Genetic diversity of Campylobacter and Salmonella*
382 ERIC-PCR and *fla*A-RFLP
383 ERIC-PCR typing of 186 *C. lanienae* isolates (obtained from 60 wild boars) revealed 81
384 *C. lanienae* different profiles. Typing of 70 *C. coli* isolates (from 21 wild boars) by *fla*A-
385 RFLP revealed 27 different profiles. Most wild boars carried a single *Campylobacter*
386 genotype (*C. lanienae* and/or *C. coli* profile), different from those carried by other
387 individuals.

Typing of 16 *Salmonella* isolates (from four wild boars) by ERIC-PCR revealed three different profiles, with each wild boar carrying a single genotype. Each different profile corresponded to one of the abovementioned serovars. The four non-typeable isolates from one wild boar host corresponded to the serovar Bardo.

Some *Campylobacter* and *Salmonella* isolates were not typeable with these methods (supplementary file 5).

PFGE of *Campylobacter*

Among the 82 *C. lanienae* isolates analysed, we obtained 22 different PFGE pulsotypes with the restriction enzyme SmaI and eight with the restriction enzyme KpnI. Among the 31 *C. coli* isolates analysed, we obtained 15 different pulsotypes with SmaI and 14 with KpnI. We obtained one pulsotype with each enzyme for the single *C. hyointestinalis* isolate. With both restriction enzymes combined, PFGE typing revealed 22 different pulsotypes, five for *C. lanienae*, 16 for *C. coli* and one for *C. hyointestinalis* (Figure 2). However, as happened with previous typing techniques, not all isolates were typeable by PFGE (see supplementary file 5).

The combined dendrogram of SmaI and KpnI PFGE profiles grouped the *C. coli* and *C. lanienae* isolates separately (Figure 2). The *C. hyointestinalis* and all but one *C. lanienae* isolates were grouped in one cluster with a 63.1% similarity. Within this group, the *C. hyointestinalis* strain clustered with a single *C. lanienae* strain (< 68% similarity). Similarly, all but one *C. coli* isolates were grouped in one cluster with a 62.6% similarity. The remaining two isolates (a *C. lanienae* and a *C. coli*), were grouped separately with a similarity of 57.9% (Figure 2).

Twenty-one out of 29 (72.4%) wild boars carried a single *Campylobacter* species and pulsotype. Only one individual carried two different *Campylobacter* species

and pulsotypes (one *C. hyointestinalis* and one *C. lanienae*, SK17 and SK19, respectively), and three others carried two different *C. coli* pulsotypes each (SK8 and SK14; SK9 and SK11; and SK11 and SK12). Despite this high genetic diversity, we found two pulsotypes in wild boars from different study areas (SK13 in Collserola and UAB, and SK16 in Collserola and Barcelona; Figure 2).

MLST *of Campylobacter*

For *C. lanienae*, all but four alleles from two different genes (uncA 7, uncA 12, glnA 12, glyA 21) were novel, whilst all the alleles were novel for *C. hyointestinalis*. Therefore, all *C. lanienae* and *C. hyointestinalis* isolates belonged to non-previously described STs. For *C. coli*, we obtained the complete MLST profile and therefore the corresponding ST for 19 out of the 21 *C. coli* isolates (Table 2).

Overall, we found 12 different *C. coli* STs, four of which were new (Table 2, Figure 3). These novel STs consisted of new allele combinations (in the case of ST 9236, ST 9237, and ST 9238) or new allele sequences (ST 9235, new allele uncA 588). All the *C. coli* isolates with existing ST and the novel ST 9236 and ST 9238 belonged to the same clonal complex (CC 828). The most frequent ST among *C. coli* isolates was ST 854, which we found in five individuals from all three study areas. However, most STs belonged to single isolates and were distributed heterogeneously among areas (Figure 2). For *C. coli*, when comparing the PFGE and MLST techniques, we obtained a higher number of pulsotypes (16) than STs (12) (Figure 2 and Table 2). We found different pulsotypes grouped as the same ST (e.g. ST 827 in SK13 and SK14) and vice versa (e.g. SK1 in ST 2814 and ST 9237).

According to the information available in the PubMLST database for *C. coli*, most of the STs found in the present study had been previously isolated from a wide

range of domestic animals worldwide such as sheep, cattle or pigs, but most frequently from chickens, and all of them had been isolated from human stools (Table 3).

The first maximum likelihood tree we constructed (with the *C. lanienae* and *C. hyointestinalis* isolates) showed that three *C. lanienae* isolates were identical (SS15069-C2, SS15127-C1 and SS15190-C1), while two other isolates (SS15102-C1 and SS15132-C2, also from different areas) were closely related and differed only in six nucleotides (Figure 3). The isolate SS15102-C4 (the *C. hyointestinalis* isolate initially misidentified as *C. lanienae*) was the most divergent (Figure 3). The second maximum likelihood tree we constructed that included different *Campylobacter* species grouped all our isolates in a *C. lanienae* cluster except for SS15102-C4, which clustered with the *C. hyointestinalis* isolates from the PubMLST database. This isolate was then identified as *C. hyointestinalis* by PCR as abovementioned.

3.3. Virulence-associated genes of Campylobacter

We tested the presence of 14 virulence-associated genes in those same *Campylobacter* isolates analysed by MLST. One *C. coli* isolate from Collserola (low urbanization) and another one from UAB (medium degree of urbanization) showed the highest number of virulence determinants (12 in total). Six other isolates, five *C. coli* and one *C. lanienae*, carried between 10 and 11 virulence determinants. Some isolates from different areas showed the same virulotype, mainly *C. coli* (Figure 2). The Kruskal-Wallis test indicated that there were no significant differences among the different degrees of urbanization regarding the number of virulence-associated genes detected in the *Campylobacter* isolates (Kruskal-Wallis chi-squared = 2.4, df = 2, $P > 0.05$).

The results on the frequency of virulence-associated genes detected in the analysed *Campylobacter* isolates are summarized in Table 4 and Figure 2. The single

471 virulence-associated gene that was not detected in any of the *Campylobacter* isolates
472 was *wla*N.
473
474 *3.4. Antimicrobial susceptibility of Campylobacter and Salmonella*
475 All the *Campylobacter* isolates tested were resistant to at least one antimicrobial agent
476 (Figure 2). However, we could not determine the antimicrobial susceptibility of one *C.*
477 *lanienae* and two *C. coli* isolates that poorly grew in the culture medium. While the *C.*
478 *lanienae* isolates tested (5/5) were resistant to nalidixic acid only, most of the *C. coli*
479 isolates showed resistance to quinolones (nalidixic acid and ciprofloxacin, 14/15),
480 tetracycline (13/15) or both (13/15), and 10/15 (66.67%) were MDR. Regarding the
481 influence of the degree of urbanization, we did not find significant differences on the
482 number antimicrobial agents to which the *Campylobacter* isolates showed resistance
483 (Kruskal-Wallis chi-squared = 5, df = 2, p-value > 5).
484 Regarding *Salmonella*, three out of the four isolates tested (*Salmonella*
485 Enteritidis, *Salmonella* Bardo and one not typeable *Salmonella*, from Barcelona and
486 Collserola) were pansusceptible, whereas the fourth isolate (monophasic *Salmonella*
487 Typhimurium, from Collserola) was MDR (ampicillin (A), streptomycin (S),
488 sulphametoxazole (Su) and tetracycline (T); ASSuT resistant profile).
489
490 **4. Discussion**
491 In the present study, we report the carriage by wild boars of the two most
492 relevant zoonotic bacteria in Europe (EFSA and ECDC, 2021). The overall
493 *Campylobacter* prevalence found here (over 60%) agrees with previous studies
494 reporting high *Campylobacter* prevalence in wild boar faecal samples in Spain and Italy
495 (66%, 95% CI: 60-71, Díaz-Sánchez et al., 2013; 83%, 95% CI: 74-93, Marotta et al.
496 2020). However, compared with previous reports we found a higher prevalence of *C.*
497 *lanienae* (46%) in wild boars, and detected *C. hyointestinalis* for the first time in wildlife
498 in Spain. These two *Campylobacter* species also have zoonotic potential, although they

499 are less frequent in literature than *C. coli* and *C. jejuni* (Jay-Russell et al., 2012; Man,
500 2011), probably because many *Campylobacter* species are uncommon and not all the
501 isolates are identified at the species level (Díaz-Sánchez et al. 2013, Navarro-
502 González et al. 2013). With regards to *Salmonella*, the low prevalence found in wild
503 boars from this study suggests that the wild boar is not a reservoir of this species in our
504 study area. Out of the three serovars identified in wild boars, the serovars Enteritidis
505 and monophasic Typhimurium are particularly relevant for public health (EFSA and
506 ECDC, 2021), especially the latter, which showed a resistance profile (ASSuT) of a
507 clone that has emerged in several European countries (Dionisi et al., 2009; Lucarelli et
508 al., 2010; Mossong et al., 2007).
509
510 *Campylobacter*
511 Overall, *C. lanienae* was more frequently isolated from wild boars than *C. coli*
512 and *C. hyointestinalis*. This species is predominantly found in wild boars, domestic pigs
513 and feral swine, although it has also been reported in other domestic animals (cattle,
514 sheep) (Guévremont et al., 2008; Jay-Russell et al., 2012; Navarro-González et al.,
515 2014; Oporto and Hurtado, 2011). The *C. lanienae* prevalence found in this study
516 (46%) is, to our knowledge, the highest reported to date in wild boars (27%, 95% CI:
517 19-35, Spain, Carbonero et al., 2014; 10%, 95% CI: 6-16, Spain, Navarro-González et
518 al., 2014; 1%, 95% CI: 0-17, Japan, Tomino et al., 2020). This high prevalence is
519 relevant, since this species causes human diarrhoeal disease (Lévesque et al., 2016;
520 Logan et al., 2000). Regarding *C. coli*, the prevalence found here (16%) is among
521 previously reported prevalence in wild boars or feral pigs: up to 6% in Spain, Germany
522 or Texas (< 1%, 95% CI: 0-4, Atanassova et al., 2008; 6%, 95% CI: 2-11, Carbonero et
523 al., 2014; 2%, 95% CI: 0-3, Cummings et al., 2018; 2%, 95% CI: 0-10, Navarro-
524 González et al., 2014; 5%, 95% CI: 1-17, Navarro-González et al., 2013), 46.9% (95%
525 CI: 43-51) in the Czech Republic (Hulankova et al., 2019) and 76.7% (95% CI: 66-87)
526 in Italy (Marotta et al. 2020). The finding of *C. coli* in wild boars from a highly populated

area such as the MAB is of public health concern, since *C. coli* is relevant in veterinary and human medicine (Horrocks et al., 2009) and it is the second most common causative agent of campylobacteriosis in humans (EFSA and ECDC, 2021). In addition, we found *C. hyointestinalis* for the first time in wild animals in Spain, but it has previously been detected in farm animals (cattle, sheep and swine) in this country (Oporto and Hurtado, 2011). This emerging *Campylobacter* species (Man, 2011) is mostly isolated from healthy cattle and humans presenting gastroenteritis, the main reservoirs are probably cattle and pigs (Wilkinson et al., 2018), and has been reported before in wild boars and feral swine (Jay-Russell et al., 2012; Sasaki et al., 2013; Tomino et al., 2020). To the best of our knowledge, this is the first report of *C. lanienae* and *C. hyointestinalis* in wild animals from urbanized areas.

Regarding the influence of the degree of urbanization on the carriage of *Campylobacter* by wild boars, we only found a relationship between urbanization and one of the *Campylobacter* species, *C. lanienae* (more frequently isolated from wild boars in the area with low urbanization). This could suggest that *C. lanienae* infection may be related to a diet based on natural components (and not human-derived, Castillo-Contreras et al. 2021b). Other variables not directly evaluated here (e.g., infection sources, places where wild boars aggregate, or wild boar density; Carbonero et al., 2014; Castillo et al. 2011) could also enhance the infection risk by *C. lanienae* for wild boars in Collserola. The lack of effects of the degree of urbanization on the overall carriage of *Campylobacter* or *C. coli* could be due to insufficient sample size, or to the sampling scheme of our study, which could have limited the ability of the statistical models to detect any patterns. Despite these limitations, an anthropogenic source of *C. coli* infection (e.g. human-derived food resources including rubbish; Castillo-Contreras et al., 2021b) would agree with the *C. coli* prevalence observed in the two most urbanized areas (doubling the *C. coli* prevalence found in the less urbanized area). The

554 monitoring of wild boar movements prior to sample collection could help in identifying
555 possible sources of infection.
556
557 Based on the results with the different genotyping techniques used, we
558 observed a high genetic diversity among our *Campylobacter* isolates. This genetic
559 diversity was expected and might be attributable to the presence of different sources of
560 infection, but mainly to the frequent genetic rearrangements that occur in the
561 *Campylobacter* genome (de Boer et al., 2002; Wassenaar et al., 1998). Moreover, we
562 detected the same *C. coli* and *C. lanienae* genotypes in different study areas,
563 suggesting that these strains might be circulating among areas. This might be a
564 consequence of wild boar movements (Jerina et al., 2014; Podgórski et al., 2014), as
565 well as the existence of a common source of infection including other wildlife species
566 (Jones, 2001).
567
568 As for the *Fla*A-RFLP, the lack of amplification of the *fla*A gene in the case of *C.*
569 *lanienae* and *C. hyointestinalis* isolates could be due to the absence of the flagellar
570 gene *fla*A in these isolates, or to a lack of recognition of the target region by the
571 primers used. As for PFGE, the restriction enzyme SmaI was more suitable than KpnI
572 for typing *C. coli* and *C. lanienae*, which is opposite to previous reports (Kérouanton et
573 al., 2015; Schweitzer et al., 2011). However, in those studies less isolates were typed
574 and results might be less representative. In our case, the worse performance of
575 restriction enzymes with *C. lanienae* isolates might be related to the lack of restriction
576 sites, similarly to what has been reported previously for some *C. jejuni* isolates
577 (Oyarzabal et al., 2008).
578
579 As for the MLST, we found no coincidences between the *C. lanienae* and *C.*
580 *hyointestinalis* MLST allele profiles from wild boars and those published in the
581 PubMLST database, probably due to the few STs available in this database (172 for *C.*

582 *lanienae* and 135 for *C. hyointestinalis*), compared with that of *C. coli* and *C. jejuni*
583 (over 11,300 STs). According to the information available in the PubMLST database,
584 besides the novel *C. coli* STs, all other STs obtained in our study have also been
585 isolated from human stools, mainly in Europe (Table 3). Five of the STs found in this
586 study (ST 827, ST 828, ST 854, ST 2097, and ST 3020) have been reported in Spain
587 (in human stools, environmental waters, or livestock), and two STs (ST 827 and ST
588 854) have been previously found in our study region, Catalonia (ST 854 in pig slurry,
589 ST 827 in a human clinical sample from the Vall d'Hebron Hospital in Barcelona, and
590 both STs also in sewage waters). In addition, we found four new *C. coli* STs, two of
591 which (ST 9237 and ST 9238) have been reported in 2019 in the USA. Our findings,
592 together with previous studies, highlight the possible role that wild boar may play in the
593 spread of *Campylobacter* and in human infection.
594
595 In relation to the virulence potential of the *Campylobacter* isolates, determining
596 the presence of genes encoding for putative virulence factors is a way to address their
597 potential to cause disease in people (Bang et al., 2003; Datta et al., 2003). The high
598 virulence potential found in our isolates (mainly *C. coli*) from all study areas, together
599 with the detection of certain virulotypes (mainly from *C. coli*) in different areas, also
600 agree with a possible circulation of strains among areas. The motility gene fla*A*,
601 detected only in the *C. coli* isolates, is probably the one responsible of *Campylobacter*
602 motility and intestine colonization (Koolman et al. 20515; Wassenaar et al. 1991).
603 Genes involved in adhesion (*cad*F, *rac*R, *dna*J; Brás et al., 1999; Ziprin et al., 2001)
604 and invasion (*ceu*E, *vir*B11, *cia*B and *pld*A; Bacon et al., 2000; Bang et al., 2003;
605 Konkel et al., 1999; Ziprin et al., 2001) were less conserved than other virulence genes,
606 and presented a heterogeneous distribution within the *Campylobacter* isolates, as
607 previously reported in *C. jejuni* (Datta et al., 2003). In contrast to other studies, the
608 prevalence of *vir*B11 in *C. coli* isolates (61.9%) was high (33.3%, Koolman et al.,
609 2015). Notably, the gene *hcp*, which is associated with severe forms of

610 campylobacteriosis (Bleumink-Pluym et al., 2013), was found in all but one of the
611 *Campylobacter* isolates analysed. Whilst this is the first report of *hcp* in both *C.*
612 *lanienae* and *C. hyointestinalis*, high prevalence among *C. coli* isolates has previously
613 been reported (Corcionivoschi et al., 2015). The full set of cytolethal distending toxin
614 genes (*cdt*ABC), suspected to be necessary to produce the toxin (Bang et al., 2003),
615 was highly prevalent among *C. coli* isolates (61.9%), similarly to previous reports
616 (Koolman et al., 2015). This suggests that most *C. coli* carried by wild boars can
617 potentially produce the toxin. Finally, the absence of the *wla*N gene, involved in the
618 Guillain-Barré syndrome, agrees with previous studies (Datta et al., 2003; Koolman et
619 al., 2015). The lower PCR detection of virulence factors in *C. lanienae* and *C.*
620 *hyointestinalis* than in *C. coli* could be explained by significant differences between the
621 alleles among the three *Campylobacter* species, since the primers were designed for
622 *C. jejuni* and *C. coli*.
623
624 With regards to the antimicrobial susceptibility of the *Campylobacter* isolates,
625 none of the isolates tested were pansusceptible. However, the *C. lanienae* isolates
626 were only resistant to nalidixic acid, similarly to previous studies in *C. lanienae* isolated
627 from domestic pigs (Schweitzer et al., 2011). The *C. hyointestinalis* isolate was
628 resistant to nalidixic acid and streptomycin, a phenotype previously reported in *C.*
629 *hyointestinalis* isolates from wild boars in Japan (Sasaki et al. 2013). More diverse
630 AMR profiles were found in *C. coli* isolates including nearly 67% MDR isolates. In
631 accordance with our findings, a higher frequency of *C. coli* isolates resistant to
632 ciprofloxacin, streptomycin and tetracycline, and a lower frequency of erythromycin-
633 resistant isolates have been reported in wild artiodactyls (Carbonero et al., 2014). The
634 resistance to ciprofloxacin, detected in all but one of the *C. coli* isolates from wild
635 boars, is especially relevant since it is one of the two antimicrobials regarded as
636 critically important for the treatment of human campylobacteriosis (EFSA and ECDC,
637 2020). Both the AMR profiles found in this study (particularly in *C. coli*) and the location

of the MDR isolates (mainly in the areas with high or medium degree of urbanization) points to an anthropogenic origin of these strains, as suggested in previous studies for AMR *E. coli* (Dolejska et al., 2016; Mukerji et al., 2019). However, we could not prove a relationship between the number of AMR bacteria in *Campylobacter* and the degree of urbanization, probably because of our small sample size. In addition, wild boar movements (Jerina et al., 2014; Podgórski et al., 2014) might also explain the lack of differences among areas.

Salmonella

With regards to *Salmonella*, the low prevalence found in wild boars from this study (3%) suggests that the wild boar is not a reservoir of this species in our study area. This prevalence is in accordance with previous reports in wild boar faeces in Spain, Czech Republic and Japan (0.4%, 95% CI: 0-1, Hulankova et al., 2019; 2.9%, 95% CI: 2-4, Molino et al., 2019; 5%, 95% CI: 1-17, Navarro-González et al., 2013; 7.4%, 95% CI: 3-12, Sasaki et al., 2013), but in contrast to others reporting prevalences over 20% (95% CI: 13-31, Portugal) and up to 36% (95% CI: 28-44, Spain) (Navarro-González et al., 2012; Vieira-Pinto et al., 2011). The three serovars identified here (Enteritidis, monophasic Typhimurium and Bardo) have been isolated from game animals in Europe, including wild boars (Díaz-Sánchez et al., 2013; Molino et al., 2019; Paulsen et al., 2012; Sannö et al., 2018; Zottola et al., 2013). From a public health perspective, *Salmonella* serovars Enteritidis and monophasic Typhimurium are particularly relevant, since they were the first and third most reported serovars in human cases in the EU during 2018 (EFSA and ECDC, 2021). Despite uncommon, *Salmonella* Bardo also causes disease to humans (Schmid and Baumgartner, 2013).

Regarding the antimicrobial susceptibility of the *Salmonella* isolates, one of them (serovar monophasic Typhimurium, from a hunted wild boar in Collserola) was

MDR, with the typical ASSuT resistance profile of the clone that has emerged in several European countries (Dionisi et al., 2009; Lucarelli et al., 2010; Mossong et al., 2007). Pigs are the likely reservoir of infection with the ASSuT resistant serovar for humans (Hopkins et al., 2010), and this resistant profile has been also reported in wild birds from a rehabilitation centre very close to our study areas (Molina-López et al., 2015).

4.5 Public health risk implications

Wild boars infected with zoonotic *Campylobacter* species and *Salmonella* serovars are a potential public health threat since wild boar populations are expanding and increasing their presence in urban areas (Licoppe et al., 2013; Massei et al., 2015). Although the overall influence of wild boars on human campylobacteriosis and salmonellosis may be small, hunters can be at higher risk of exposure when handling wild boar carcasses and through the consumption of contaminated meat (Brown et al., 2018; Ruiz-Fons, 2017). However, people other than hunters could be also exposed, since wild boars can contaminate the urban environment when feeding or defecating in public spaces, bringing or spreading zoonotic pathogens into cities (Fernández-Aguilar et al., 2018; Jansen et al., 2007; Wang et al., 2019). Disease transmission could also occur in the wild boar habitat, where people practise outdoor activities, provided that they come into contact with a contaminated water or food source (Bradley and Altizer, 2007; Ruiz-Fons, 2017; Stuart et al. 2010). This health risk increases when the zoonotic agents show a particular potential of virulence and/or are MDR, as is the case of several isolates found in this study.

Moreover, we suggest that wild boars might have become infected with *Campylobacter* and *Salmonella* strains from an anthropogenic source (human waste or animal products, reflecting environmental contamination), given that wild boars exploit anthropogenic food resources - including rubbish - in urbanized areas (Castillo-

694 Contreras et al., 2021b; Hafeez et al., 2011). This is further supported by the
695 characteristics of the isolates recovered, since 1) all the *C. coli* STs identified from wild
696 boars in the present study have been previously isolated from human stools; 2) two of
697 the *Salmonella* serovars detected are among the most reported in human infections in
698 the EU; and 3) the AMR profiles of *C. coli* and monophasic *S*. Typhimurium agree with
699 those most frequently found in people from Spain and Europe, respectively. Wild boars
700 can act as carriers and spread these bacteria and their antimicrobial resistance, which
701 have great impact on human health (EFSA and ECDC, 2021, 2019; Torres et al., 2020;
702 Vittecoq et al., 2016).
703
704 To control campylobacteriosis and salmonellosis, it is necessary to better
705 understand their global epidemiology, their reservoirs and the pathogenicity of the
706 different strains. Since health management is far more difficult to perform in wildlife
707 than in domestic animals (Mentaberre et al., 2013), special emphasis should be made
708 on wildlife species, in light of their potential role as reservoir for *Campylobacter* and
709 *Salmonella.* Moreover, our research highlights the need to consider a One Health
710 approach that encompasses the complexity of zoonotic bacteria and antimicrobial
711 resistance in order to prevent and mitigate public health risks.
712
713 **Conclusions**
714 Our results provide further evidence of the role of wild boars as a contributing host for
715 the maintenance of zoonotic *Campylobacter* species. We detected a high genetic
716 diversity of *C. lanienae* and *C. coli* isolates, and the same circulating clones in wild
717 boars from areas with different degrees of urbanization. In addition, we report the
718 isolation of *C. hyointestinalis* from wildlife for the first time in Spain. All the *C. coli* STs
719 identified in wild boars had previously been reported in clinical human cases, with
720 some isolates showing a high virulence potential, and all the *Campylobacter* isolates
721 being antimicrobial resistant or multidrug resistant. We also report the presence of two

722 zoonotic *Salmonella* serovars of clinical importance to humans, one of these being the
723 recently emerged and widely spread MDR monophasic *S.* Typhimurium. Wild boars
724 infected with *Campylobacter* or *Salmonella* are a potential public health threat since
725 wild boar populations are expanding worldwide and increasing their presence in
726 urbanized areas. Hence, wild boars could also be used as indicators of antimicrobial
727 resistance contamination in the environment.
728
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743
744 **Conflict of interest statement**
745 The authors declare they have no conflicts of interest.

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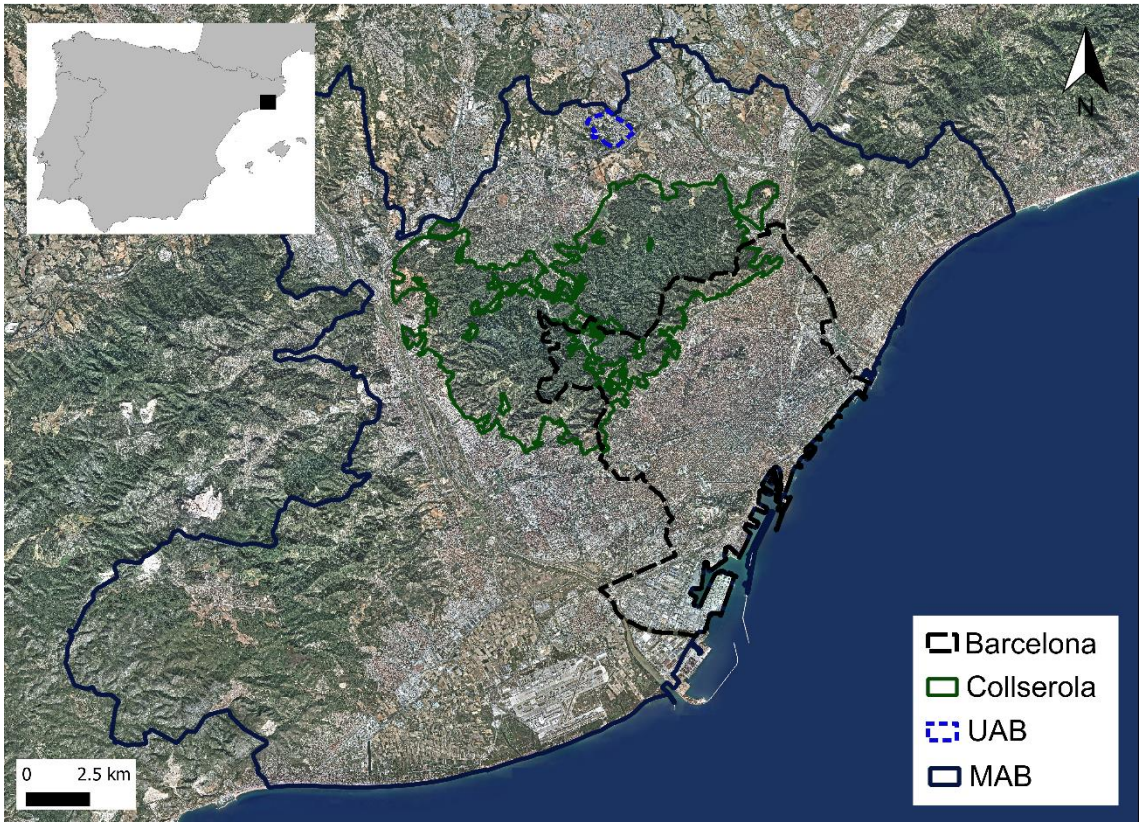


Figure 1. Study areas. Barcelona municipality (Barcelona), Serra de Collserola Natural Park (Collserola), and the Campus of the Universitat Autònoma de Barcelona (UAB), in the Metropolitan Area of Barcelona (MAB). Orthophoto from Institut Cartogràfic i Geològic de Catalunya (ICGC).

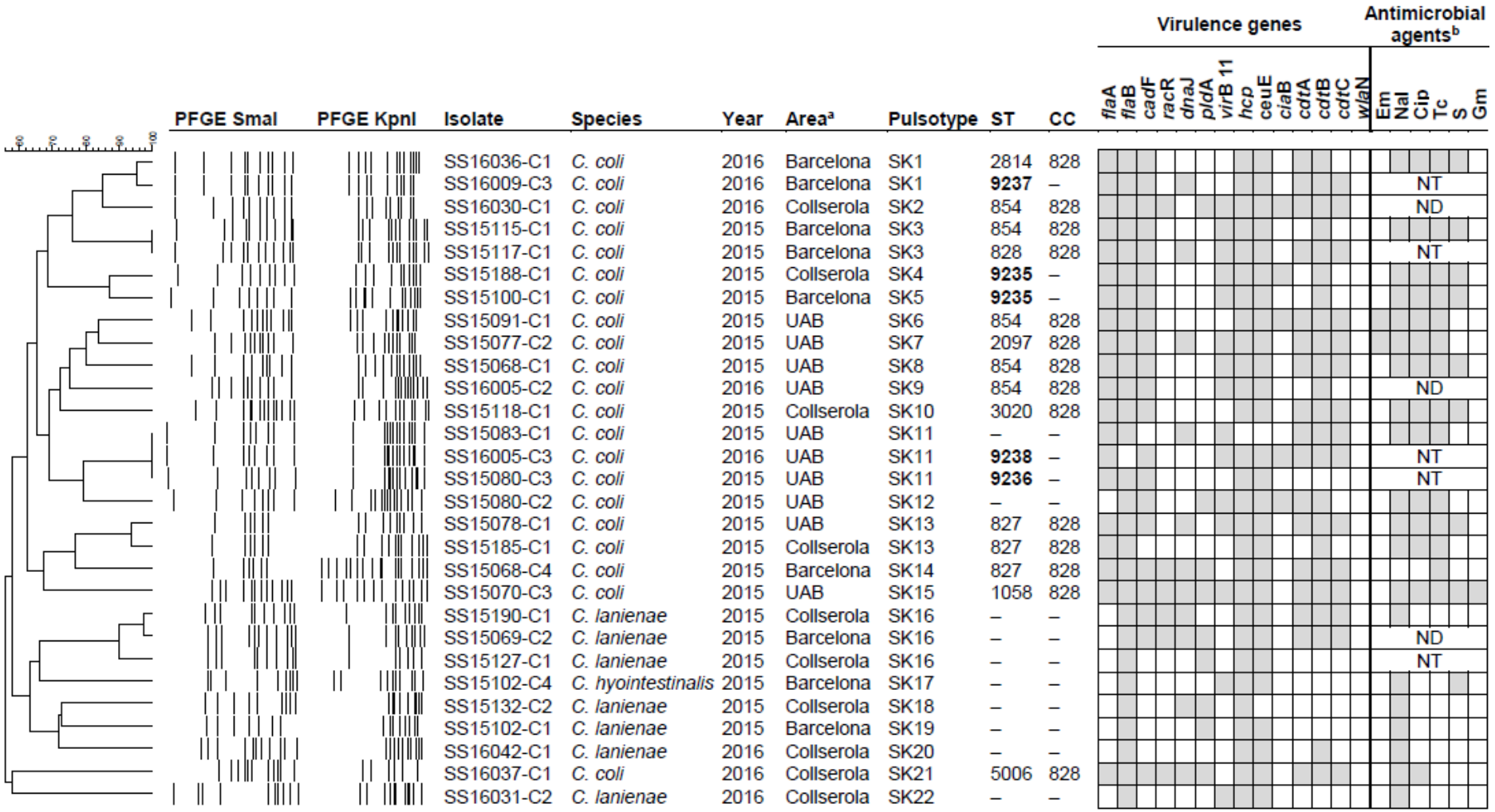
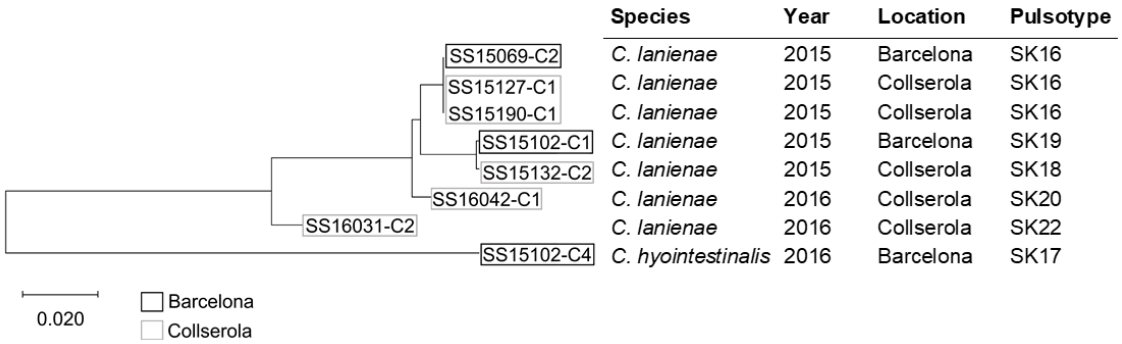

Virulence genes
Antimicrobial agents[b]
PFGE SmaI
PFGE KpnI
Isolate
Species
Year
Area[a]
Pulsotype
ST
CC
flaA flaB cadF racR dnaJ pldA virB 11 hcp ceuE ciaB cdtA cdtB cdtC wlaN
Em Nal Cip Tc S Gm
SS16036-C1 C. coli 2016 Barcelona SK1 2814 828
SS16009-C3 C. coli 2016 Barcelona SK1 9237 – NT
SS16030-C1 C. coli 2016 Collserola SK2 854 828 ND
SS15115-C1 C. coli 2015 Barcelona SK3 854 828
SS15117-C1 C. coli 2015 Barcelona SK3 828 828 NT
SS15188-C1 C. coli 2015 Collserola SK4 9235 –
SS15100-C1 C. coli 2015 Barcelona SK5 9235 –
SS15091-C1 C. coli 2015 UAB SK6 854 828
SS15077-C2 C. coli 2015 UAB SK7 2097 828
SS15068-C1 C. coli 2015 UAB SK8 854 828
SS16005-C2 C. coli 2016 UAB SK9 854 828 ND
SS15118-C1 C. coli 2015 Collserola SK10 3020 828
SS15083-C1 C. coli 2015 UAB SK11 – –
SS16005-C3 C. coli 2016 UAB SK11 9238 – NT
SS15080-C3 C. coli 2015 UAB SK11 9236 – NT
SS15080-C2 C. coli 2015 UAB SK12 – –
SS15078-C1 C. coli 2015 UAB SK13 827 828
SS15185-C1 C. coli 2015 Collserola SK13 827 828
SS15068-C4 C. coli 2015 Barcelona SK14 827 828
SS15070-C3 C. coli 2015 UAB SK15 1058 828
SS15190-C1 C. lanienae 2015 Collserola SK16 – –
SS15069-C2 C. lanienae 2015 Barcelona SK16 – – ND
SS15127-C1 C. lanienae 2015 Collserola SK16 – – NT
SS15102-C4 C. hyointestinalis 2015 Barcelona SK17 – –
SS15132-C2 C. lanienae 2015 Collserola SK18 – –
SS15102-C1 C. lanienae 2015 Barcelona SK19 – –
SS16042-C1 C. lanienae 2016 Collserola SK20 – –
SS16037-C1 C. coli 2016 Collserola SK21 5006 828
SS16031-C2 C. lanienae 2016 Collserola SK22 – –

Figure 2. Combined dendrogram of SmaI and KpnI PFGE profiles of the *C. coli*, *C. lanienae* and *C. hyointestinalis* isolates, presence of virulence-associated genes (depicted as grey squares), and antimicrobial susceptibility to six antimicrobial agents (resistance depicted as grey squares). ST: MLST sequence type (novel STs are shown in bold), CC: MLST clonal complex. [a]Barcelona: Barcelona municipality (area with high degree of urbanization), Collserola: Serra de Collserola Natural Park (area with high low degree of urbanization), UAB: Campus of the Universitat Autònoma de Barcelona (area with medium degree of urbanization). [b]Em: erythromycin, Nal: nalidixic acid, Ci: ciprofloxacin, Tc: tetracycline, S: streptomycin, Gm: gentamycin, NT: not tested; ND: could not be determined.



Species	Year	Location	Pulsotype
C. lanienae	2015	Barcelona	SK16
C. lanienae	2015	Collserola	SK16
C. lanienae	2015	Collserola	SK16
C. lanienae	2015	Barcelona	SK19
C. lanienae	2015	Collserola	SK18
C. lanienae	2016	Collserola	SK20
C. lanienae	2016	Collserola	SK22
C. hyointestinalis	2016	Barcelona	SK17

Figure 3. Maximum likelihood tree based on concatenated MLST loci of *C. lanienae* and *C. hyointestinalis* isolates. Branch lengths are based on the number of substitutions per site.

Table 1. *Campylobacter* spp. isolates from wild boars with relative frequency, prevalence and 95% confidence interval per species and area. Different superscript letters indicate differences ($P < 0.05$) among the prevalences within that column (through a 4-sample test for equality of proportions), and different superscript numbers indicate differences ($P < 0.05$) within each row (logistic regression).

	Positive wild boar/Analysed wild boar (%) [95% confidence interval]			
	Barcelona	**Collserola**	**UAB**	**Total**
C. lanienae	6/32 (**18.8**^{1}) [8.9 – 35.3]	39/73 (**53.4**^{2}) [42.1 – 64.4]	9/25 (**36**^{1,2}) [20.2 – 55.5]	54/130 (**41.5**^{a}) [33.4 – 50.1]
C. coli	6/32 (**18.8**^{1}) [8.9 – 35.3]	6/73 (**8.2**^{1}) [3.8 – 16.8]	5/25 (**20**^{1}) [8.9 – 39.1]	17/130 (**13.1**^{b}) [8.3 –19.9]
C. jejuni	0/32 (**0**) [0 – 10.7]	0/73 (**0**) [0 – 5]	0/25 (**0**) [0 – 13.3]	0/130 (**0**) [0 – 2.9]
Co-infection (*C. lanienae* and *C. coli*)	2/32 (**6.3**) [1.7 – 20.1]	1/73 (**1.4**) [0.1 – 7.4]	2/25 (**8**) [2.2 – 25]	5/130 (**3.8**^{c}) [1.7 – 8.7]
Co-infection (*C. lanienae* and *C. hyointestinalis*)	1/32 (**3.1**) [0.2 – 15.7]	0/73 (**0**) [0 – 5]	0/25 (**0**) [0 – 13.3]	1/130 (**0.8**^{c}) [0 – 4.2]
Campylobacter sp.	0/32 (**0**) [0 –10.7]	1/73 (**1.4**) [0.07 – 7.4]	1/25 (**4**) [0.2 – 19.5]	2/130 (**1.5**) [0.4 – 5.4]
Total	15/32 (**46.9**^{1}) [30.9 – 63.6]	47/73 (**64.4**^{1}) [52.9 – 74.4]	17/25 (**68**^{1}) [48.4 – 82.8]	79/130 (**60.8**) [52.2 – 68.7]

Table 2. MLST allelic profiles, sequence types (ST) and clonal complex (CC) of *C. coli* isolates. Novel STs are shown in bold. Isolates are grouped by studied areas.

Isolate	Year	Area	aspA	glnA	gltA	glyA	pgm	tkt	uncA	ST[a]	CC
SS15068-C4	2015	Barcelona	33	39	30	82	104	56	17	827	828
SS15100-C1	2015	Barcelona	33	39	44	82	118	35	588	**9235**	UN
SS15115-C1	2015	Barcelona	33	38	30	82	104	43	17	854	828
SS15117-C1	2015	Barcelona	33	39	30	82	104	43	17	828	828
SS16009-C3	2016	Barcelona	32	38	30	82	104	44	36	**9237**	UN
SS16036-C1	2016	Barcelona	32	38	30	82	104	43	36	2814	828
SS15118-C1	2015	Collserola	33	179	30	79	113	43	17	3020	828
SS15185-C1	2015	Collserola	33	39	30	82	104	56	17	827	828
SS15188-C1	2015	Collserola	33	39	44	82	118	35	588	**9235**	UN
SS16030-C1	2016	Collserola	33	38	30	82	104	43	17	854	828
SS16037-C1	2016	Collserola	33	66	30	192	189	43	17	5006	828
SS15068-C1	2015	UAB	33	38	30	82	104	43	17	854	828
SS15070-C3	2015	UAB	33	39	30	82	104	35	17	1058	828
SS15077-C2	2015	UAB	33	38	30	238	104	43	36	2097	828
SS15078-C1	2015	UAB	33	39	30	82	104	56	17	827	828
SS15080-C2	2015	UAB	33	38	30	115	104	85	–	ND	ND
SS15080-C3	2015	UAB	33	4	30	115	104	85	17	**9236**	828
SS15083-C1	2015	UAB	–	–	–	–	–	–	–	ND	ND
SS15091-C1	2015	UAB	33	38	30	82	104	43	17	854	828
SS16005-C2	2016	UAB	33	38	30	82	104	43	17	854	828
SS16005-C3	2016	UAB	33	39	30	82	118	44	17	**9238**	828

[a]ND: no sequence obtained for one or more genes, and thus no allele number, nor ST or CC could be assigned.
[b]UN: unassigned CC.

Table 3. Sequence type (ST) of *C. coli* isolates obtained from wild boars in the present study compared to the wider population of STs in the PubMLST database. The most frequent source of the most abundant STs is shown in bold. Data updated on June 2021.

ST	Total pubMLST isolates	Chicken	Chicken meat	Sheep	Farm environment	Cattle	Pig	Environmental waters	Human stool	Other sources/ Unspecified
827	1556	212 (13.6%)	74 (4.8%)	138 (8.9 %)	157 (10.1%)	82 (5.3%)	5 (0.3%)	8 (0.5%)	**690 (44.3%)**	190 (12.2%)
828	704	**310 (44.0%)**	46 (6.5%)	1 (0.1%)	–	4 (0.6%)	33 (4.7%)	2 (0.3%)	73 (10.4%)	235 (33.4%)
854	525	**150 (28.6%)**	20 (3.8%)	3 (0.6%)	–	8 (1.5%)	**183 (34.9%)**	7 (1.3%)	38 (7.2%)	116 (22.1%)
1058	109	3 (2.8%)	5 (4.6%)	–	–	–	**33 (30.3%)**	–	3 (2.8%)	**65 (59.6%)**
2097	8	3 (37.5%)	–	–	–	–	–	–	3 (37.5%)	2 (25%)
2814	4	–	–	–	–	–	1 (25%)	–	3 (75%)	–
3020	8	4 (50%)	–	–	–	–	–	–	3 (37.5%)	1 (12.5%)
5006	1	–	–	–	–	–	–	–	1 (100%)	–
9235	1	–	–	–	–	–	–	–	–	1 (100%)
9236	1	–	–	–	–	–	–	–	–	1 (100%)
9237	2	–	–	–	–	–	–	–	–	2 (100%)
9238	5	–	–	–	–	–	–	–	–	5 (100%)

Table 4. Frequency of each virulence-associated genes detected in the different *Campylobacter* species.

Virulence-associated gene		***C. lanienae* (n = 7)**	***C. coli* (n = 21)**	***C. hyointestinalis* (n = 1)**	**Total (n = 29)**
Motility	flaA	0 (0%)	20 (95.2%)	0 (0%)	20 (69%)
	flaB	7 (100%)	20 (95.2%)	1 (100%)	28 (96.6%)
Adhesion and colonization	cadF	2 (28.6%)	19 (90.5%)	0 (0%)	21 (72.4%)
	racR	2 (28.6%)	4 (19%)	0 (0%)	6 (20.7%)
	dnaJ	3 (42.9%)	8 (38.1%)	0 (0%)	11 (37.9%)
	pldA	4 (57.1%)	5 (23.8%)	0 (0%)	9 (31%)
	virB11	1 (14.3%)	13 (61.9%)	1 (100%)	15 (51.7%)
TS66	hcp	7 (100%)	20 (95.2%)	1 (100%)	28 (96.6%)
Invasion	ceuE	6 (85.7%)	20 (95.2%)	1 (100%)	27 (93.1%)
	ciaB	0 (0%)	5 (23.8%)	0 (0%)	5 (17.2%)
Toxin production	cdtA	2 (28.6%)	15 (71.4%)	0 (0%)	17 (58.6%)
	cdtB	3 (42.9%)	20 (95.2%)	0 (0%)	23 (79.3%)
	cdtC	2 (28.6%)	13 (61.9%)	0 (0%)	15 (51.7%)
	wlaN	0 (0%)	0 (0%)	0 (0%)	0 (0%)

Supplementary file 1. *C. coli* primer sets used for MLST.

Locus	Primer	Forward 5' – 3'	Primer	Reverse 5' – 3'	Reference
aspA	aspAF1	GAGAGAAAAGCWGAAGAATTTAAAGAT	aspAR1	TTTTTTCATTWGCRSTAATACCATC	Miller et al. (2005)
	aspA_Cjc-L	CAACTKCAAGATGCWGTACC	aspA_Cjc-R	ATCWGCTAAAGTATRCATTGC	Korczak et al. (2009)
atpA	atpAF	GWCAAGGDGTTATYTGTATWTATGTTGC	atpAR	TTTAADAVYTCAACCATTCTTTGTCC	Miller et al. (2005)
	atpA_Cjc-L	CAAAAGCAAAGYACAGTGGC	atpA_Cjc-R	CTACTTGCCTCATCYAAATCAC	Korczak et al. (2009)
glnA	glnAF	TGATAGGMACTTGGCAYCATATYAC	glnAR	ARRCTCATATGMACATGCATACCA	Miller et al. (2005)
	glnA_Cjc-L	ACWGATATGATAGGAACTTGGC	glnA_Cjc-R	GYTTTGGCATAAAAGTKGCAG	Korczak et al. (2009)
gltA	gltAF	GARTGGCTTGCKGAAAAYAARCTTT	gltAR	TATAAACCCTATGYCCAAAGCCCAT	Miller et al. (2005)
	gltA_Cjc-L	TATCCTATAGARTGGCTTGC	gltA_Cjc-R	AAGCGCWCCAATACCTGCTG	Korczak et al. (2009)
glyA	glyAF	ATTCAGGTTCTCAAGCTAATCAAGG	glyAR	GCTAAATCYGCATCTTTKCCRCTAAA	Miller et al. (2005)
	glyA_Cjc-L	AGGTTCTCAAGCTAATCAAGG	glyA_Cjc-R	CATCTTTTCCRCTAAAYTCACG	Korczak et al. (2009)
pgm	pgmF1	CATTGCGTGTDGTTTTAGATGTVGC	pgmR1	AATTTTCHGTBCCAGAATAGCGAAA	Miller et al. (2005)
	glmM_Cjc-L	GCTTATAAGGTAGCWCCKACTG	glmM_Cjc-R	AATTTTCHGTTCCAGAATAGCG	Korczak et al. (2009)
tkt	tktF1	GCAAAYTCAGGMCAYCCAGGTGC	tktR1	TTTTAATHAVHTCTTCRCCCAAAGGT	Miller et al. (2005)
	tkt_Cjc-L	AAAYCCMACTTGGCTAAACCG	tkt_Cjc-R	TGACTKCCTTCAAGCTCTCC	Korczak et al. (2009)

Supplementary file 2. *C. lanienae* primer sets used for MLST (Miller et al. 2012).

Locus	Primer	Forward 5'-3'	Primer	Reverse 3'-5'
aspA	LANaspF	TTTAGCCACAGCTATGGAGTATCTCAA	LANaspR	ATATGGGTTRAAWGCTGTAACRATACC
	*HFLaspXF	AAYATGAAYGCAAACGAAGTTATAGC	LANaspR	ATATGGGTTRAAWGCTGTAACRATACC
atpA	LANatpF	AACCAAAAAGGTCAAGATGTTATATG	LANatpR	ATTTTCTACTGGAAGTGGGCTATAAGG
glnA	LANglnF	TGGCAYCAYGTATCWTATAATATAAAAGC	LANglnR	ATGGACRTGCATACCRCTWCCATTATC
	*HFLglnXF	TTTYGAATWTTGTRAWGAAAATGAAGT	*HFLglnXF	AGAGTAWGTWAGAATGCTTGGKGCTTC
gltA	LANgltF	ATGCATAGMGGMTATGATATAGCGTGG	LANgltR	CATCAACTCTATCTGGAGTWCCKATCA
glyA	LANglyF	TGCWAATGTTCAGCCAAATAGCG	LANglyR	CAAGAGCGATATCRGCRTCTTTACC
pgm	LANpgmF	GCTTACYTTAAAAGGCCTRMGAGTTGT	LANpgmR	AAGAAGCAGYCTAATCAAATTYTCTGT
tkt	HFLtktXF	AATAAGATTTTTRTGTGCVGATATGGT	HFLtktXR	AAGAGTGAATTTARMAGCTCTTTTTTA
	*LANtktF	CATCTAAAKCAYAATCCMAAAAATCC	*LANtktR	ATCTCWKCGCCAAGMGGAGC

*Alternative MLST primer.

Supplementary file 3. Table 1. PCR primers and annealing temperatures used for the detection of virulence-associated genes.

Locus	Primer	Sequence 5'- 3'	Annealing T (°C)	Reference
flaA	flaA664 flaA1494	AATAAAAATGCTGATAAAACAGGTG TACCGAACCAATGTCTGCTCTGATT	55	Datta et al. (2003)
flaB	flaB-F flaB-R	AAGGATTTAAAATGGGTTTTAGAATAAACACC GCTCATCCATAGCTTTATCTGC	55	Goon et al. (2003)
cadF	cadF-F2B cadF-R1B	TTGAAGGTAATTTAGATATG CTAATACCTAAAGTTGAAAC	55	Datta et al. (2003)
ceuE (Cc)	COL1 COL2	ATGAAAAAATATTTAGTTTTTGCA GATCTTTTTGTTTTGTGCTGC	55	Bang et al. (2003)
racR	racR-25 racR-593	GATGATCCTGACTTTG TCTCCTATTTTTACCC	40	Datta et al. (2003)
dnaJ	dnaJ-299 dnaJ-1003	AAGGCTTTGGCTCATC CTTTTTGTTCATCGTT	40	Datta et al. (2003)
virB11	virB-232 virB-701	TCTTGTGAGTTGCCTTACCCCTTTT CCTGCGTGTCCTGTGTTATTTACCC	45	Datta et al. (2003)
ciaB	ciaB-403 ciaB-1373	TTTTTATCAGTCCTTA TTTCGGTATCATTAGC	45	Datta et al. (2003)
pldA	pldA-84 pld-981	AAGCTTATGCGTTTTT TATAAGGCTTTCTCCA	45	Datta et al. (2003)
cdtA	DS-18 DS-15	CCTTGTGATGCAAGCAATC ACACTCCATTTGCTTTCTG	55	Hickey et al. (2000)
cdtB	cdtB-113 cdtB-713	CAGAAAGCAAATGGAGTGTT AGCTAAAAGCGGTGGAGTAT	55	Datta et al. (2003)
cdtC	cdtC-192 cdtC-351	CGATGAGTTAAAACAAAAAGATA TTGGCATTATAGAAAATACAGTT	55	Datta et al. (2003)

wlaN	wlaN-DL 39 wlaN-DL 41	TTAAGAGCAAGATATGAAGGTG CCATTTGAATTGATATTTTTG	53	Linton et al. (2000)
hcp	hcp-F hcp-R	CAAGCGGTGCATCTACTGAA TAAGCTTTGCCCTCTCTCCA	56	Corcionivoschi et al. (2015)
	*gltA-F *gltA-R	GCCCAAAGCCCATCATGCACA GCGCTTTGGGGTCATGCACA	56	Corcionivoschi et al. (2015)

* Internal positive control of the PCR.

Supplementary file 4. Tables with AIC values of the logistic regression models. Δi: difference in AIC value between a given model and the model with the lowest AIC value.

Response variable	**Explanatory variables**	**AIC value**	**Δi**
Campyobacter (any species)	–	176.2	0.0
Campyobacter (any species)	Degree of urbanization	176.8	0.6
Campylobacter lanienae	Degree of urbanization	170.6	0.0
Campylobacter lanienae	–	178.5	7.9
Campylobacter coli	–	102.9	0.0
Campylobacter coli	Degree of urbanization	103.6	0.7

Supplementary file 5. Typeability, with different genotyping techniques, of the *Campylobacter* and *Salmonella enterica* subsp. *enterica* isolates recovered from wild boar.

Method (restriction enzyme)	***Campylobacter coli***	***Campylobacter lanienae***	***Campylobacter hyointestinalis***	***Salmonella enterica***
FlaA-RFLP	62/70 (100%)	NT[a]	ND	ND
ERIC-PCR	ND	184/186 (98.9%)	1/1 (100%)	12/16 (75%)
PFGE (SmaI)	31/31 (100%)	35/82 (42.7%)	1/1 (100%)	ND
PFGE (KpnI)	23/31 (74.2%)	17/82 (20.7%)	1/1 (100%)	ND
MLST	21/21 (100%)	7/7 (100%)	1/1 (100%)	ND

NT: not typeable. ND: not done. [a] We initially attempted the typing of the *C. lanienae* isolates by flaA-RFLP with a subset of isolates (out of the total 186), but most isolates were not typeable.