



Urban yellow-legged gull (*Larus michahellis*) and peri-urban Audouin's gull (*Larus audouinii*) as a source of *Campylobacter* and *Salmonella* of public health relevance

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HIGHLIGHTS

- Urban gulls are significant sources of *C. jejuni* and *Salmonella* of public health risk.
- *Salmonella* Typhimurium (and its monophasic variant) was the most frequent serovar.
- Yellow-legged gull and Audouin's gull carried strains potentially highly virulent.
- A high proportion of *C. jejuni* strains carried the marker for Guillain-Barré syndrome.
- Urban yellow-legged gull showed a high prevalence of AMR *C. jejuni* and *Salmonella*.

GRAPHICAL ABSTRACT



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ABSTRACT

Campylobacter spp. and *Salmonella* spp. are the leading cause of human enteric infections in the European Union. Some gull species act as reservoirs and play an important role in the epidemiology of these zoonotic agents. To gain insight into *Campylobacter* and *Salmonella* epidemiology we studied colonies of Audouin's gull (*Larus audouinii*) and yellow-legged gull (*Larus michahellis*) in Barcelona metropolitan area, Catalonia (north-eastern Spain). We assessed the occurrence, genetic diversity, virulence potential, and antimicrobial susceptibility of *Campylobacter* and *Salmonella* isolates recovered from gull faeces in different time periods within 2009–2018. The occurrence of *Campylobacter* was higher compared to *Salmonella* in both gull species. Also, the occurrence of

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Zoonosis
Wild birds

both pathogens was significantly higher in Audouin's gull (45 % for *Campylobacter*, 20 % for *Salmonella*), than in yellow-legged gull (13 % and 7 %, respectively). All but one individual carried *C. jejuni*; the remaining positive yellow-legged gull carried *C. lari*. *Salmonella* serovar Typhimurium (including its monophasic variant) was the most frequent in both hosts followed by ser. Bredeney. Other serovars frequently associated with human salmonellosis (Infantis, London, Virchow) were only isolated from yellow-legged gulls. Multilocus Sequence Typing analyses showed that yellow-legged gull and not Audouin's gull carried several *Campylobacter* genotypes associated with human enteritis. *Campylobacter* isolates from both gull species revealed a high virulence potential, as opposed to *Salmonella* isolates which showed a lower prevalence of virulence-associated genes, particularly in Audouin's gull. Overall, a moderate to high frequency of antimicrobial resistance (including multidrug resistance) was found in both pathogens from both gull species. *Campylobacter* and *Salmonella* from yellow-legged gull showed a higher frequency of isolates resistant to antimicrobials of relevance in human medicine. Overall, our results highlight the potential public health threat associated with these gull species, particularly yellow-legged gull, in densely human populated areas.

1. Introduction

Bacterial zoonoses caused by *Campylobacter* spp. and *Salmonella* spp. constitute a serious health risk both in developing and in developed countries (Chlebicz and Śliżewska, 2018) and are the main cause of foodborne disease worldwide (Havelaar et al., 2015). Their incidence is increasing even in countries with adequate public health surveillance. In the European Union, both zoonoses remain the most reported in humans since 2007 (EFSA-ECDC, 2022). In 2022 campylobacteriosis accounted for >62 % of all the reported and confirmed human cases of zoonotic disease while salmonellosis has stabilized over the last years after a long period of a declining trend (EFSA and ECDC, 2023b). Complications due to *Campylobacter* infections occur in the form of serious sequelae like peripheral neuropathies, including the Guillain – Barré syndrome, an autoimmune disease resulting in paralysis (Guirado et al., 2020). In addition, reactive arthritis (Reiter's syndrome), a type of arthritis that occurs because of an infection, can be associated with intestinal *Salmonella* infection (Indrieš, 2014).

Both enteropathogens can be transmitted to humans through the consumption of contaminated food and water, as well as through the contact with infected animals (Rukambile et al., 2019). Animals usually act as asymptomatic carriers of *Campylobacter* and *Salmonella* and their presence in these hosts is well documented (Chlebicz and Śliżewska, 2018). Particularly, *Campylobacter* spp. are considered as a part of the normal intestinal microbiota of domestic livestock (Haruna et al., 2013), though there are reports pointing to adverse effects in poultry colonized by these bacteria (Humphrey et al., 2014). *Salmonella* spp. live in the intestinal tract of vertebrates and under stressful conditions can cause disease or death (Tizard, 2004).

Campylobacter and *Salmonella* are widely distributed in aquatic environments and have been isolated from diverse asymptomatic wildlife. *Campylobacters* are thought to be environmentally fragile compared with *Salmonella*. They do not tolerate dry environments or heat but can often survive in moist environments for up to several weeks (Whiley et al., 2013). *Salmonella* can survive for longer periods in the environment (Literák and Čížek, 1996). The risk of infection of wildlife depends on the survival of these pathogens outside their hosts, but also on their feeding habits and their proximity to farms or livestock pastures that can increase the likelihood of becoming infected (Antilles et al., 2015; Gargiulo et al., 2014; Jurado-Tarifa et al., 2016). Particularly, opportunistic species of gulls (*Larus* spp) are one of the most documented carriers of both enteropathogens. Their increased use of anthropogenic food subsidies has led to their exponential growth in the last decades and to a greater risk of becoming infected with pathogens, including zoonotic agents (Vidal et al., 1998). By shedding pathogens in faeces, gulls contaminate agricultural lands and surface waters used for drinking, recreation or irrigation and may also contaminate farm environments (Reed et al., 2003). Also, with their foraging movements they can spread pathogens over long distances (Navarro et al., 2019). In southern Europe, there are large colonies of yellow-legged gulls (*Larus michahellis*) and Audouin's gulls (*Larus audouinii*) that are reservoirs of both *Campylobacter* and *Salmonella*

(Antilles et al., 2021).

Audouin's gull has breeding populations endemic to the Mediterranean Sea. It was considered Near Threatened until 2012 but the species is now considered Least Concern with a population estimated around 42,000 mature individuals (Morera-Pujol et al., 2018). Although the species has specialist feeding traits, in the last few decades it has been documented exploiting fishery discards and freshwater food resources (Morera-Pujol et al., 2018).

The yellow-legged gull breeds around the entire Mediterranean basin and the NE Atlantic. Its European population is about 1,000,000 individuals. Its feeding plasticity and an increase of human-related food subsidies has resulted in a substantial increase in its population size in recent decades (Morera-Pujol et al., 2018). In several areas, the species is considered a nuisance because of interactions with protected species, disturbances in urban areas or its potential role in the transmission of pathogens. Hence, population control measures have been implemented in some breeding sites along its range (Pedrocchi et al., 2002).

The worrying increase in the number of bacterial pathogens showing resistance to antimicrobials has become a major public health problem globally (Roca et al., 2015). Besides humans and domestic animals, wildlife can get infected and contribute to the spread of antimicrobial resistant (AMR) bacteria (Swift et al., 2019). Notably, gulls have been reported as reservoirs and spreaders of AMR bacteria in the environment (Antilles et al., 2021; Moré et al., 2017; Zeballos-Gross et al., 2021). Exponential growth of gull colonies and their role as carriers of zoonotic agents, including AMR bacteria, raises public health concern for the potential transmission of zoonotic bacteria to the community, particularly in densely populated areas such as big cities where a more direct contact with humans is likely to occur. Wild birds living in cities, particularly opportunistic species that frequently feed on refuse, can be more prone to carry zoonotic agents and AMR bacteria and have indeed been reported to harbour *Campylobacter*, *Salmonella* and AMR strains (Martín-Maldonado et al., 2020; Martín-Vélez et al., 2024; Vergara et al., 2017). The yellow-legged gull is generally assumed to be more opportunistic in its feeding regime compared to Audouin's gull. As such, the former is expected to be a more likely carrier of zoonotic agents and AMR strains.

Here we aimed to assess the occurrence, the genetic diversity, the virulence potential, and the antimicrobial susceptibility of *Campylobacter* and *Salmonella* from Audouin's gull (*Larus audouinii*) and yellow-legged gull (*Larus michahellis*), in the Metropolitan Area of Barcelona (MAB, Catalonia, north-eastern Spain).

2. Materials and methods

2.1. Study area and sampling

Sampling was carried out in June 2013 for Audouin's gull in the MAB and from May to July 2009, 2010, and 2018 for yellow-legged gull in the city of Barcelona, which included the breeding period of both gull species. Audouin's gull bred in the Llobregat Delta (10 Km south Barcelona

city, MAB) a common breeding area in recent years, and did so for the first time in the city of Barcelona in 2013 with 69 nests, and the sampling was conducted during the ringing campaign of chicks conducted that year. The sampling program of yellow-legged gull was part of the sanitary and epidemiological surveillance that is carried out by the Agència de Salut Pública de Barcelona (ASPB), in Barcelona city, which is the institution responsible for the supervision and surveillance of the species. The sampling sites were chosen according to citizens' reports regarding the species nesting on their terraces or high roofs of the city (Fig. 1). One gull chick from each nest found was sampled. All nests were independent from each other since the urban structure of cities promotes isolated instead of colonial nesting. The sampling sites and size are shown in Table 1. We aseptically obtained faecal samples from the cloaca using sterile swabs that were subsequently placed in Amies transport medium containing charcoal (Deltalab, Barcelona, Spain). All samples were kept refrigerated until arrival and processing at the laboratory, which occurred within 24 h.

2.2. *Campylobacter* and *Salmonella* isolation and identification

Campylobacter isolation from the swabs was performed as described by Urdaneta et al. (2015). Briefly, direct streaking onto *Campylobacter* blood-free selective agar medium (mCCDA) was performed and the plates were incubated at 42 °C for 48 h in microaerophilic conditions. Up to three *Campylobacter*-typical colonies were subcultured onto blood agar plates (bioMérieux, France) at 37 °C for 48 h in microaerophilic conditions. *Campylobacter* species identification was performed by DNA extraction (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, USA) followed by a multiplex PCR using specific primer pairs (Klena et al., 2004): forward primers lpxA-*C. coli* 5'-AGA CAA ATA AGA GAG AAT CAG-3', lpxA-*C. jejuni* 5'-ACA ACT TGG TGA CGA TGT TGT A-3', lpxA-*C. lari* 5'-TRC CAA ATG TTA AAA TAG GCG A-3' and a common reverse primer lpxA-RKK2m 5'-CAA TCA TGD GCD ATA TGA SAA TAH GCC AT-3' that produced a 391-bp amplicon for *C. coli*, of 331-pb for *C. jejuni*, and 233-pb for *C. lari*.

For *Salmonella* isolation and identification, samples were pre-enriched in buffered peptone water (Oxoid®, Basingstoke, UK). Selective enrichment was carried out in Rappaport-Vassiliadis (Biomérieux, Marcy l'Etoile, France) and Muller-Kauffmann Tetrathionate-Novobiocin *Salmonella* enrichment broths (Biomérieux, Marcy l'Etoile, France). After incubation, selective broths were plated on Xylose Lysine Deoxycholate agar (Oxoid®, Basingstoke, UK) and ASAP agar (Biomérieux, Marcy l'Etoile, France) for isolation and confirmation according to ISO 6579:2002. One isolate per positive bird was picked for further analysis.

Serotyping was performed on each *Salmonella* isolates by slide agglutination following the Kauffmann-White scheme (Grimont and Weill, 2007). If required, identification of monophasic *Salmonella* ser. Typhimurium was performed by DNA extraction (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA, USA) followed by a PCR using a previously described protocol (Tennant et al., 2010).

2.3. Molecular typing

To assess the genetic diversity of *Campylobacter*, we used three different typing methods. We initially used restriction fragment length polymorphism of the *flaA* gene (*flaA*-RFLP) (see section 2.3.1). We considered as the same strain those isolates showing identical band patterns and selected one isolate from each positive individual, as well as non-typeable isolates for further typing by pulse-field gel electrophoresis (PFGE) (see section 2.3.2). Finally, we typed a subset of *C. jejuni* strains by multi locus sequence typing (MLST) using PubMLST protocols, based on PFGE results and isolate selection (see section 2.3.4).

All *Salmonella* Typhimurium and its monophasic variant were typed by PFGE (see section 2.3.2).

2.3.1. *flaA*-RFLP

We carried out RFLP of the *flaA* gene following the standard CAMPYNET protocol as described previously (Harrington et al., 2003). We amplified the *flaA* gene using the forward A1 (5'-GGA TTT CGT ATT AAC ACA AAT GGT GC-3') and reverse A2 (5'-CTG TAG TAA TCT TAA AAC ATT TTG-3') primers (Nachamkin et al., 1993). The PCR amplified product of 1.7 kb was digested using the restriction enzyme *DdeI* (*HypF3I*; FastDigest®, Thermo Fisher Scientific, Waltham, MA, USA). Digest products were separated by electrophoresis in a 2.5 % agarose gel in 1 × TAE buffer at 90 V for three h.

2.3.2. PFGE

We typed by PFGE the selected *Campylobacter* isolates according to the standard operating procedure of PulseNet (pulsenetinternational.org). We digested the genomic DNA embedded in agarose with the restriction enzyme *KpnI* (Roche Applied Science, Indianapolis, IN). Electrophoresis was performed in a CHEF-DR II System (Bio-Rad laboratories, Hercules, CA, USA). Representative isolates from each PFGE pattern (pulsotype) were further characterized by MLST.

Salmonella PFGE typing was performed following the standard operating procedure of PulseNet, using the restriction endonuclease *XbaI* (and the secondary enzyme *BlnI* / *AvrII* when needed) and a CHEF Mapper XA System (Bio-Rad laboratories, Hercules, CA, USA).

2.3.3. Analysis and comparison of band patterns

The *flaA*-RFLP and PFGE band patterns of *Campylobacter* isolates were analysed using Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). The PFGE patterns of *Salmonella* isolates were analysed with the InfoQuest™ FP v.5.4 software (Bio-Rad laboratories). Similarity matrices were calculated using the Dice coefficient with tolerance and optimization values of 1.0 %. Dendrograms were constructed based on an unweighted-pair group method with arithmetic mean (UPGMA) cluster analysis. A cut-off of 90 % was used for the determination of the different pulsotypes (PFGE types) of *Campylobacter* and 85 % for *Salmonella*.

Campylobacter and *Salmonella* pulsotypes were compared with those present in our own PFGE database, from other regions and hosts (mostly of avian origin, wild and domestic, and humans, in the case of *Salmonella*). The database contains almost 1900 *Campylobacter* isolates and over 1700 *Salmonella* isolates, both databases span over a decade.

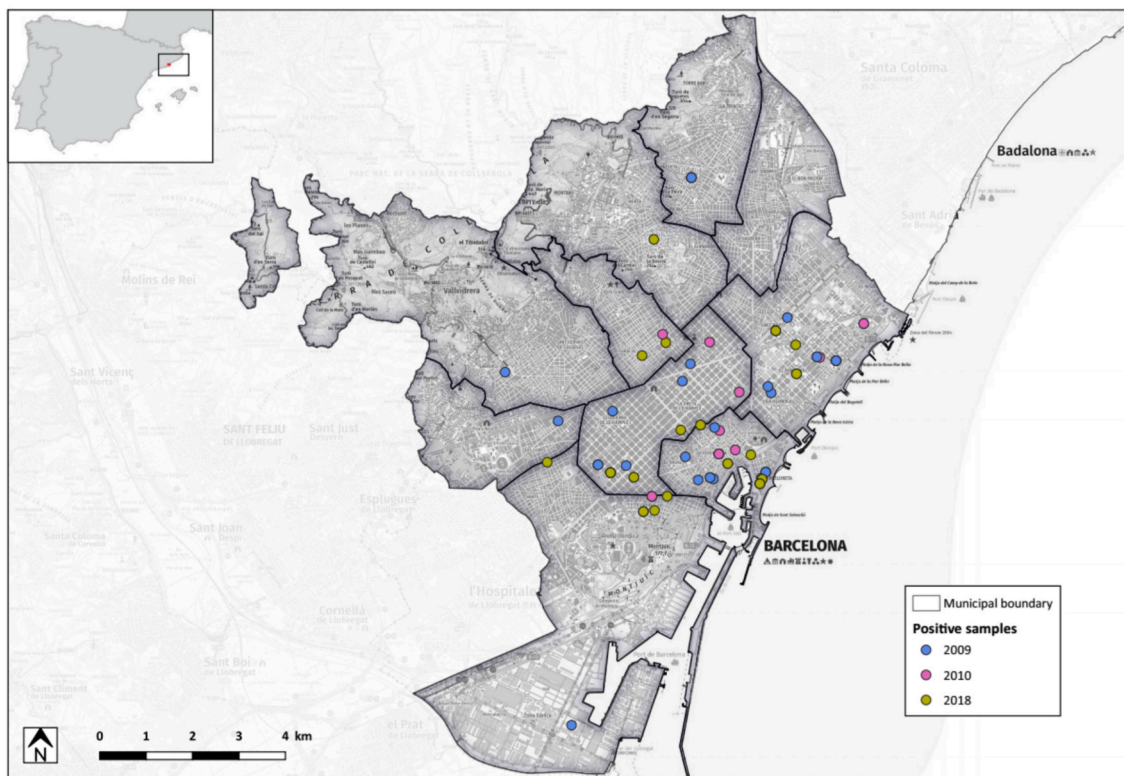
2.3.4. Multilocus sequence typing (MLST)

Based on PFGE results and the selection of the isolates we typed *C. jejuni* strains by MLST according to the procedures of PubMLST (<http://pubmlst.org/campylobacter>), Miller et al. (2012) and Korczak et al. (2009). Sanger sequencing of the PCR purified products was performed by MacroGen Spain (Madrid, Spain). The obtained sequences were imported, checked for quality, and analysed using the Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA) and assigned alleles, sequence types (STs) and clonal complexes (CC) were based on the MLST scheme provided on the *Campylobacter* PubMLST database. CC are defined as those STs that match the central genotype (ST) at four or more loci; that is, CC groups STs that are closely related. We also searched the most frequent source (human stool, chicken, or environmental waters, among others) of the *Campylobacter* STs found in this study, already reported in the PubMLST database (Table 2).

2.4. Virulence – associated genes of *Campylobacter* and *Salmonella*

The presence of 14 virulence-associated genes was assessed by PCR in selected *Campylobacter* isolates. These included genes related to motility (*flaA* and *flaB*), adhesion and colonization (*cadF*, *dnaj*, *racR*, *pldA*, *virB11*), invasion (*ceuE*, *ciaB*), cytotoxin production (*cdtA*, *cdtB*, *cdtC* and *wlaN*) and the type VI secretion system (T6SS) (Koolman et al., 2015). Primers and annealing temperatures used are shown in Supplementary table 1.

A.



B.

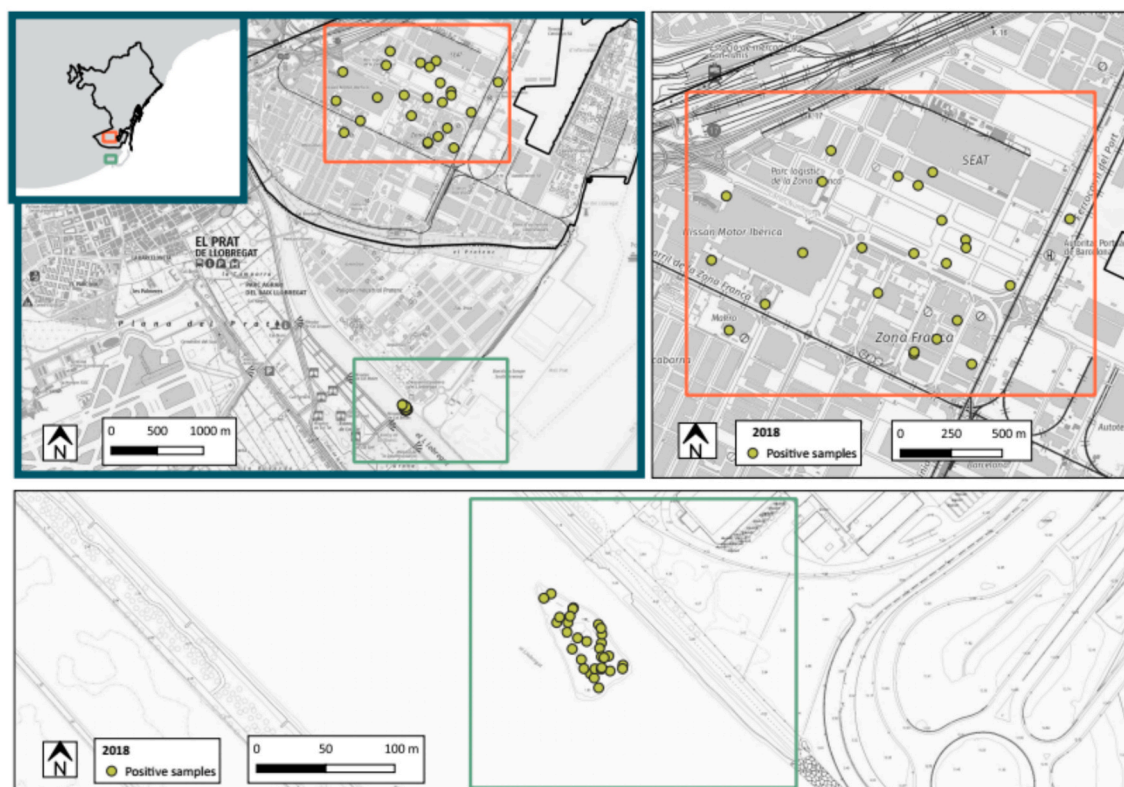


Fig. 1. Sampling sites indicating the positive individuals. A) *Larus michahellis* (yellow-legged gull). B) *Larus audouinii* (Audouin's gull): red square, Zona Franca area (south Barcelona); green square, Llobregat Delta.

Table 1
Occurrence of *Campylobacter* and *Salmonella* in Audouin's gull (*L. audouinii*) and yellow-legged gull (*L. michahellis*) according to sampling site and year.

Host	Date	Location	N ^a	<i>Campylobacter</i>	<i>Salmonella</i>	Coinfections ^c
Audouin's gull	2013	Barcelona	48	22 (45,8) ^b	6 (12,5)	4 (8,3)
	2013	Delta Llobregat	50	22 (44)	14 (28)	6 (12)
		Total	98	44 (44,9)	20 (20,4)	10 (10,2)
Yellow-legged gull	2009	Barcelona	121	20 (16,5)	11 (9,2)	0
	2010	Barcelona	110	6 (5,5)	5 (4,5)	0
	2018	Barcelona	100	18 (18)	9 (9)	1(1)
		Total	331	44 (13,3)	25 (7,6)	1(0,3)

^a N: total number of birds sampled.
^b Number of positive birds (% positive birds).
^c Prevalence of birds coinfecting with *Campylobacter* and *Salmonella*.

Table 2
Most common sources of the ST found in *C. jejuni* isolates from Audouin's gull and yellow-legged gull, according to PubMLST database. The most frequent source and the most abundant STs are shown in bold. Data updated on July 2024.

ST (n) ^a	Total PubMLST isolates	Human Stool	Environmental waters	Wild Birds	Chicken	Chicken offal or meat	Broiler environment	Other sources
45 (5)	4680	1585 (33,9)^b	119 (2,5)	72 (1,5)	692 (14,8)	691 (14,8)	20 (0,5)	1501 (32,1)
220 (1)	28	6 (21,4)	3 (10,7)	7 (25)	1 (3,6)	–	–	11 (39,3)
257 (1)	2800	1838 (65,6)	3 (0,1)	–	378 (13,5)	174 (6,2)	2 (0,1)	405 (14,5)
441 (1)	255	176 (69)	2 (0,8)	–	6 (2,4)	5 (2)	–	66 (25,8)
637 (2)	79	2 (2,5)	30 (38)	42 (53,2)	3 (3,8)	–	–	2 (2,6)
1223 (3)	76	3 (3,9)	14 (18,4)	51 (67,1)	6 (7,9)	–	–	2 (2,6)
1268 (7)	23	2 (8,7)	5 (21,7)	13 (56,5)	–	–	–	3 (15)
2197 (1)	20	7 (35)	4 (20)	–	5 (25)	–	3 (15)	1 (5)
2209 (1)	28	1 (3,6)	–	20 (71,4)	4 (14,3)	1 (3,6)	–	2 (7,1)
2654 (1)	12	1 (8,3)	5 (41,7)	6 (50)	–	–	–	–
6862 (1)	1	–	–	–	1 (100)	–	–	–
9239 (1)	3	–	1 (33,3)	2 (66,6)	–	–	–	–
9855 (1)	3	–	1 (33,3)	2 (66,6)	–	–	–	–
10,177 (1)	1	–	–	1 (100)	–	–	–	–
10,178 (1)	1	–	–	1 (100)	–	–	–	–
10,179 (1)	2	–	–	2 (100)	–	–	–	–

^a n: total number of isolates from this study belonging to each ST.
^b Total isolates (%).

For *Salmonella* we assessed by PCR the presence of 15 virulence-associated genes harboured in *Salmonella* pathogenicity island (SPI)-1 (*sitC*, *invA*), SPI-2 (*spiC*), SPI-5 (*sopB*), pathogenicity islets (*iroN*, *cdtB*), fimbrial operons (*agfA*, *lpfC*, *sefC*), plasmids (*spvC*, *pefA*) and phages (*sopE*, *gipA*, *gogB* and *gtgB*). The primers and annealing temperatures are shown in Supplementary Table 2.

2.5. Antimicrobial susceptibility testing of *Campylobacter* and *Salmonella*

We evaluated the antimicrobial susceptibility of one *Campylobacter* isolate per pulsotype and all *Salmonella* isolates (one per positive bird). Antibiotics tested where those included in the EU monitoring program of AMR for food-producing animals and foodstuffs derived thereof. We used a minimum inhibitory concentration (MIC)-based broth micro-dilution method (plate EUCAMP2 for *Campylobacter*, and EUVSEC for *Salmonella*, Sensititre®, ThermoFisher Scientific, Spain).

Campylobacter isolates were tested against the following six antimicrobials: erythromycin (1–128 mg/L), ciprofloxacin (0,12–16 mg/L), tetracycline (0,5–64 mg/L), gentamicin (0,12–16 mg/L), nalidixic acid (1–64 mg/L) and streptomycin (0,25–16 mg/L). *C. jejuni* ATCC 33560 was used as control strain. Plates were incubated in microaerophilic conditions at 37 °C for 48 h.

For *Salmonella* isolates, the antimicrobials tested were: ampicillin (1–64 mg/L), azithromycin (2–64 mg/L), cefotaxime (0,25–4 mg/L), ceftazidime (0,5–8 mg/L), chloramphenicol (8–128 mg/L); ciprofloxacin (0,06–8 mg/L), colistin (1–16 mg/L), gentamicin (0,5–32 mg/L), meropenem (0,12–16 mg/L), nalidixic acid (4–128 mg/L), sulfamethoxazole (8–1024 mg/L), tetracycline (2–64 mg/L), tigecycline (0,25–8 mg/L), and trimethoprim (0,25–32 mg/L). We used *E. coli* ATCC 25922 as control strain. Plates were incubated at 37 °C for 24 h.

An isolate was considered multidrug resistant (MDR) when showing resistance to three or more nonrelated antimicrobials. Isolates were susceptible or resistant based on epidemiological cut-off values according to EUCAST guidelines (www.eucast.org). When reporting data using EUCAST epidemiological cut-off values, bacteria should be reported as “wild-type” (WT) or “non-wild-type” (non-WT) (Schwarz et al., 2010). For simplicity of the terms, susceptible and resistant have been used here.

2.6. Statistical analysis

To compare the overall prevalence of *Campylobacter* and *Salmonella* in both gull species and to determine the differences in the prevalence across years in yellow-legged gull, we used the chi square test.

We used the R software (Version 3.6.3; R Core Team, 2020) for all statistical analyses. For the chi square test calculation, we used the chisq.test function from the stats package which is included by default in RStudio. 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* occurrence

Overall, 429 seagulls were sampled (98 Audouin's gull and 331 yellow-legged gull). The prevalence of *Campylobacter* and *Salmonella* was significantly higher in Audouin's gull compared to yellow-legged gull ($\chi^2 = 44,404$, $df = 1$, $P = 2,67e-11$ for *Campylobacter* prevalence

and $X^2 = 12,828$, $df = 1$, $P = 0,0003415$ for *Salmonella* prevalence). For *Campylobacter* 44,90 % (44/98, $CI_{95\%}$: 35,4–54,8 %) vs 13,29 % (44/331, $CI_{95\%}$: 10,1–17,4 %) and for *Salmonella* 20,41 % (20/98, $CI_{95\%}$: 13,6–29,4 %) vs 7,55 % (25/331, $CI_{95\%}$: 5,2–10,9 %) (Table 1). Notably, *Campylobacter* prevalence in Audouin's gull was similar in the two sampling sites, contrary to *Salmonella* that doubled in prevalence in Llobregat Delta. Significant differences were found in the prevalence of *Campylobacter* in yellow-legged gull across years, with the highest prevalence of *Campylobacter* in 2009 and 2018 compared to that of 2010 ($X^2 = 70,517$, $df = 2$, $P = 0,02943$). On the contrary, no significant differences were observed in the prevalence of *Salmonella* between the sampled years ($X^2 = 16,517$, $df = 2$, $P = 0,4379$). A low prevalence of birds coinfecting with both pathogens was observed in both gull species, being anecdotal in yellow-legged gull (0,3 %) compared to Audouin's gull (10,2 %).

Among the 88 *Campylobacter*-positive gulls (44 Audouin's gull and 44 yellow-legged gull), all Audouin's gull and yellow-legged gull carried *C. jejuni* species except one yellow-legged gull that was positive for *C. lari*.

Among the 45 *Salmonella*-positive gulls (20 Audouin's gull and 25 yellow-legged gull), we have observed a diversity of serovars (Supplementary table 3). The most frequent serovar in both gull species was Typhimurium (including the monophasic variant) with a prevalence of 51 %, followed by Bredeney (8,8 %). Other serovars were recovered only from 1 to 3 individuals: Chester, Goldcoast, Sandiego and Wangata were exclusively found in Audouin's gull, and serovars Corvallis, Infantis, London, Mons and Virchow, were limited exclusively to yellow-legged gull (Table 3, Supplementary table 3).

3.2. Genetic diversity of *Campylobacter* and *Salmonella*

The flaA-RFLP analysis of 121C. *jejuni* isolates from Audouin's gull revealed 19 different profiles, with three profiles being the most abundant, clustering isolates from five to 12 birds. In most cases (all but four birds) isolates from the same individual showed identical flaA-RFLP profile and therefore only one isolate per bird was further analysed by PFGE. Also, the same RFLP profile was found among several birds. From

yellow-legged gull there was only one isolate per individual and therefore we analysed them directly by PFGE together with the selected Audouin's gull isolates.

Overall, 73 *Campylobacter* isolates were genotyped by PFGE, 71 isolates of *C. jejuni* and two of *C. lari* (both isolates from the same individual). PFGE analysis revealed a high genetic diversity with each bird carrying a single pulsotype (PFGE profile), although several pulsotypes were identified in two individuals. Fig. 2 shows the *Campylobacter* PFGE dendrogram including only one representative isolate per pulsotype and bird. The *C. lari* isolate grouped separately from the *C. jejuni* isolates resulting in 29 different pulsotypes (28 of *C. jejuni* and one of *C. lari*). Audouin's gull and yellow-legged gull isolates grouped separately (i.e., formed host-specific clusters). In some instances, we noticed the same pulsotype in different individuals from the same gull species. Yellow-legged gull was sampled in three different years with one pulsotype (PT) found across years: PT10 with isolates from 2009 and 2010 (09_12393, 10_11056). Additionally, we compared the strains with those present in our PFGE database (data not shown) and observed the same (PT22) or closely related pulsotypes (>86 % similarity) (PT15, PT21 yellow-legged gull; PT18, Audouin's gull) in poultry (chickens, hens, ducks) from Catalonia (northeastern Spain).

In total 31C. *jejuni* isolates were further analysed by MLST. Complete MLST profile was obtained (Supplementary table 4) for the 28 out of the 31C. *jejuni* isolates analysed. For the remaining three isolates the ST could not be determined since some genes could not be PCR-amplified and sequenced. Overall, we found 16 different STs, three of which were novel (Table 2). These novel STs consisted of new allele combinations (for ST10177 and ST10178) or new allele sequences (ST10179, new allele tkt 808). The most frequent ST was ST1268, recovered from seven individuals from both gull species (BA53-C4, 10_17620, 18_13771-C1, 18_13773-C1, 18_13773-C2, 18_13774-C1, 18_14410-C1) across years, followed by ST45 (5 individuals) which was isolated only from yellow-legged gull. The most common CC was CC1275.

We obtained a higher number of different pulsotypes for *C. jejuni* isolates ($n = 28$) than STs ($n = 16$) (Fig. 2). Hence, we detected isolates with different pulsotypes belonging to the same ST (e.g., ST1223 in PT1, 12 and 20; ST1268 in PT2, 4, 6, 7, 16 and 27). In contrast, no isolates

Table 3

Distribution of 15 virulence genes in *Salmonella* serovars (other than Typhimurium and its monophasic variant) from Audouin's gull (*L. audouinii*) and yellow-legged gull (*L. michahellis*).

Host	<i>Salmonella</i> serovar	N ^b	Fimbrial operon			SPI-1 ^a		SPI-2	SPI-5	Chromosome		Plasmid		SopEΦ prophage	GIFS-1 prophage		GIFS-2 prophage
			agfA	lpfC	sefC	sitC	invA	spiC	sopB	iroN	cdtB	spvC	pefA	sopE	gipA	gogB	gtgB
Audouin's gull	Bredeney	2	–	–	–	–	+	–	+	–	+	–	–	–	–	–	–
	Chester	1	+	–	–	–	+	–	–	+	+	–	–	–	+w	–	–
	Chester	1	+w	–	–	–	–	–	–	–	+	–	–	–	–	–	–
	Goldcoast	1	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	Goldcoast	1	+	–	–	–	+w	–	+	–	+	–	–	–	–	–	–
	Goldcoast	1	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	Sandiego	2	+	–	–	–	+	–	+	–	+	–	–	–	–	–	–
	Sandiego	1	+w	–	–	–	+	–	–	–	+	–	–	–	–	–	–
	Wangata	1	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	Wangata	1	+w	–	–	–	+	–	+	–	–	–	–	–	–	–	–
Subtotal (%) ^c		12	66,7	0	0	0	100	0	50	8,3	66,7	0	0	0	8,3	0	0
Yellow-legged gull	Bredeney	2	+	–	–	+	+	+	+	+	+	–	–	–	–	–	–
	Corvallis	3	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–
	Infantis	1	+	+	–	+	+	+	+	+	–	+	+	–	+	+	+
	Infantis	1	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–
	London	1	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–
	Mons	1	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–
	Virchow	1	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–
Subtotal (%)		10	100	50	0	70	100	70	100	70	20	10	10	0	10	10	10
Total (%)		22	81,8	23	0	31,8	100	31,8	72,7	36,4	45,5	4,5	4,5	0	9,1	4,5	4,5

^a SPI: *Salmonella* pathogenicity island.

^b N: Number of *Salmonella* isolates per serovar.

^c Prevalence of virulence genes according to gull species.

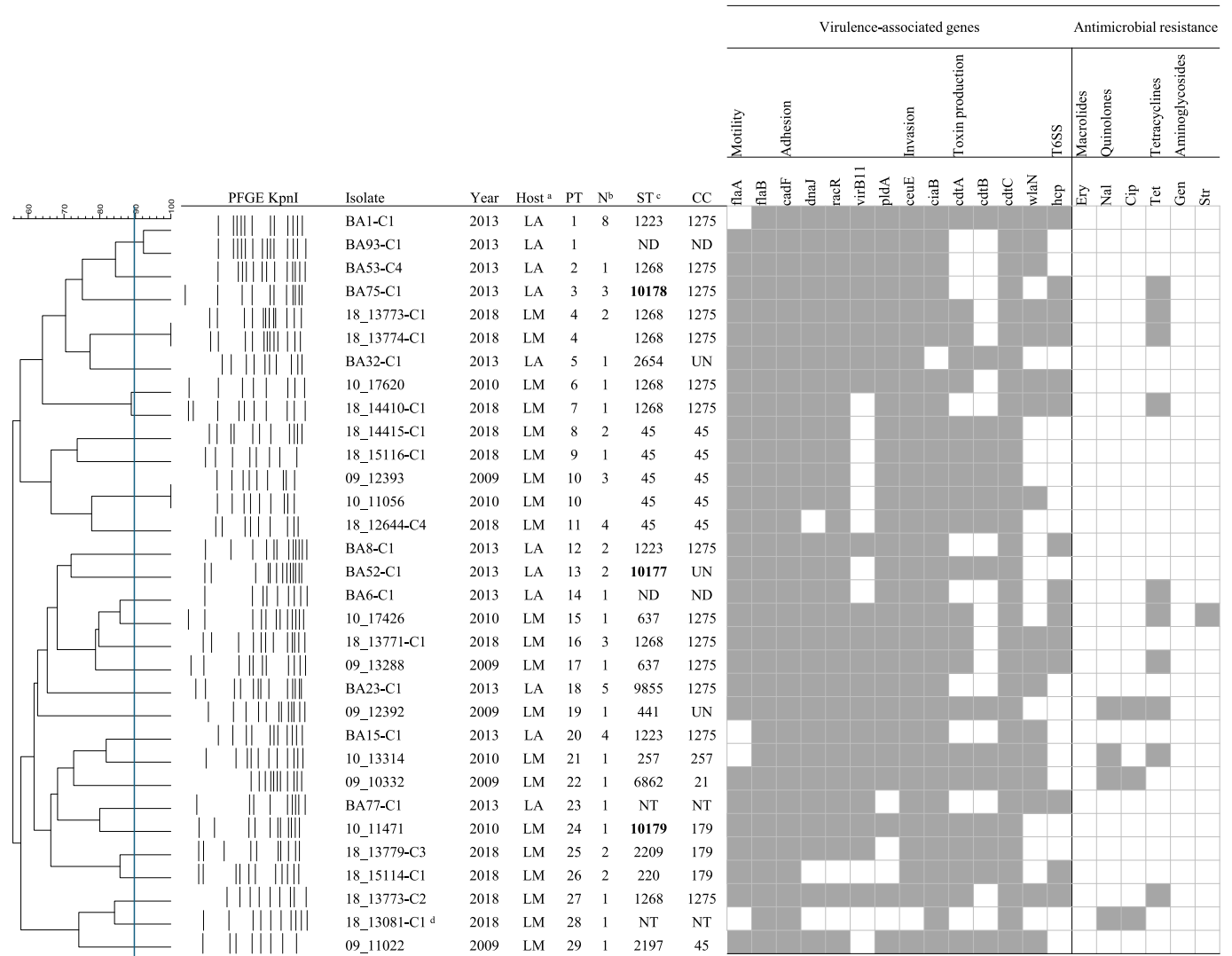


Fig. 2. Dendrogram of KpnI PFGE profiles of *C. jejuni* isolates (and a *C. lari*) showing the different pulsotypes (PT), MLST types (ST, Sequence type and CC, Clonal complex), virulence-associated genes (depicted as grey squares), and antimicrobial resistance profiles (depicted as grey squares; Ery, erythromycin; Nal, nalidixic acid; Cip, ciprofloxacin; Tet, tetracycline; Gen, gentamicin; Str, streptomycin). ^a LA: *Larus audouinii*, LM: *Larus michahellis*; ^b Number of individuals carrying the same pulsotype. ^c ND: Could not be determined, NT: Not tested, UN: Unassigned. ^d *C. lari*. Blue line: cut off determining the different pulsotypes.

with different ST grouped as the same pulsotype. According to the *C. jejuni* PubMLST database, most of the STs obtained in this study had been isolated previously from different sources, the most common from human stool, chicken, environmental waters and wild birds (Table 2).

Among *Salmonella*, the 23 isolates of ser. Typhimurium and monophasic Typhimurium were genotyped by PFGE (Fig. 3). Similar to *Campylobacter*, *Salmonella* strains from Audouin's gull and yellow-legged gull grouped separately (formed host-specific clusters). We found eight different pulsotypes; PT5 was the most frequent and included eight different isolates of ser. Typhimurium from yellow-legged gulls from three different years (2009, 2010, 2018).

Ser. Typhimurium and its monophasic variant were also compared with our PFGE database (data not shown) and revealed the same pulsotype (PT4 and PT5, yellow-legged gulls) in a domestic pigeon and a hen from Catalonia, and in other yellow-legged gulls from northern Catalonia (Medes Island) and a chicken (PT3). Notably, we also observed one pulsotype (PT1) in common with a human isolate.

3.3. Virulence-associated genes of *Campylobacter* and *Salmonella*

We assessed the presence of 14 virulence-associated genes in those

C. jejuni and *C. lari* isolates analysed by PFGE (Fig. 2). Overall, we found a high prevalence of virulence-associated genes in *C. jejuni* isolates from both gull species (Supplementary table 5). The highest number of virulence determinants ($n = 13$) was detected in one and seven *C. jejuni* isolates from Audouin's gull and yellow-legged gull, respectively.

The *flaB*, *cadF*, *cdtC* and *ceuE* genes, which are involved in motility, adhesion, cytotoxin production and invasion, respectively, were present in all *C. jejuni* isolates from both gull species. Other genes, such as *dnaJ* and *racR* (adhesion) were detected in all Audouin's gull isolates (100 %), and in almost all yellow-legged gull isolates (90 % and 95 %, respectively). The gene *ciaB* (invasion) was present in all yellow-legged gull isolates, and in almost all Audouin's gull isolates (94,1 %). Other genes were less prevalent (e.g., *virB11* 82,3 % in Audouin's gull, 60 % in yellow-legged gull isolates). The less prevalent virulence-associated genes in Audouin's gull were *cdtA* and *cdtB* (29,4 % each, cytotoxin production), followed by *hcp* gene (35,3 %) which was also the less prevalent in yellow-legged gull (45 %). Finally, the *wlaN* gene responsible of the Guillain-Barré syndrome, was present in over half of the isolates from both gull species.

The most frequent virulence profile of *C. jejuni* isolates from Audouin's gulls included all the virulence-associated genes except the

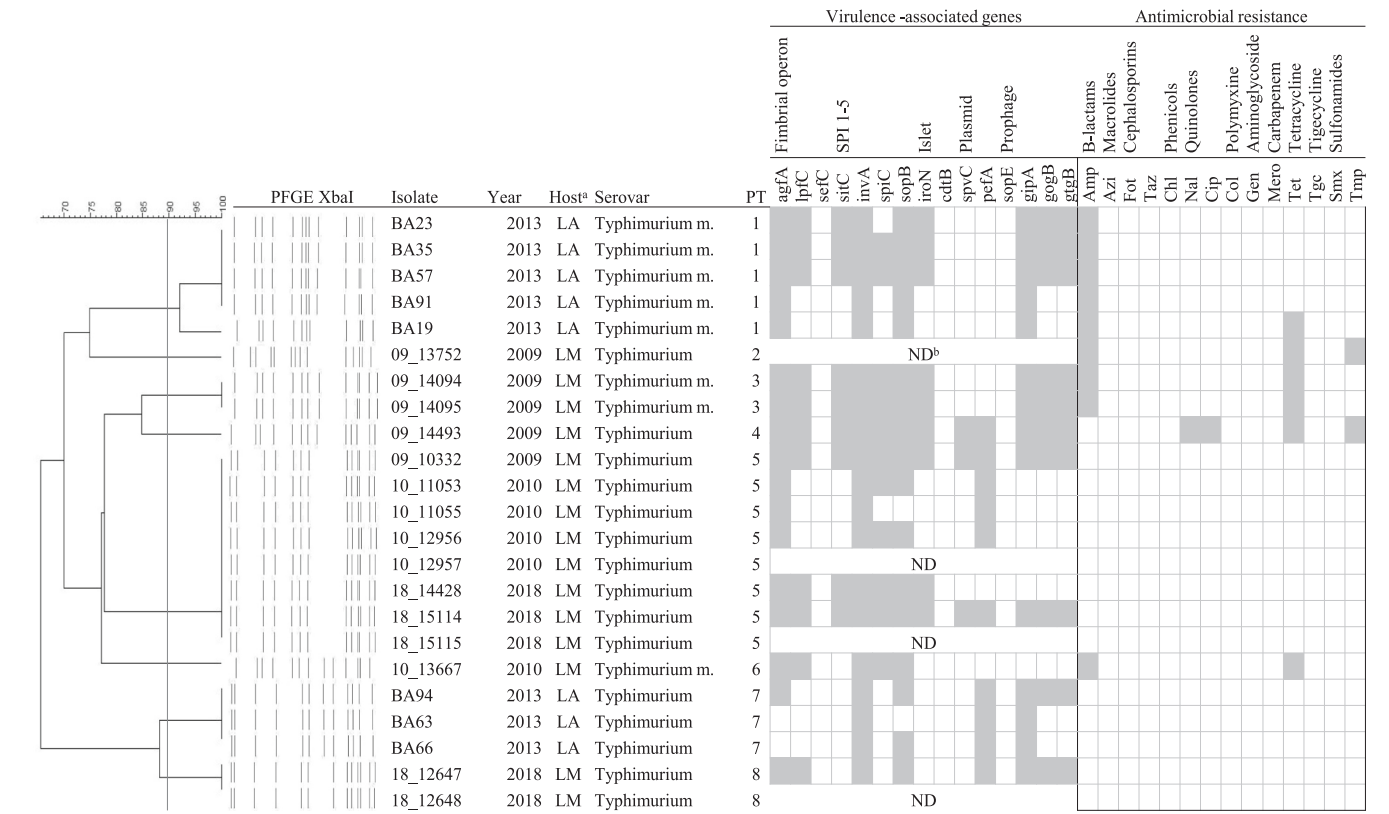


Fig. 3. Dendrogram of *XbaI* PFGE profiles of *Salmonella* Typhimurium and monophasic *S. Typhimurium* (Typhimurium m.) isolates showing pulsotypes (PT), virulence-associated genes (depicted as grey squares) and antimicrobial resistance (AMR) genes (depicted as grey squares; Amp: ampicillin; Azi: azitromicin; Fot: Cefotaxime; Taz: Tazobactam; Chl: chloramphenicol; Nal: nalidixic acid; Cip: ciprofloxacin; Col: colistin; Gen: gentamicin; Mero: meropenem; Tet: tetracycline; Tgc: tigecycline; Smx: sulfamethoxazole; Tmp: trimethoprim). ^a LA: *Larus audouinii*, LM: *Larus michahellis*. ^b ND: Not determined. Blue line: cut off determining the different pulsotypes.

cdtA, *cdtB* and *hcp* genes. This virulence profile was observed in four different strains from three different animals. The most frequent virulence profile from yellow-legged gull isolates included all the virulence-associated genes except the *cdtB* gene. This profile was found across all years in six different strains from five different birds.

We also assessed the prevalence of 15 virulence-associated genes for *Salmonella*, (Table 3, Fig. 3). Overall, isolates from yellow-legged gulls carried a higher number of virulence determinants compared with those from Audouin's gulls with seven out of fifteen genes present with a prevalence over 50 %. Furthermore, few virulence-associated genes (*sefC* and *sopE*) were not detected in *Salmonella* isolates from either gull species; other genes (e.g., *spiC*, *spvC*) were undetected only in Audouin's gulls. The *invA* gene (SPI) was present in all *Salmonella* isolates tested, followed in frequency by the genes *agfA* (fimbrial operon) and *sopB* (SPI). The prevalence of the genes *gipA*, *gogB* and *gtgB* was also low in both gull species.

The *Salmonella* serovars which carried the highest number of virulence determinants ($n = 12$) were ser. Infantis and ser. Typhimurium from yellow-legged gull isolates (Fig. 3 and Table 3). The most frequent virulence profiles (virulotypes) were noticed in ser. Corvallis and ser. Typhimurium isolates from yellow-legged gulls, with three isolates from three different animals in both cases. The same virulotypes were observed in different serovars from both gull species. In addition, for Audouin's gull, different virulotypes were found in most of the serovars but in single isolates, except for ser. Bredeney and Sandiego with two isolates each showing the same virulotype.

3.4. Antimicrobial susceptibility of *Campylobacter* and *Salmonella*

We detected a moderate occurrence of AMR *Campylobacter* isolates

(Supplementary fig. 1) with 62,5 % of the isolates being pansusceptible (Table 4, Fig. 2). The 81,8 % of *C. jejuni* isolates from Audouin's gulls were susceptible to all six antimicrobials tested and only two isolates possessed resistance to tetracycline (BA6-C1 and BA75-C1). Among the 21 *Campylobacter* isolates (20 *C. jejuni*, 1 *C. lari*) from yellow-legged gulls, the 47,6 % of *C. jejuni* isolates were pansusceptible and none were MDR. The resistance to tetracycline was the most frequent and with a moderate prevalence (38,1 %), followed by resistance to ciprofloxacin and nalidixic acid (19,1 % and 14,3 %, respectively). The *C. lari* isolate (18_13081-C1) was resistant to quinolones (nalidixic acid and ciprofloxacin).

Table 4
Antimicrobial resistance patterns of *C. jejuni* from Audouin's gull and yellow-legged gull.

Host	AMR pattern ^a	N atb ^b	N ^c
Audouin's gull	Pansusceptible	0	9
	Tet	1	2
Yellow-legged gull	NalCip	1	1
	Pansusceptible	0	11
	NalCipTet	2	1
	NalTet	2	1
	TetStr	2	1
	Tet	1	5
	NalCip ^d	1	1

^a Antimicrobial resistance (AMR) pattern: Cip, ciprofloxacin; Nal, nalidixic acid; Tet, tetracycline; Str, streptomycin.
^b N atb: number of classes of antimicrobial agents; resistance to three or more classes of antimicrobial agents, indicative of multidrug resistance.
^c N: Number of *Campylobacter* isolates per antimicrobial resistance pattern.
^d *C. lari*.

The antimicrobial susceptibility patterns were also determined for the 45 *Salmonella* isolates (20 from Audouin’s gulls and 25 from yellow-legged gulls). Similar to *Campylobacter*, a low occurrence of AMR *Salmonella* isolates was observed with 57,8 % being pansusceptible. Among Audouin’s gulls, 65 % of the *Salmonella* isolates were pansusceptible. On the contrary, 48 % of yellow-legged gull isolates showed resistance to at least one antimicrobial tested. Results for ser. Typhimurium and its monophasic variant are shown in Fig. 3, while those of *Salmonella* serovars other than Typhimurium are shown in Table 5. Resistance to ampicillin was the most common among isolates from Audouin’s gull. Over half of the 20 *Salmonella* isolates from Audouin’s gulls ($n = 13$) were susceptible to all 14 antimicrobials tested while two isolates of ser. Bredeney were MDR, showing resistance to four different classes of antimicrobials. A lower frequency of pansusceptible isolates was found in yellow-legged gulls (52 %) than in Audouin’s gulls (65 %). A higher frequency of MDR strains ($n = 4$) was found in yellow-legged gulls, that belonged to ser. Infantis, Typhimurium and Virchow, showing resistance to three different classes of antimicrobials.

4. Discussion

Campylobacter spp. and *Salmonella* spp. have been identified as the most common causes of human gastroenteritis worldwide. Many efforts have been taken to reduce human incidence of both zoonosis though they still remain the first and second most reported in the EU (EFSA and ECDC, 2023b). To control these zoonoses, it is necessary to better understand the global epidemiology of these pathogens, their reservoirs, and information on the pathogenicity of the strains. For this, a One Health approach with particular emphasis on the role of wildlife as an important reservoir for *Campylobacter* and *Salmonella* spp. is envisaged. In this study we investigated the occurrence, genetic diversity, virulence potential and antimicrobial susceptibility of these zoonotic agents from Audouin’s gull and yellow-legged gull from a densely populated area in north-eastern Spain.

4.1. *Campylobacter* and *Salmonella* occurrence

Campylobacter occurrence was high to low depending on the gull species (44,9 % for Audouin’s gulls and 13,3 % for yellow-legged gulls), compared with previous studies reporting a low *Campylobacter* prevalence (5 %–10 %) in gulls from southern Europe (Antilles et al., 2021; Ramos et al., 2010). Based on the feeding plasticity of yellow-legged gulls, being prone to feed on refuse given the chance or preying on urban birds, mainly pigeons (Méndez et al., 2020), a higher prevalence of these zoonotic bacteria would be expected. On the contrary, a higher

prevalence of *Campylobacter* in Audouin’s gulls compared to yellow-legged gulls was noticed. Further studies are needed to explore particularly the mobility associated with the feeding habits of Audouin’s gulls in the MAB.

Similarly, a higher prevalence of *Salmonella* than *Campylobacter* was expected as the former can persist in the environment for a longer time, including its survival on the soil of gull nesting colonies between breeding seasons (Literák and Čížek, 1996). This increases the likelihood of infection of gulls. However, *Salmonella* prevalence was moderate to low in both gull species, but still higher in Audouin’s gulls (20,4 % in Audouin’s gull vs 7,6 % in yellow-legged gull). It should be noted that a large part of the diet of the yellow-legged gull corresponds to pigeons (Méndez et al., 2020), and this species has a low prevalence of *Salmonella* (<1 %) as opposed to *Campylobacter* which reaches higher values (ASPB Technical report, 2016). A total of 11 different *Salmonella* serovars were identified and the most frequent was Typhimurium (including its monophasic variant) in both gull species. Both serovars are among the most frequent in gulls from southern Europe (Antilles et al., 2021; Ramos et al., 2010) including urban gulls (Martín-Maldonado et al., 2020). Furthermore, *Salmonella* Typhimurium is the second more important serovar involved in human infections after ser. Enteritidis (EFSA and ECDC, 2023a). Other serovars also frequently reported in human infections were those identified in yellow-legged gulls, such as ser. Infantis or Virchow. Ser. Infantis is also included in the top five *Salmonella* serovars involved in human infection by EFSA (EFSA-ECDC, 2022) and has also been reported in urban gulls in central Spain (Martín-Maldonado et al., 2020). Ser. Virchow has been involved in persistent cross-border outbreaks in the European Union (EFSA and ECDC, 2023a). The overall diversity of serovars identified in both gull species may be indicative of different feeding habits and consequently, different sources of infection and likelihood of getting infected by diverse serovars (Morera-Pujol et al., 2018).

Despite the higher prevalence of *Salmonella* and *Campylobacter* in Audouin’s gull compared with yellow-legged gull, their different habitat uses and exposure to anthropic environments may play an important role in the transmission of these zoonotic agents. Since yellow-legged gulls use highly humanized urban environments they pose a greater risk of pathogen transmission to humans, as reflected by their higher carriage of *Campylobacter* genotypes and *Salmonella* serovars associated with human infections. Also, while only chicks were sampled in the present study, we assume that the transmission risk can be extended to juvenile and adult individuals since infected birds can continue shedding the bacteria for long periods of time. For *Campylobacter*, once a broiler chicken is colonized (usually at 2–3 weeks of age), it will remain *Campylobacter*-positive until slaughter age, shedding the bacteria continuously (Urdaneta et al., 2023). For *Salmonella*, adults can also easily maintain the infection, although in this case they may shed the bacteria intermittently (Van Immerseel et al., 2004).

4.2. Genetic diversity

The genetic diversity of *Campylobacter* isolates was initially assessed by flaA-RFLP, followed by PFGE. The analyses revealed a high genetic diversity of *C. jejuni* isolates with 29 pulsotypes identified (Fig. 2). It further indicates a host specificity since none of the strains from Audouin’s gull were detected in yellow-legged gull, and vice versa. In some instances, we observed the same pulsotype in different individuals from the same gull species. Yellow-legged gull was sampled in different years and one pulsotype (PT10) was recovered in 2009 and 2010 (corresponding to the widespread ST45). This suggests there are strains continuously shed by these gulls which subsequently infect new individuals, maintaining such strains in the environment. Alternatively, there might be a common source of infection maintained along the time such as the pigeons they feed on in Barcelona city, which have moderate prevalences of *Campylobacter* (ASPB Technical report, 2016). On the other hand, comparing the PTs obtained in this study with our PFGE

Table 5
Antimicrobial resistance patterns of *Salmonella* serovars (other than Typhimurium and its monophasic variant) from Audouin’s gull and yellow-legged gull.

Host	Serovar	AMR pattern ^a	N atb ^b	N ^c
Audouin’s gull	Bredeney	AmpFotTazChlTet	4	2
	Chester	Pansusceptible	0	2
	Goldcoast	Pansusceptible	0	3
	Sandiego	Pansusceptible	0	3
	Wangata	Pansusceptible	0	2
	Bredeney	TmpTet	2	2
Yellow-legged gull	Corvallis	Pansusceptible	0	3
	Infantis	NalCipTmpTet	3	2
	London	TmpTet	2	1
	Mons	NalCip	1	1
	Virchow	NalCipTmpTet	3	1

^a Antimicrobial resistance (AMR) pattern: Amp: ampicillin; Chl: chloramphenicol; Nal: nalidixic acid; Tet: tetracycline; Tmp: trimethoprim.
^b N atb: number of classes of antimicrobial agents; resistance to three or more classes of antimicrobial agents, indicative of multidrug resistance.
^c N: Number of *Salmonella* isolates per serovar and antimicrobial resistance pattern.

database, we have observed several PTs matched with those of isolates from poultry, suggesting an anthropic origin of these isolates, as also supported by MLST results (see below).

MLST analysis of *Campylobacter* isolates showed that most of the isolates, particularly from Audouin's gull, belonged to STs (and the corresponding CC, specifically CC1275) associated with wild birds and/or environmental waters according to the pubMLST database (www.pubmlst.org/protocols/pfge/), pointing to a non-anthropogenic origin of these isolates. However, the STs (ST45, ST257, ST441) of three isolates from yellow-legged gull, indicate an anthropogenic origin since they are mostly associated with humans and with food animals. Also, one isolate belonged to a ST (ST6862 and CC21) associated with poultry, although it is represented only by one isolate in the pubMLST database. According to the information available in the PubMLST database (Table 2), besides the novel *C. jejuni* STs, all other STs obtained in our study have been mostly isolated from human stool, chicken, chicken products and broiler environment, in addition to wild birds. Comparatively, there were more isolates from yellow-legged gull belonging to STs of anthropic origin than from Audouin's gull. This suggests that the foraging habit of yellow legged gull makes it more likely that they become infected with these *Campylobacter* strains and their increasing presence in cities could mean that they are also a potential source of infection for humans.

The genetic diversity of *Salmonella* Typhimurium and its monophasic variant assessed by PFGE (Fig. 3) showed seven different pulsotypes. Audouin's gull and yellow-legged gull isolates formed host-specific clusters, suggesting a different source of infection. However, PT7 which includes Audouin's gull isolates from 2013 and yellow-legged gull isolates from 2018, show a similarity close to 90 % indicating closely related strains from different years and hosts.

The comparison of isolates of ser. Typhimurium and its monophasic variant obtained in this study with those of our PFGE database revealed that several PTs matched with those of isolates from poultry, gulls (from other colonies) and one of them with a human clinical isolate. This suggests an anthropic origin of these isolates, but also that gulls with their movements might be spreading *Salmonella* strains between colonies (Medes Island and Barcelona, separated over 140 km apart). Knowing the ecology of the movement of the two gull species could help identifying the feeding areas and therefore the possible sites where the gulls acquire the bacteria. This would help urban and public health management. A recent study where movements of yellow-legged gull from MAB have been tracked, revealed a maximum dispersal distance of 167 km for immature gulls versus 53 km for adults (Martín-Vélez et al., 2024).

4.3. Virulence potential

This study also provides information on the virulence potential of *C. jejuni*, *C. lari* and *Salmonella* isolates by determining the presence of genes encoding for putative virulence factors. Regarding *Campylobacter* colonization, it is required that the bacteria have the capacity of motility, adhesion to intestinal mucosa, invasion, and production of toxins (Bang et al., 2004). In general, most of the virulence genes associated with motility, adhesion and invasion were widely distributed in isolates from both gull species, similarly to what has been reported in poultry and wild birds (Iglesias-Torrens et al., 2018; Koolman et al., 2015). Notably, the *wlaN* gene involved in toxin production, encoding a b-1,3 galactosyltransferase involved in cell wall synthesis, and responsible for the Guillain-Barré syndrome showed a high prevalence (55–58 %) in both gull species. This is in contrast to what has previously been reported (Guirado et al., 2020) where the presence of *wlaN* was relatively rare in *C. jejuni* from gulls (6 % prevalence). We do not have an explanation for these differences, since in both cases the study involves yellow-legged gull from colonies with scavenging feeding habits (feeding on waste), and similar likelihood of getting infected from anthropogenic sources.

The cytolethal distending toxin (CDT) genes (*cdtABC*) showed a high prevalence in *C. jejuni* isolates from yellow-legged gull (90,5 % *cdtA*, 57,1 % *cdtB*, 100 % *cdtC*) compared with that from Audouin's gull isolates (29,4 % *cdtA* and *cdtB*, 100 % *cdtC*). Many strains lacked one or two of the three genes from the *cdtABC* operon, a phenomenon that has been reported earlier (Bang et al., 2004; Koolman et al., 2015). Bang et al. (2004) demonstrated that isolates without either *cdtA* or *cdtC* genes produced little or no CDT. Therefore, we assume that isolates from yellow-legged gull from the studied area may have a higher CDT activity, and therefore higher virulence, than those from Audouin's gull.

The gene *hcp* is a component of the type VI secretion system (T6SS) and it is associated in more severe forms of campylobacteriosis as it can confer cytotoxicity towards red blood cells (Harrison et al., 2014). Moreover, *hcp*⁺ strains have increased abilities to adhere and invade the host intestine, conferring them higher virulence. The prevalence of this gene was moderately high in both gull species (35,3 % in Audouin's gull and 42,9 % in yellow-legged gull). These prevalences are higher than what has previously been reported for non-urban yellow-legged gull in Spain (Iglesias-Torrens et al., 2018). However, similar (49 %) and a much higher prevalence (72 %) has been described in other wild birds or game bird species in Finland (Kovanen et al., 2019). It has been suggested that T6SS might provide an evolutionary advantage in the environment, e.g. higher rate of host colonization, ability of a faster growth rate when competing for the intestinal niche with other pathogens (Gabbert et al., 2023). However, more studies are needed to test this potential evolutionary advantage.

For *Salmonella* to establish an infection and cause illness, it requires bacterially expressed virulence factors that interact with the host cells and promote the pathogenicity (Siddiky et al., 2022). One example of these virulence factors are the genes that contribute to the production of the fimbriae like *agfA*, *sefC* and *lpfC* (Bäumler and Heffron, 1995; Clouthier et al., 1993; Mashayekh et al., 2022); fimbriae are long filamentous polymeric protein structures located at the surface of bacterial cells that enable the bacteria to bind to specific receptors. The *agfA* gene has properties related to auto-aggregation and to bacterial adhesion, and therefore is considered important for biofilm formation and the environmental maintenance of the bacteria (Mashayekh et al., 2022). In our study, most of the *Salmonella* isolates presented the *agfA* gene (73,7 % from Audouin's gull isolates and 100 % yellow-legged gull isolates). On the contrary, all *Salmonella* isolates studied were negative to *sefC*. This is in contrast to previous studies in chicks where this gene was detected in >90 % of the isolates (Castilla et al., 2006). The *lpfC* gene, important in the recognition and invasion of the host tissues (Krawiec et al., 2015), was more frequent in isolates from yellow-legged gull than Audouin gull (61,9 % and 15,8 %, respectively).

Infectivity is also related to different *Salmonella* pathogenicity islands (SPI) (Siddiky et al., 2022). We have studied the presence of *invA* and *sitC* genes, related to the SPI-1, and *spcC* and *sopB* genes related to SPI-2 and SPI-5, respectively. The *invA* gene, which is related to the host identification and the access of the bacterium during the invasion of epithelial cells (Mashayekh et al., 2022), is widely distributed, and as such, all isolates from this study carried this gene. The *sitC* gene which encodes for an important transporter of iron (Lozano-Villegas et al., 2023) was absent in Audouin's gull isolates, while 70 % of yellow-legged gull isolates were *sitC* positive. The *spcC* gene required for *Salmonella* survival within macrophages and systemic infection (Uchiya et al., 2009) was observed in most yellow-legged gull isolates (76,2 %), contrary to Audouin's gull isolates which were mostly negative (89,5 %). Similarly, the *Salmonella* outer protein (*sopB*) gene was much more prevalent (95,2 %) in yellow-legged gull isolates, compared with those from Audouin's gull (68,4 %).

Overall, *Salmonella* ser Typhimurium and its monophasic variant from both gull species possessed similar number of genes encoding for putative virulence factors. However, for the remaining serovars, strains from yellow-legged gull carried more virulence genes compared with Audouin's gull, suggesting a higher virulence potential for the former.

These differences in the carriage of virulence-associated genes suggest potential differences in the origin of the isolates from these gull species. Those recovered from yellow-legged gull might have an anthropogenic origin, contrary to Audouin's gull that might feed on more natural resources where *Salmonella* can also be present. This potential different origin of the isolates is also supported by the differences in the AMR of isolates from both gull species (see below, section 4.4).

4.4. Antimicrobial resistance

A low frequency of AMR would be expected for free-living wild birds, since they do not naturally encounter antibiotics. Accordingly, most of *Campylobacter* isolates from both gull species were pansusceptible and no MDR strains were found. However, a higher frequency of AMR was noticed in yellow-legged gull, particularly to antimicrobials of relevance in human medicine (quinolones and tetracycline). This might indicate an anthropic origin of the isolates, associated with the more insalubrious feeding habits of yellow-legged gull compared with Audouin's gull as reported by others (Iglesias-Torrens et al., 2018). It should be noted that AMR *E. coli* producing extended-spectrum β -lactamases (ESBL) (51.5 %) or carbapenemases (1.5 %) have also been reported in yellow-legged gull from Barcelona city (Martín-Vélez et al., 2024; Vergara et al., 2017). Our findings highlight the role of yellow-legged gull from Barcelona as reservoirs and potential spreaders of AMR bacteria. As mentioned above (section 4.2), gulls from MAB can cover long distances.

Considering the potential relationship between genotypes and virulence potential of the strains, it is noteworthy that most *C. jejuni* isolates harbouring the *wlaN* and *hcp* genes belonged to CC 1275 that is associated with wild birds. Hence, not only those genotypes of anthropic origin (STs belonging to CC21, CC45 and CC257) carried these genetic markers associated with important virulence traits, but also those from wild birds. Both gull species carried these STs, thus suggesting a much relevant virulence potential (i.e., potentially can cause more severe forms of campylobacteriosis) of isolates from wild birds.

Looking at the association of STs with AMR, we observed that *C. jejuni* isolates of CC 1275 (i.e., associated with wild birds) and isolated from both gull species were resistant to tetracycline. On the contrary, those isolates associated to humans and poultry (ST257, ST441 and ST6862) showed resistance to tetracyclines and fluoroquinolones and were recovered from yellow-legged gull.

Salmonella isolates were mostly pansusceptible, particularly those from Audouin's gull, while a higher frequency of AMR and MDR was found among isolates from yellow-legged gull. This might be related to different feeding habits of these gull species, pointing to an anthropic origin of such isolates, as indicated above for *Campylobacter*. A similar proportion of *Campylobacter* and *Salmonella* isolates showed resistance to at least one antimicrobial agent (37.5 % and 42.2 % respectively) and no MDR-*Campylobacter* were observed, opposite to the 15.5 % of MDR-*Salmonella* isolates (serovars Bredeney, Infantis, Typhimurium and Virchow). A high frequency of *Salmonella* AMR strains in yellow-legged gull has been previously reported in different colonies in Spain, which included among others these same serovars, together with a high prevalence of strains resistant to tetracycline and quinolones as we have also reported here (Antilles et al., 2021; Migura-García et al., 2017). AMR of *Salmonella* strains, including a high prevalence of tetracycline resistant isolates has also been reported in other gull species in Europe (Masarikova et al., 2016). On the contrary, a diversity of free-living birds from Portugal carried *Salmonella* isolates susceptible to all the antimicrobials tested, including tetracycline, nalidixic acid and ciprofloxacin, among others (Batista et al., 2022). The most frequent serovars (Typhimurium and its monophasic variant) we have reported in this study showed a moderate prevalence of AMR and MDR strains compared to the higher frequency in other gull colonies in Spain with high anthropic pressure (Antilles et al., 2021; Migura-García et al., 2017). Because of this high frequency of AMR strains in different gull species, they have been suggested as sentinel species for antibiotic pressure in

the environment (Antilles et al., 2021; Stedt et al., 2014).

In summary, gull species from MAB are a source of *C. jejuni* and of different *Salmonella* serovars of public health relevance, because of their high occurrence and virulence potential. It is also of relevance the high prevalence of the marker for Guillain – Barré syndrome in *C. jejuni* from both gull species and the high prevalence of AMR in *Campylobacter* and *Salmonella* isolates from yellow-legged gull. The finding of the same *Salmonella* genotypes in yellow-legged gull from Barcelona and in an important colony over 140 km northern Barcelona highlights the potential of this gull species as spreader through long distances of both zoonotic agents and AMR bacteria. Hence, this study underlines the need to work with a One Health approach that allows integrating environmental, animal and human health in order to improve global welfare.

CRedit authorship contribution statement

Alicia Manzanares-Pedrosa: Writing – original draft, Visualization, Investigation, Formal analysis. **Teresa Ayats:** Methodology. **Noelia Antilles:** Writing – review & editing, Methodology. **Sara Sabaté:** Writing – review & editing, Methodology. **Raquel Planell:** Methodology. **Raquel González:** Methodology. **Tomás Montalvo:** Writing – review & editing, Visualization, Formal analysis. **Marta Cerdà-Cuellar:** Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.178227>.

Data availability

Data will be made available on request.

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