Epidemiology and antimicrobial resistance of *Salmonella* spp. and *Campylobacter* spp. from wild birds and poultry reared outdoors

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Epidemiology and antimicrobial resistance of *Salmonella* spp. and *Campylobacter* spp. from wild birds and poultry reared outdoors

Tesi doctoral presentada per na **Noelia Antillés Silva** per optar al grau de Doctora en Veterinària dins del programa de doctorat de Medicina i Sanitat Animals del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra **Marta Cerdà Cuéllar** i la tutoria de la Dra. **Natàlia Majó i Masferrer.**

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La Dra. MARTA CERDÀ CUÉLLAR, investigadora del Centre de Recerca en Sanitat Animal (CReSA), i la Dra. NATÀLIA MAJÓ i MASFERRER, professora titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigadora adscrita al CReSA,

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Dra. Marta Cerdà Cuéllar	Dra. Natàlia Majó i Masferrer	Noelia Antillés Silva
Directora	Tutora	Doctoranda

Al meu avi,

continua lluitant amb força

i al meu estimat Eloi

Érase una Gallina que ponía
un huevo de oro al dueño cada día.
Aun con tanta ganancia mal contento,
quiso el rico avariento
descubrir de una vez la mina de oro,
y hallar en menos tiempo más tesoro.
Matóla, abrióla el vientre de contado;
pero, después de haberla registrado,
¿qué sucedió? que muerta la Gallina,
perdió su huevo de oro y no halló la mina.

¡Cuántos hay que teniendo lo bastante
enriquecerse quieren al instante,
abrazando proyectos
a veces de tan rápidos efectos
que sólo en pocos meses,
cuando se contemplaban ya marqueses,
contando sus millones,
se vieron en la calle sin calzones.

Félix María de Samaniego. 1804.
Fábulas en verso para el uso del Real Seminario Bascongado.
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Abbreviations

AR	Antimicrobial resistance
BB	Bolton broth
BGA	Brilliant green agar
Bp	Base pairs
BPW	Buffered peptone water
C	Cytidine
CCDA	charcoal cefoperazone deoxycholate agar
CDT	Cytolethal distending toxin
CEB	Campylobacter enrichment broth
DNA	Deoxyribonucleic acid
DT	Definitive type
EC	European Commission
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ERIC	enterobacterial repetitive intergenic consensus
EU	European Union
G	Guanine
GALT	Gut associated lymphoid tissue
HE	Hektoen enteric
HSPs	Heat shock proteins
Kb	Kilobase
LPS	Lipopolysaccharide
MDR	Multidrug resistance
MLST	multilocus sequence typing
MS	Member States
MSRV	Rappaport Vassiliadis semisolid medium
PB	Preston broth
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis

PT	Phage type
REP	Repetitive extragenic palindromic
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
SS	*Salmonella-Shigella*
ST	Sequence type
USA	United States of America
VBNC	Viable but non-cultivable cells
WHO	World Health Organization
XLD	Xylose lysine deoxycholate agar
XLT4	Xylose lysine tergitol 4

Summary

Campylobacter and *Salmonella* are the most important foodborne enteropathogenic bacteria worldwide. Infections caused by these bacteria are of significant economic and public health concern. Both bacteria have the ability to infect a variety of domestic and wild animal species. The close contact between humans, domestic and wild animals is an important factor contributing to human infections with these bacteria. However, limited data exists on the occurrence, antimicrobial susceptibility and genetic diversity of *Campylobacter* and *Salmonella* in wild birds and poultry reared outdoors in southern Europe. Therefore, a wide sampling was performed in order to assess the contribution of domestic (poultry reared outdoors) and wild birds (waterfowl and seagulls) in the epidemiology and antimicrobial resistance of *Salmonella* spp. and *Campylobacter* spp. in the western Mediterranean and eastern Atlantic Ocean.

In these studies, we found that poultry reared outdoors, as well as certain waterfowl species and seagulls (particularly Audouin's gulls) are an important reservoir for *Campylobacter*. The most common *Campylobacter* species isolated from poultry reared outdoors and seagulls was *C. jejuni*, while from waterfowl was *C. coli*. On the contrary, poultry reared outdoors and waterfowl seems not to be an important reservoir of *Salmonella* spp, while seagulls (yellow-legged gulls and Audouin's gulls) are important carriers of this pathogen. A great diversity of *Salmonella* serotypes was isolated from seagulls, some of them of important public health concern. The two most important serotypes causing human food-borne disease, *S.* Enteriditis and *S.* Typhimurium, were isolated in most of the studied seagull colonies. It is noteworthy the differences of *Campylobacter* or *Salmonella* carriage among different birds species. Those with coprophagic and/or scavenging habits showed a high occurrence of these bacteria.

Wild birds do not naturally come into contact with antimicrobials. Thus, it was not surprising the susceptibility to all of the antimicrobials tested of all *Campylobacter* isolates from waterfowl. However, *Salmonella* and *Campylobacter* strains isolated from seagulls and poultry reared outdoors showed resistance to several antimicrobial

agents. The main resistances found were to fluoro(quinolones) and tetracycline, which is of public health concern, since these agents are the ones of choice to treat enteric infections in humans.

Genetic diversity of *Campylobacter* and *Salmonella* was assessed by ERIC-PCR and PFGE. The high diversity of *Campylobacter* and *Salmonella* strains found in wild birds suggests bird infections by multiple sources. On the other hand, several common *Salmonella* strains were detected in different seagull colonies and different seagull species (yellow-legged gull and Audouin's gull). This finding suggests a common origin of infection or the contribution of seagulls to the spread of *Salmonella* strains by dispersal or migrating movements. The detection of the same PFGE pattern of *Salmonella* Kottbus in poultry reared outdoors and seagulls suggests a circulation of the bacteria between farm and wild birds. However, more studies are needed in order to confirm this.

The data provided in this thesis highlights the importance of domestic and wild birds as carriers and dispersal agents of *Campylobacter* and *Salmonella* and antibiotic resistance traits to the environment, in southern Europe. An improvement of surveillance efforts and development of appropriate control strategies are needed in order to reduce *Campylobacter* and *Salmonella* exposure to humans.

Resumen

Campylobacter y *Salmonella* son las bacterias enteropatógenas transmitidas por los alimentos más importantes a nivel mundial. Las infecciones causadas por dichas bacterias representan un grave problema económico y de salud pública. Ambas bacterias tienen la capacidad de infectar distintas especies de animales domésticos y silvestres. El estrecho contacto entre éstos y el hombre constituye un riesgo de infecciones humanas. Sin embargo, hay pocos datos sobre la incidencia, susceptibilidad antimicrobiana y diversidad genética de *Campylobacter* y *Salmonella* en aves silvestres y aves de corral criadas al aire libre en el sur de Europa. Por ello, se realizó un amplio muestreo con el fin de evaluar cuál es la contribución de las aves domésticas (aves de corral criadas al aire libre) y silvestres (ánades y gaviotas) en la epidemiología y resistencia antimicrobiana de *Salmonella* spp. y *Campylobacter* spp. en el área del Mediterráneo occidental y Atlántico oriental.

En estos estudios encontramos que las aves de cría al aire libre y algunas especies de ánades y gaviotas (en especial las gaviotas de Audouin) son un reservorio importante de *Campylobacter*. La especie de *Campylobacter* aislada con más frecuencia en aves domésticas y gaviotas fue *C. jejuni*, mientras que en las ánades fue *C. coli.* Por el contrario, ni las aves de cría al aire libre ni las ánades constituyen un reservorio importante de *Salmonella* spp, mientras que las gaviotas (gaviota de Audouin y gaviota patiamarilla) son reservorios importantes de este patógeno. Se aislaron una gran diversidad de serotipos de *Salmonella* procedentes de gaviotas, algunos de ellos de gran importancia para la salud pública. En la mayoría de las colonias de gaviotas estudiadas se aislaron *S.* Enteriditis y *S.* Typhimurium, los dos serotipos más importantes que causan enfermedades transmitidas por los alimentos en humanos. Es destable la diferencia entre las distintas especies de aves portadoras de *Campylobacter* o *Salmonella*. Las especies con hábitos coprófagos y/o carroñeros presentaron una alta prevalencia de estas bacterias.

Las aves silvestres no entran en contacto con antimicrobianos de manera natural. No es pues sorprendente que todos los aislados de *Campylobacter* procedentes de ànades fuesen susceptibles a todos los antimicrobianos estudiados. Sin embargo, las cepas de

Salmonella y *Campylobacter* aisladas de gaviotas y aves de cría al aire libre presentaron resistencia a varios antimicrobianos. Las principales resistencias fueron a fluoro(quinolonas) y a tetraciclinas, lo que representa un problema de salud pública importante puesto que éstos son los agentes más usados para tratar infecciones entéricas humanas.

Se evaluó la diversidad genética de *Campylobacter* y *Salmonella* mediante ERIC-PCR y PFGE. La gran diversidad de cepas de *Campylobacter* y *Salmonella* encontradas en aves silvestres sugiere la existencia de más de una fuente de infección. Por otro lado, en diversas ocasiones se detectó la misma cepa de *Salmonella* en diferentes colonias y especies de gaviotas. Este resultado sugiere un origen común de la infección o una dispersión de cepas de *Salmonella* mediante movimientos migratorios o de dispersión de las gaviotas. La detección del mismo patrón PFGE de *Salmonella* Kottbus tanto en aves domésticas como en gaviotas sugiere que existe una circulación de estas bacterias entre aves de granja y aves silvestres. Sin embargo, se necesitan más estudios para confirmarlo.

Los datos proporcionados en esta tesis confirman la importancia de las aves domésticas y silvestres como portadoras y diseminadoras de *Campylobacter* y *Salmonella* así como de resistencias antimicrobianas al ambiente, en el sur de Europa. Es necesario mejorar los esfuerzos de vigilancia y desarrollar estrategias de control adecuadas para reducir la exposición del ser humano a *Campylobacter* y *Salmonella*.

Resum

Campylobacter i Salmonella són els bacteris enteropatogens transmesos pels aliments més importants a nivell mundial. Les infeccions causades per aquests bacteris representen un greu problema econòmic i de salut pública. Ambdós bacteris tenen la capacitat d'infectar diferents espècies d'animals domèstics i silvestres. El contacte proper entre aquests animals i l'home és un factor de risc d'infeccions humanes. Tot i així, hi ha poques dades sobre la incidència, susceptibilitat antimicrobiana i diversitat genètica de *Campylobacter* i *Salmonella* en aus silvestres i aus de corral criades a l'aire lliure al sud d'Europa. Per aquest motiu es va realitzar un ampli mostreig amb la finalitat d'avaluar quina és la contribució de les aus domèstiques (aus de corral criades a l'aire lliure) i silvestres (ànecs i gavines) en l'epidemiologia i resistència antimicrobiana de *Salmonella* spp. i *Campylobacter* spp. a l'àrea del Mediterrani occidental i Atlàntic oriental.

En aquests estudis vam trobar que les aus de cria a l'aire lliure i algunes espècies d'ànecs i gavines (en especial la gavina corsa) són un reservori important de *Campylobacter*. L'espècie de *Campylobacter* aïllada amb més freqüència en aus domèstiques i gavines va ser *C. jejuni,* en canvi en els ànecs ho va ser *C. coli*. D'altra banda, ni les aus de cria a l'aire lliure ni els ànecs són reservoris importants de *Salmonella* spp., mentre que les gavines (gavina corsa i gavià argentat) són reservoris importants d'aquest patogen. Es van aïllar una gran diversitat de serotips de *Salmonella* procedents de gavines, alguns d'ells de gran importància per a la salut pública. A la majoria de les colònies de gavines estudiades s'hi van aïllar *S.* Enteriditis i *S.* Typhimurium, els dos serotips més importants que causen malalties transmeses per aliments a l'home. És destacable la diferència entre les diferents espècies d'aus portadores de *Campylobacter* o *Salmonella*. Les espècies amb hàbits copròfags i/o carronyers van presentar una gran prevalença d'aquests bacteris.

Les aus silvestres no entren en contacte amb antimicrobians de manera natural. No és sorprenent, doncs, que tots els aïllats de *Campylobacter* procedents d'ànecs fossin susceptibles a tots els antimicrobians estudiats. En canvi, les soques de *Salmonella* i

Campylobacter aïllades de gavines i aus de cria a l'aire lliure van presentar resistència a diversos antimicrobians. Les resistències principals van ser a fluoro(quinones) i a tetraciclines, cosa que representa un problema de salut pública important ja que són els agents més utilitzats per a tractar infeccions entèriques humanes.

Es va avaluar la diversitat genètica de *Campylobacter* i *Salmonella* mitjançant ERIC-PCR i PFGE. La gran diversitat de soques de *Campylobacter* i *Salmonella* trobades en aus silvestres suggereix l'existència de més d'una font d'infecció. Per altra banda, en diverses ocasions es va detectar la mateixa soca de *Salmonella* en diferents colònies i espècies de gavines. Aquest resultat suggereix un origen comú de la infecció o una dispersió de soques de *Salmonella* mitjançant moviments migratoris o de dispersió de les gavines. La detecció del mateix patró PFGE de *Salmonella* Kottbus tant en aus domèstiques com en gavines suggereix l'existència d'una circulació d'aquests bacteris entre aus de granja i aus silvestres. Tot i així, calen més estudis per a confirmar-ho.
Les dades proporcionades en aquesta tesi confirmen la importància de les aus domèstiques i silvestres com a portadores i disseminadores de *Campylobacter* i *Salmonella* així com de resistències antimicrobianes a l'ambient, al sud d'Europa. Cal millorar els esforços de vigilància i desenvolupar estratègies de control adequades per a reduir l'exposició de l'ésser humà a *Campylobacter* i *Salmonella*.

Introduction

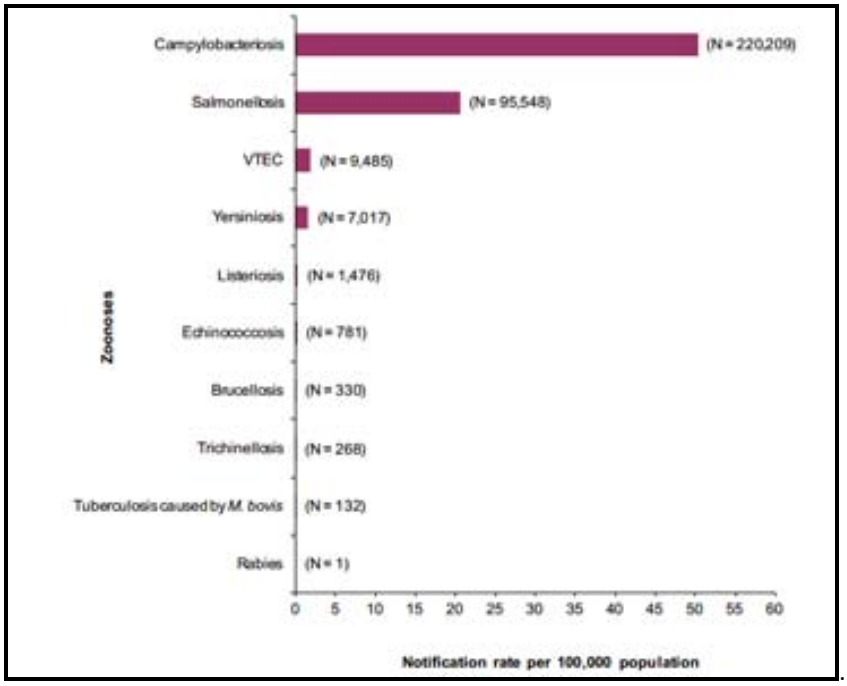
1. Zoonoses

Zoonoses are infections or diseases that can be transmitted directly or indirectly between animals and humans. Between one third and one half of all human infectious diseases have a zoonotic origin. In the past 10 years around 75.00 % of the new diseases that have affected humans have originated from animals or products of animal origin (EFSA, 2013a).

There are multiple ways of zoonosis transmission and depending on these ways the infections are divided in two groups: food-borne zoonotic diseases and nonfood-borne zoonotic diseases. The former include diseases caused by pathogens transmitted through consumption of contaminated food or drinking water, such as *Salmonella*, *Campylobacter*, *Trichinella* and hepatitis A virus. The latter include diseases that are mainly transmissible through direct contact or close proximity with infected animals, such as avian influenza and Q fever, or by vectors such as malaria, West-Nile virus and Lyme disease.

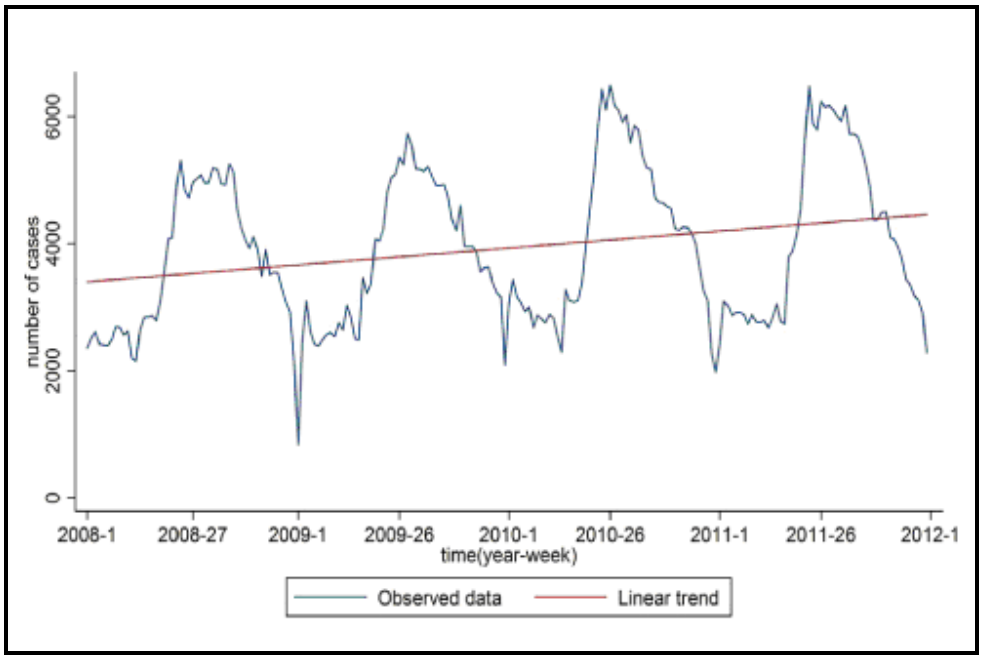
Nowadays, food-borne diseases have acquired considerable importance worldwide. The most important zoonotic pathogens causing food-borne diseases are *Campylobacter*, *Salmonella* and *Escherichia coli* O157:H7 (Figure 1). (Cloeckaert, 2006; Humphrey et al., 2007; Frederick and Huda, 2011). Infections caused by these bacteria are of significant public health concern. The economic impact of *Campylobacter* infections in the European Union has been estimated at approximately 2.4 billion euros per year. For *Salmonella* infections, the estimated impact was around 3 billion euros per year. Based on such economic impact and statistics there is a worldwide interest in lowering *Campylobacter* and *Salmonella* infections.

Figure 1. Reported notification rates of zoonoses in confirmed human cases in the EU, 2011. Note: Total number of confirmed cases is indicated in parenthesis at the end of each bar. EFSA Journal 2013;11(4):3129



In the last 5 years, the Community Zoonoses Reports of the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) confirm campylobacteriosis as the most commonly reported zoonosis in the EU (EFSA, 2013a), with a continuous increase in the last years. A total of 220.209 *Campylobacter* confirmed cases were reported in humans in 2011, which was an increase of 2.20 % compared to 2010 (Figure 2). However, according to EFSA (EFSA, 2013a), a relevant number of clinical cases of campylobacteriosis are under-reported in the EU (27 Member States (MS)). It is estimated that there might be between 2 million and possibly as high as 20 million cases of clinical campylobacteriosis per year in the EU 27 MS. The reported food-borne outbreaks of campylobacteriosis are limited but may be more common than previously suspected. Outbreak investigations suggests chicken as the source of the outbreak in over 25.00 % of *Campylobacter* cases, while in 33.00 % of the cases the source was unknown (EFSA, 2010).

Figure 2. Trend in reported confirmed cases of human campylobacteriosis in the EU, 2008-2011. Source: data for EU trend 24 MSs: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Poland, Romania, Slovakia, Slovenia, Spain, Sweden, and United Kingdom. Bulgaria is excluded because only monthly data were reported. EFSA Journal 2013;11(4):3129.

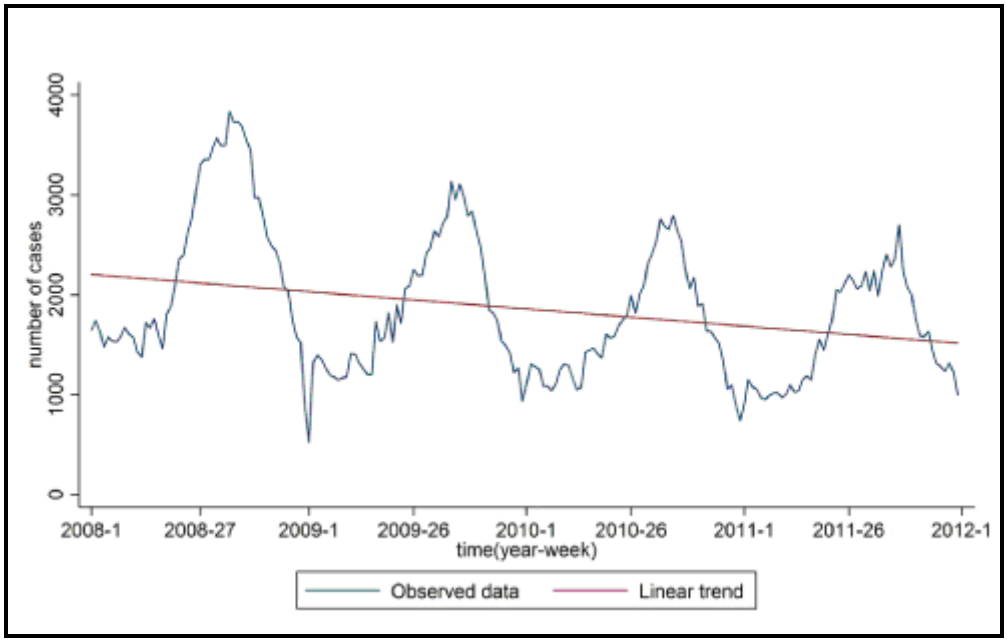


EFSA has emphasized the importance and recommended the establishment of an active surveillance of campylobacteriosis in all MS, including efforts to determine the uncertain and unreported campylobacteriosis cases. Storage and genotyping of human and putative reservoirs of *Campylobacter* isolates in all MS have also been recommended.

Salmonellosis has declined significantly in the last years, despite there is a worldwide increase in the dissemination of *Salmonella enterica*, serotypes Enteritidis and Typhimurium (Figure 3) (EC, 2004). It was still the second most frequently reported zoonotic disease in humans in 2011, with 95.548 reported cases and continued to be the most frequently reported cause of outbreaks of known origin (26.60 % of all outbreaks), followed by bacterial toxins (12.90 %) and *Campylobacter* (10.60 %). The continued decrease in *Salmonella* human cases is a result of the introduction of *Salmonella* control programmes by EU MS and the European Commission, which have

led to a decline in *Salmonella* infections in poultry populations, particularly laying hens and chickens. *Salmonella*, which can cause fever, diarrhoea and abdominal cramps, was most often found in fresh chicken meat, as well as minced chicken meat and chicken meat preparations.

Figure 3. Trend in reported confirmed cases of human salmonellosis in the EU, 2008-2011. Source: data from 25 MSs: Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, and United Kingdom. Bulgaria and Poland are excluded as they reported only monthly data. EFSA Journal 2013;11(4):3129.



A factor contributing to the appearance of these zoonotic pathogens in human populations is the increased contact between humans and wildlife (Daszak et al., 2001). This can be caused either by encroachment of human activity into wilderness areas or by movement of wild animals into areas of human activity. Highly mobile animals such as birds may present a greater risk of zoonotic transmission than other animals due to the ease with which they can move into areas of human habitation. It is therefore of upmost importance to gain insight into the epidemiology of zoonotic pathogens in wildlife, especially wild birds, to determine their role and significance as reservoirs and vectors of disease.

2. *Campylobacter*

2.1. Taxonomy of *Campylobacter*

In 1886 Theodor Escherich described non-culturable spiral-shaped bacteria isolated from the colonic contents of neonates and kittens that have died as a result of "cholera infantum" (Figure 4) (Escherich, 1886). It has been suggested that this was the first reported observation of the bacterium that we now know as *Campylobacter* (Skirrow and Butzler, 2000; Park, 2002). Until the early 1900's, it was not possible culture the bacteria. It was in 1909 when the first pure culture of the bacteria was obtained from the aborted ovine fetuses (McFadyean and Stockman, 1913). Due to their striking morphological similarity to *Vibrio cholerae*, the bacteria were classified as members of *Vibrio* genus. Five years later, Smith discovered spiral bacteria in aborted bovine fetuses and concluded that these strains and the vibrios of McFadyean and Stockman belonged to the same species (Smith and Taylor, 1919), for which he proposed the name *Vibrio fetus*. Closely related organisms were later described as *V. jejuni* isolated from the jejunum of cattle, and *V. coli* from pigs (Doyle, 1944; Jones et al., 1931).

Figure 4. First publication of non-culturable spiral-shaped bacteria isolated from the colonic contents of neonates and kittens (Escherich, 1886).


MÜNCHENER

MEDICINISCHE WOCHENSCHRIFT

(FRÜHER ÄRZTLICHES INTELLIGENZ-BLATT)

ORGAN FÜR AMTLICHE UND PRAKTISCHE ÄRZTE.

Originalien.

In the 1950's Elizabeth King suggested that "vibrios" could be associated with human enteric disease, and was the first to study human strains in detail. She discriminated between *V. fetus* and the thermo-tolerant *V. jejuni* and *V. coli*, though she kept the

provisional names referred as “related-vibrios” (King, 1957). Sebald and Verón formally separated the “related-vibrios” from the *Vibrionaceae* family due to the low G and C base composition of DNA of these microorganisms, their microaerophilic growth requirements, their non-fermentative metabolism and their elevated optimum growth temperature. They proposed the term *Campylobacter*, which is derived from two Greek words meaning “curved rod” (Sebald and Veron, 1963). Ten years later, Véron and Chatelain clarified the taxonomy and considered four distinct species in the genus *Campylobacter*: *Campylobacter fetus, Campylobacter coli, Campylobacter jejuni* and *Campylobacter sputorum* (Véron and Chatelain, 1973).

The failure to culture campylobacters from faeces had been attributed to the overgrowth of competing coliforms and the fastidious nature of campylobacters. This problem was overcomed by the use of a filtration method coupled with growth on selective media (Dekeyser et al., 1972; Butzler et al., 1973). The filtration step allowed the smaller and vigorously motile campylobacters to pass through a 0.65 µM filter while other larger organisms were held back. After the use of this method, certain campylobacters were recognised as potential causative agents of foodborne enteric disease and were successfully cultured from human faeces (Dekeyser et al., 1972; Slee, 1972). In 1977, the isolation of campylobacters on selective agar supplemented with a mixture of vancomycin, polymyxin B, and trimethoprim was improved to the point where a filtration step was no longer needed, thus enabling the routine examination of human faecal samples (Skirrow, 1977).

The adequate isolation procedures allowed the recovery of *Campylobacter* from a variety of human, animal and environmental sources, and gradually new species were proposed. As a result of the description of new species the taxonomy was revised and a new bacterial family was proposed, the *Campylobacteriaceae* family (Vandamme and De ley, 1991). This family contains four genera: *Campylobacter*, the closely related phylogenetic neighbour *Arcobacter*, *Sulfurospirillum* and *Dehalospirillum*.

2.1.1. The genus *Campylobacter*

The taxonomic structure of the genus *Campylobacter* has experienced extensive changes and even some parts of the current genus taxonomy remain a matter of controversy and require further investigation (Debruyne et al., 2005; On, 2001).

Presently, in the genus *Campylobacter* there are 25 described species and 10 subspecies (Table 1).

Members of this genus are small (0.2-0.8 µm x 0.5-5 µm) Gram-negative, thin spirally curved rods. When two or more bacterial cells are grouped together, they form an "S" or a "V" shape of gull-wing. However, aged cells or cells exposed to atmospheric oxygen can take on a coccoid form (Rollins and Colwell, 1986; Bovill and Mackey, 1997). With the exception of *C. gracilis*, they achieve motility by means of a single polar unsheathed flagella at one or both ends, which together with their helical shape, generates a corkscrew-like motion (Ferrero and Lee, 1988). *C. showae* has multiple flagella (Debruyne et al., 2005). All species are nonsporeforming and nonsaccharolytic bacteria with microaerobic growth requirements.

The majority of the species have oxidase activity with the only exception of *C. gracilis*. The relatively small genome of campylobacters explains their inability to ferment or oxidise carbohydrates and in turn their requirement for rich growth media (Griffiths and Park, 1990). Energy is obtained from amino acids or tricarboxylic acid cycle intermediates.

Table 1. *V*alidated species within the genus *Campylobacter*.

***Campylobacter* species**	**References**
C. avium	Rossi et al., 2009
C. canadensis	Inglis et al., 2007
C. coli	Véron and Chatelain, 1973
C. concisus	Tanner et al., 1981
C. cuniculorum	Zanoni et al., 2009
C. curvus	Vandamme et al., 1991
C. fetus subsp. fetus	Véron and Chatelain, 1973
C. fetus subsp. venerealis	Véron and Chatelain, 1973
C. gracilis	Vandamme et al., 1995
C. helveticus	Stanley et al., 1992
C. hominis	Lawson et al., 2001
C. hyointestinalis subsp. hyointestinalis	On et al., 1995
C. hyointestinalis subsp. lawsonii	On et al., 1995
C. insulaenigrae	Foster et al., 2004
C. jejuni subsp. doylei	Steele and Owen, 1988
C. jejuni subsp. jejuni	Steele and Owen, 1988
C. lanienae	Logan et al., 2000
C. lari subsp. concheus	Debruyne et al., 2009
C. lari subsp. lari	Debruyne et al., 2009
C. mucosalis	Roop et al., 1985
C. peloridis	Debruyne et al., 2009
C. rectus	Vandamme and De ley, 1991
C. showae	Etoh et al., 1993
C. sputorum subsp. bubulus	Véron and Chatelain, 1973
C. sputorum subsp. sputorum	Véron and Chatelain, 1973
C. subantarcticus	Debruyne et al., 2010a
C. upsaliensis	Sandstedt and Ursing, 1991
C. ureolyticus	Vandamme et al., 2010
C. volucris	Debruyne et al., 2010b

The optimum growth temperature is 30°C to 37°C. Under unfavourable growth conditions, these microorganisms have the ability to form viable but non-cultivable cells (VBNC) (Portner et al., 2007). Thermophilic *Campylobacter* species are able to grow between 37°C and 42°C, but are incapable to grow below 30°C, due to the

absence of cold shock protein genes which play a role in low-temperature adaption. Thermophilic *Campylobacter* species include *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*, which are of public health importance since they are the causal agents of campylobacteriosis. Among them, the most commonly species associated with human infection are *C. jejuni* followed by *C. coli*. These species account for the 90.00 % of human campylobacteriosis (EFSA, 2013a). It has been suggested that the elevated optimum growth temperature of thermophilic Campylobacters has been developed as a result of the bacteria adapting to warm blooded animals, especially bird hosts (Ketley, 1997). This suggestion is supported by the fact that the temperature of the avian gut is 42°C and that exposure and consumption of poultry is a dominant source of human infection (Friedman et al., 2000; Park, 2002; Manning et al., 2003).

2.2. Isolation and identification

The sensitivity of *Campylobacter* spp. to oxygen and oxidizing radicals has led to the development of several selective media containing one or more oxygen scavengers and selective agents, particularly antibiotics. Depending on the matrix from where *Campylobacter* isolation is attempted, methods may involve a pre-enrichment step in a liquid medium, followed by plating onto an agar medium.

There are several selective broths, e.g., Bolton broth (BB), *Campylobacter* enrichment broth (CEB) and Preston broth (PB). Also, several selective agars have been formulated, such as Preston, charcoal cefoperazone deoxycholate (CCDA) and Butzler agars. The use of CCDA and incubation at 42°C rather than 37°C is usually the methodology of choice since it allows for the isolation of more *Campylobacter* strains (Zanetti et al., 1996).

Alternative and rapid methods have been developed for detecting and confirming *Campylobacter* spp., e. g. those that include fluorescence in situ hybridization (FISH; (Lehtola et al., 2006)), latex agglutination and a physical enrichment method (filtration) that permits the separation of *Campylobacter* from other organisms present in the food matrix (Baggerman and Koster, 1992).

Perhaps the most effective confirmation methods are those based on the PCR, since phenotypic reactions are often atypical and difficult to read. Several species-specific PCR protocols have been developed for the detection and identification of thermophilic campylobacters. Some of them are indicated in Table 2. More recently, real-time PCR methods have been developed that show the potential of detecting as few as 1 cfu in chicken samples, and in less than 2 h (Debretsion et al., 2007).

Table 2. PCRs developed for detection of thermophilic *Campylobacter* spp.

PCR target	Target gene	Primer	Primer sequence	Reference
Campylobacter spp.	16S rRNA	C412F	5'-GGA TGA CAC TTT TCG GAG C-3'	Katzav at al.,2008
		campR2	5'-GGC TTC ATG CTC TCG AGT T-3'	
C. coli and *C. jejuni*	16S rRNA	MD16S1Upper	5'-ATC TAA TGG CTT AAC CAT TAA AC-3'	Denis et al., 2001
		MD16S2Lower	5'-GGA CGG TAA CTA GTT TAG TAT T-3'	
C. coli and *C. jejuni*	*ceuE* (*C. coli*)	COL3Upper	5'-AAT TGA AAA TTG CTC CAA CTA TG-3`	Denis et al., 1999
		MDCOL2Lower	5'-TGA TTT TAT TAT TTG TAG CAG CG-3'	
	mapA (*C. jejuni*)	MDmapA1Upper	5'-CTA TTT TAT TTT TGA GTG CTT GTG-3'	
		MDmapA2Lower	5'-GCT TTA TTT GCC ATT TGT TTT ATT A-3'	
C. lari	16S rRNA	CL594F	5'-CAA GTC TCT TGT GAA ATC CAA C-3'	Linton et al., 1996
		CL1155R	5'-ATT TAG AGT GCT CAC CCG AAG-3'	
C. upsaliensis	16S rRNA	CHCU146F	5'-GGG ACA ACA CTT AGA AATGAG-3'	Linton et al., 1996
		CU1024R	5'-CAC TTC CGT ATC TCT ACA GA-3'	
C. coli, C. jejuni, C. lari and C. upsaliensis	23S rRNA	THERM1	5'-CTT CGC TAA TGC TAA CCC-3'	Fermer and Engvall, 1999
		THERM4	5'-TAT TCC AAT ACC AAC ATTAGT-3'	
C. coli, C. jejuni, C. lari and C. upsaliensis	*lpxA*	Forward primers: lpxAF9625		Klena et al., 2004
		0301	5'-TGC GTC CTG GAG ATA GGC-3'	
		lpxA*C. coli,*	5'-CTT AAA GCN ATG ATA GTR GAY AAR-3'	
		lpxA*C.Jejuni*	5'-AGA CAA ATA AGA GAG AAT CAG-3'	
		lpxA*C. Lari,*	5'-ACA ACT TGG TGA CGA TGT TGT A-3'	
		lpxA*C. upsaliensis*	5'-TRC CAA ATG TTA AAA TAG GCG A-3'	
			5'-AAG TCG TAT ATT TTC YTA CGC TTG TGT G-3'	
		Reverse primers:		
		lpxAR0025	5'-TAG GCA TTA TTT TTA CCC CTA TAG ACA G-3'	
			5'-ACA GGR ATT CCR CGY TTT GTY TC-3'	
		0304	5'-CAA TCA TGD GCD ATA TGA SAA TAH GCC	
		lpxARKK2m	AT-3'	

2.3. Clinical aspects

Infection with enteric campylobacters ranges from a severe inflammatory diarrhoea to a generally mild, non-inflammatory, watery diarrhoea (Butzler and Skirrow, 1979; Walker et al., 1988; van Vliet and Ketley, 2001). The infection usually begins with a prodrome of characteristic acute abdominal pain, often with fever and general malaise.

Campylobacteriosis affects mostly young adults and children, but is also found in older people. In general the infective dose of *Campylobacter* is low. Infections have been induced with as few as 500-800 bacteria. The incubation period prior to the appearance of symptoms usually ranges from 1 to 7 days. Although infection can result in a severe illness lasting more than a week, it is usually self-limiting and the complications are uncommon (Skirrow and Blaser, 1992). Perhaps the most notable complication is Guillain-Barré syndrome, a serious autoimmune disorder of the peripheral nervous system and one of the most common causes of acute flaccid paralysis (Kuroki et al., 1991; Nachamkin et al., 1998).

Most patients infected with *Campylobacter* spp. will recover without any specific treatment other than replacing lost fluids and electrolytes. In more severe cases the treatment of choice are antibiotics, generally macrolides, (fluoro)quinolones, cephalosporins and tetracyclines.

2.4. *Campylobacter* epidemiology

Thermophilic campylobacters are commonly found in food-producing animals and have also been detected in wild birds and in environmental water sources (Humphrey et al., 2007; EFSA, 2013a). In 2011, while *Campylobacter* prevalence in poultry was reported by all MS, only few countries reported data of *Campylobacter* prevalence on animals other than poultry, which included pigs, cattle, sheep and goats. Positive findings in cats and dogs, as well as positive samples from foxes and other unspecified wild animals have also been reported (EFSA, 2013a). The highest mean *Campylobacter*

prevalence reported by EFSA in these animal species was detected in pigs (52.32 %) followed by sheep, cattle and goats with prevalences of 13.45 %, 7.55 % and 3.84 %, respectively (EFSA, 2013a).

Animals rarely succumb to disease caused by thermophilic *Campylobacter*. These human pathogens are considered to be part of the natural intestinal microbiota of a wide range of domestic and wild birds. Also, the digestive tract of healthy cattle has been demonstrated to be a significant reservoir for a number of *Campylobacter* species (Atabay and Corry, 1998), with prevalences ranging from 0 % to 80.00 %.

Contaminated shellfish have also been implicated as a vehicle in the dissemination of campylobacteriosis. Harvesting shellfish from *Campylobacter*-contaminated waters would appear to be the most likely cause of infection (Wilson and Moore, 1996). Consumption of untreated water or rainwater has also been considered as a risk factor for campylobacteriosis (Schorr et al., 1994; Eberhart-Phillips et al., 1997). Other sources include raw milk and contact with domestic animals (Potter et al., 1983; Studahl and Andersson, 2000). However, avian species are the most common carriers of *Campylobacter* due to their higher body temperature (Skirrow, 1977).

Campylobacter prevalence in poultry production depends on the kind of production system. Positive flocks are generally more frequent among organic and free-range chickens than among intensively reared birds, probably due to increased environmental exposure (Hendrixson and DiRita, 2004). The environment is considered to be the most likely source of *Campylobacter* spp. to birds. The transmission within a flock occurs rapidly once individual birds are colonized by *Campylobacter* (Carrillo et al., 2004; Horrocks et al., 2009). Once established, it is very difficult to eliminate. High flock size, environmental water supplies, litter, insects, wild birds, rodents, faecal contact, personnel and other animals, may increase the risk of colonization and dissemination (Aarts et al., 1995; Adkin et al., 2006; Horrocks et al., 2009). Consistent with exposure of the chickens to different environmental sources is the finding that organic and free-range chickens can be colonized with multiple genotypes of *Campylobacter* spp. (Newell and Wagenaar, 2000).

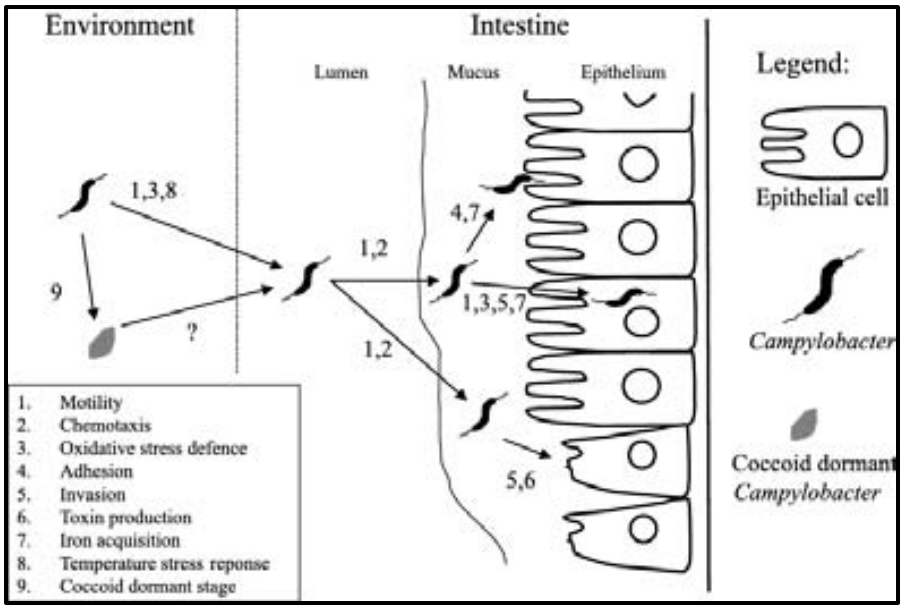
2.5. Pathogenesis

Campilobacteriosis severity depends on the virulence of the strain as well as the host's immune condition. The motility elements of *Campylobacter* (flagella) and chemotaxis capacity (capacity to detect and move up or down chemical gradients) are essential for *Campylobacter* colonization of the small intestine. The flagella are also important for bacterial survival in the various ecological niches encountered in the gastrointestinal tract (Jagannathan and Penn, 2005). Upon infection, *Campylobacter* crosses the mucus layer covering the epithelial cells and adheres to these cells, and a subpopulation subsequently invades the epithelial cells. This invasion can lead to the mucosal damage and inflammation often seen in C*ampylobacter* infection. The essential lesion is an acute inflammatory enteritis, that later affect the colon, the target organ (Poly and Guerry, 2008). Enteritis is probably also consequence of cytotoxins production. Cytolethal distending toxin (CDT) is the best characterized of the toxins produced by *Campylobacter* spp. It has been described as an important virulence factor of this pathogen (Asakura et al., 2008).

Upon entering into the organism, *Campylobacter* gets in contact with the host immune defences. Also, it has to deal with toxic oxygen metabolites produced during normal metabolism and with the limitation of free iron in host tissues. *Campylobacter* spp. is able to use the siderophores ferrichrome and enterochelin produced by other organisms and also haem compounds, which might be released at the site of inflammation (Pickett et al., 1992). The ability of these bacteria to acquire the essential nutrient iron from the host contributes to bacterial pathogenesis.

Campylobacter spp. must be able to respond to a change in temperature. The thermal stress response of bacteria is mostly carried out by the induction of the expression of heat shock proteins (HSPs). These HSPs have an important function in thermotolerance as well as in the response to other stresses by acting as chaperones to promote the folding of most cellular proteins and proteolysis of potentially deleterious, misfolded proteins (van Vliet and Ketley, 2001). All the steps involved in the pathogenesis of *Campylobacter* spp. causing enteritis are shown in the Figure 5.

Figure 5. Overview of the different phases of *Campylobacter* colonization of the intestine. Putative virulence factors are indicated, together with the phase(s) in which these are thought to be expressed (van Vliet and Ketley, 2001).



3. *Salmonella*

3.1. Taxonomy of *Salmonella*

In 1880, Karl Joseph Eberth, observed for the first time *Salmonella spp.* in spleen sections and mesenteric lymph nodes from a patient who died from typhoid fever. Five years later, *Salmonella* was first described by Daniel Elmer Salmon (1850-1914) and Theobald Smith (1859-1934) that discovered a new bacteria isolated from pig with Classical Swine Fever (Salmon and Smith, 1886). The organism was originally called "*bacillus choleraesuis"* that was subsequently changed to "*Salmonella choleraesuis"* by Joseph Léon Marcel Ligniéres (1868-1933), in 1900, in honour to Daniel E. Salmon, who first isolated the bacteria.

The nomenclature of *Salmonella* is complex and continually evolving. Almost a century ago, the Kauffman and White classification system was established and was based on studies on antibody interactions with surface antigens of *Salmonella* organisms. All antigenic formulae of recognized *Salmonella* serotypes are listed in a document named

the Kauffmann-White scheme. *Salmonella* serovars identified after 1966 were designated mainly by their antigenic formula and some clinically important salmonellae identified before 1966 had been given specific names either according to the disease and/or the animal from which the organism was isolated.

On the basis of DNA-DNA hybridization experiments it was demonstrated that all *Salmonella* strains should belong to a single hybridization group, with six subspecies (Crosa et al., 1973). In 1982, Le Minor et al. proposed the name "*Salmonella choleraesuis*" for the single *Salmonella* species (Le Minor et al., 1982). In 1989, due to differences demonstrated by DNA relatedness, one of the subspecies, *Salmonella choleraesuis* subsp. *bongori*, was separated from the other subspecies as a unique *Salmonella* species (Reeves et al., 1989). The Judicial Commission approved in 2002 that from January 2005, "*Salmonella enterica*" would replace "*Salmonella choleraesuis*" to become the type species of the genus *Salmonella* (Anonymous, 2005).

Currently, the nomenclature system used at the Centers for Disease Control and Prevention (CDC) for the genus *Salmonella* is based on recommendations from the World Health Organization Collaborating Centre (WHO). This Centre is responsible for the updating of the scheme. Every year newly recognized serotypes are reported.

3.1.1. The genus *Salmonella*

Salmonella is a genus of rod-shaped, Gram-negative, non-spore forming, non-lactose fermenting, with diameters around 0.7 to 1.5 µm, lengths from 2 to 5 µm. With the exception of the serotypes *S.* Pullorum and *S.* Gallinarum, they are motile with peritrichous flagellae. *Salmonella* can multiply under various environmental conditions outside the living hosts. They are chemoorganotrophs, obtaining their energy from oxidation and reduction reactions using organic sources, and are facultative anaerobes; most species produce hydrogen sulphide. *Salmonella* is oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmons citrate positive, H_2S producing and urease negative. Some of these characteristics are used for biochemical confirmation of *Salmonella*.

Salmonella spp. can grow at a temperature range of 5.5°C to 45.6°C, with an optimum temperature of 35°C to 37°C (Angelotti et al., 1961; Matches and Liston, 1968). Some bacteria of this genus can grow at temperatures as low as 3.5°C (Morey and Singh, 2012). Nevertheless *Salmonella* is able to survive for extended periods in chilled and frozen foods. These bacteria can grow in the presence of 0.40 % to 4.00 % of sodium chloride, in the pH range of 4 to 9 (optimum 6.5 to 7.5) and requires high water activity (0.99-0.94) (Silliker, 1982; Sperber, 1983). *Salmonella* is sensitive to heat (temperature higher than 70°C), although, some rare serotypes such as *S.* Senftenberg are much more heat resistant (54°C). The complete inhibition growth occurs at temperatures < 7°C, pH < 3.8 and/or water activity < 0.94 (Hanes, 2003; Bhunia, 2008).

Salmonella genus comprises two species, *Salmonella bongori* and *Salmonella enterica* (Penner, 1988; Reeves et al., 1989). Both species contain the pathogenicity island SP1, than encodes a number of *Salmonella* virulence traits, only *Salmonella enterica* has acquired a second pathogenicity island SP2 (Bäumler, 1998). *S. enterica* is divided into six subspecies (Grimont and Weill, 2007) (Table 3). Serotypes of the subspecies *enterica*, cause 99.00 % of *Salmonella* infections in humans and higher animals (Uzzau et al., 2000).

Table 3. Species and subspecies of *Salmonella* genus

1. *Salmonella enterica*
Salmonella enterica subsp. *enterica* (I)
Salmonella enterica subsp. *salamae* (II)
Salmonella enterica subsp. *arizonae* (IIIa)
Salmonella enterica subsp. *diarizonae* (IIIa)
Salmonella enterica subsp. *houtenae* (IV)
Salmonella enterica subsp. *indica* (VI)
2. *Salmonella bongori* (V)

Notably, the genus contains over 2600 different serotypes or serovars which are serologically identified by antigenic variation in the O (Lipopolysaccharide, somatic), H (Flagella) and Vi (Capsular) antigens (Madigan et al., 1997; Brenner et al., 2000) .

While some serotypes of *Salmonella* such as *S.* Typhi and *S.* Pullorum have a restricted host range, most serotypes infect a broad range of warm-blooded animals and are capable of causing disease in humans. There are different degrees of adaptation to the host and also the pathogenic potential of the serotypes can differ among humans and animals. *Salmonella* Typhi and *Salmonella* Parathyphi are two serotypes nonpathogenic for animals. However, in humans both have high levels of pathogenicity causing septic syndrome and typhoid fever respectively. On the other hand, two serotypes that produce none or mild symptomatic infections in humans, *S.* Gallinarum and *S.* Abortus-ovis, are responsible of avian typhoid and abortions in small ruminants respectively. *S.* Choleraesuis is a serotype pathogenic to humans and animals, causing severe disease in swine, but also systemic disease in humans. In 2011, the two most commonly reported *Salmonella* serovars were *S.* Enteritidis and *S.* Typhimurium, representing 44.40 % and 24.90 % respectively, of all reported serovars in human-confirmed cases (EFSA, 2013a). Both serotypes are non-symptomatic in birds.

For more detailed studies on taxonomy and pathogenesis, serotypes are further classified by determination of biotypes and phagotypes. The biotype refers to the biochemical variation between organisms belonging to the same serotype, while the phagotype refers to the variation in susceptibility to lyses by bacteriophages between organisms of the same serotype. In *S.* Enteritidis, a phagotype is denoted PT (Phage type), while in *S.* Typhimurium it is called DT (definitive type). *S.* Typhimurium has been debated, in the last decade, due to its ability to become multiresistant to antibiotics and *S.* Enteritidis PT 4 was responsible of the pandemic that has swept over the world during the 90's.

3.2. Isolation and identification

Salmonella detection and isolation is usually performed using a non-selective preenrichment, followed by a combination of two selective enrichments and plating onto two selective media. In samples from hostile environments, bacteria may be feeble or sub-lethally injured and will require a pre-enrichment stage for successful recovery. The use of non-selective preenrichment like buffered peptone water (BPW)

or universal broth is necessary in order to diminish the risk of obtaining false negative results (Gaillot et al., 1999; Maddocks et al., 2002). Following pre-enrichment a selective enrichment stage is typically employed. Some selective enrichments are tetrathionate broth, selenite broth and Rappaport Vassiliadis broth. The latter is the most commonly used. And alternative is the use of Rappaport-Vassiliadis Semisolid Medium (MSRV), which is a modification of Rappaport-Vassiliadis enrichment broth for detecting motile *Salmonella* spp (Hoorfar and Mortensen, 2000; Voogt et al., 2001).

There are a high variety of solid selective media: MacConkey agar, Xylose lysine deoxycholate agar (XLD), Xylose-Lysine-Tergitol 4 (XLT4), Hektoen-Enteric (HE), *Salmonella- Shigella* (SS), Brilliant Green agar (BGA) are some of them. All of these media favour the growth of *Salmonella* and inhibit the growth of undesired bacteria, while favour the visual identification of *Salmonella*.

After *Salmonella* spp. isolation by using selective media, identification is usually performed by phenotypic methods, such as Analytical Profile Index (API) test, Vitek (Biomerieux), or Mucap test. PCR-based methods can also be used, such as that based on the *invA* gene (Malorny et al., 2003).

3.3. Clinical aspects

In humans, *Salmonella* are the cause of two diseases called salmonellosis: enteric fever (typhoid), resulting from bacterial invasion of the bloodstream, and acute gastroenteritis, resulting from a food-borne infection/intoxication. Gastroenteritis is the most common pattern typically caused by Non-typhoidal serotypes. After 8 to 72 h of incubation *Salmonella* produces clinical signs of colitis during five days. The most common symptoms are watery diarrhoea and abdominal pain. Salmonellosis is usually a self-limiting diarrhoeal disease, rehydration with clean drinking water is usually sufficient to remove the bacteria from the site of infection. Most patients recover without antibiotic treatment. However, if the diarrhoea is severe, hospitalization may be required. Children, elderly and patients with immunodeficiency are more susceptible to infection.

Occasionally, bacteria can enter the bloodstream after passing through the intestinal barrier and produce seeding of bacteria in other organs. *Salmonella* Typhi and Paratyphi A, B and C have the ability of causing life threatening systemic infections (Huang and DuPont, 2005). Enteric fever pattern is caused by both serotypes where approximately 10.00 % of patients may relapse, die or encounter serious complications such as encephalopathy, gastrointestinal bleeding and intestinal perforation. This serious pattern is rare and scarce. However, those cases may be fatal if the infection is not controlled by an effective antibiotic therapy (Scherer and Miller, 2001; Hanes, 2003). Ciprofloxacin is often administered at the first sign of severe gastroenteritis whereas ceftriaxone is given to children with systemic salmonellosis.

3.4. *Salmonella* epidemiology

Nontyphoidal salmonellosis has increased worldwide but *Salmonella* typhoid cases are stable with low numbers in developed countries due to improvements in sanitation and water supply, whilst numbers of cases remain high in developing countries. Typhoid fever is endemic throughout Africa and Asia. It also persists in the Middle East, some eastern and southern European countries and central and South America. Typhoid incidence in endemic areas is typically low in the first few years of life, peaking in school-aged children and young adults and then falling in middle age. It usually causes mortality in 5.00 % to 30.00 % of typhoid-infected individuals in the developing world. The World Health Organization (WHO) estimates 16 to 17 million cases occur annually, resulting in about 600.000 deaths.

In 2011, a total of 95.548 confirmed cases were reported by the 27 EU MSs. However, a significant decreasing trends were observed in 10 MSs (Austria, Denmark, Finland, Germany, Greece, Italy, Portugal, Slovakia, Slovenia and Sweden), only one country, France, had a significant increasing trend in salmonellosis cases, could be due to two very large outbreaks of the monophasic variant of *S*. Typhimurium and an increased proportion of *Salmonella* isolates sent to the national reference center for *Salmonella* from 2008 (EFSA, 2013a). It should be noted that the proportion of travel related cases was as usual very high, >70.00 %, in the Nordic countries Finland, Sweden and Norway.

Normally, only large outbreaks are investigated whereas sporadic cases are under-reported.

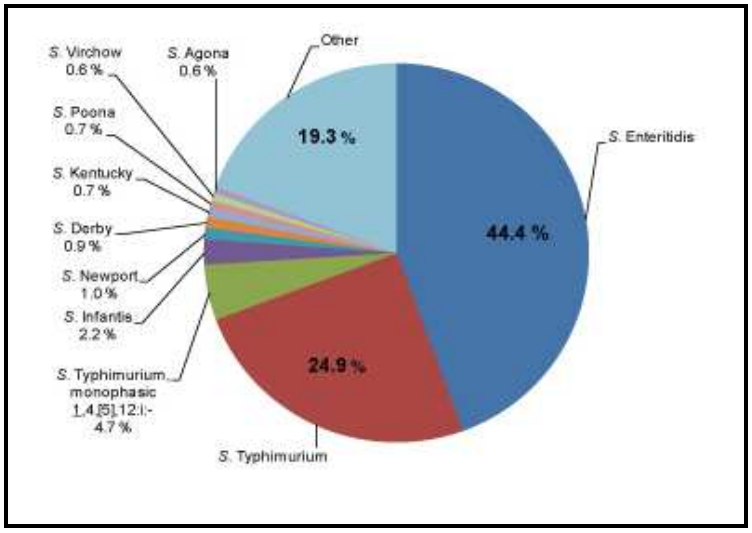
Salmonella spp. are widely distributed in the environment such as in water and soil where it can survive for a long time. Animal reservoirs are infected orally by the environment and/or contaminated feed. Interestingly, *Salmonella* has been isolated from wild birds, demonstrating the ease of routes of transmission and cross-contamination into the environment. The intestinal tract of a wide range of domestic and wild animals is a common reservoir of *Salmonella* which results in a variety of foodstuffs as sources of infections. It can be transmitted to vectors such as rats, flies and birds where *Salmonella* can be shed in their faeces for weeks and even months. The high diversity of environments that could be potential sources of *Salmonella* and also its presence in animals allows for a number of routes of transmission. Surveillance of *Salmonella* infection in wild animals and also in food producing animals is vitally important as these last animals are an important route of transmission into the human food-chain. *Salmonella* infection is complex and difficult to control due to the many areas of exposure.

One of the major routes of *Salmonella* infection in humans is via contaminated food (undercooked food or food that is not washed thoroughly before consumption), especially poultry meat, eggs and egg products. Also, human to human transmission and direct transmission from animals to humans can occur (Darwin and Miller, 1999).

Of the approximately 2500 different *Salmonella* serotypes identified, only a small number are reported with significant frequency. *Salmonella* Enteritidis and *S.* Typhimurium are among the most common serotypes of non-typhoidal salmonellosis in the EU and are in addition the major serotypes in poultry and poultry products (Gurakan et al., 2008; EFSA, 2013a). *S.* Typhimurium is the serotype most often associated with the consumption of contaminated pig, poultry and bovine meat, while *S.* Enteritidis is the most commonly serotype associated with the consumption of contaminated eggs and broiler meat. The latter is the cause of the food-borne salmonellosis pandemic in humans, in part because it has the unique ability to

contaminate eggs without causing discernible illness in the birds infected (Guard-Petter, 2001). However, these both serotypes represent more than 70.00 % of the human confirmed cases in the EU in 2011, other serovars were also involved in human cases, a distribution of the 10 most common serovars detected in humans in the EU in 2011 is shown in Figure 6.

Figure 6. Distribution of the 10 most common *Salmonella* serovars in humans in the EU, 2011.



3.5. Pathogenesis

Pathogenesis in humans depends on the serovar, the strain, the infectious dose, the nature of the contaminated food and the host status.

The bacteria enter in the human digestive tract typically via oral route. A dose of 10^3 - 10^7cells is necessary to cause infection. When *Salmonella* enters the stomach the low pH generally eliminates approximately 99.00 % of *Salmonella* cells (Carter and Collins, 1974). The remaining 1.00 % passes into the small intestine where the bile salts contain surfactant molecules which are broadly antimicrobial. This bacterial genus has adapted to survive in these conditions. The motion of peristalsis in the small intestine maintains *Salmonella* in the gut lumen and approximately 15.00 % of the residual *Salmonella* are retained here, whereas the rest are expelled in the faeces. The survivor bacteria attach to the apical epithelial surface of enterocytes by flagella, apical

appendages and long polar fimbriae and penetrate the intestinal wall to reach the gut associated lymphoid tissue (GALT)(Carter and Collins, 1974). After that, bacteria penetrate the submucosa and establish on the lamina propria. Following adhesion, pathogens invade and destroy M cells located in the Peyer's patches, which triggers an inflammatory response.

Non-typhoidal serotypes multiply in the Peyer's patch tissue where they are drained into the mesenteric lymph nodes. If the host is unable to contain the infection clinical gastroenteritis is presented. The clinical symptoms are due to enterotoxins produced by the bacilli (Chopra et al., 2003).

In *Salmonella* typhoid cases, the bacteria are stopped in the mesenteric lymph nodes, where bacterial multiplication occurs. From there, viable bacteria and LPS (endotoxin) may be released into the bloodstream resulting in septicaemia. The release of endotoxin is responsible for cardiovascular collapse due to its action on the ventriculus neurovegetative centers.

Patients infected with *Salmonella* can become asymptomatic carriers and these individuals excrete large numbers of the bacteria in their faeces, therefore, having the potential to re-infect (Ruby et al., 2012). The carrier state has also been described in livestock animals and is responsible for food-borne epidemics.

4. Antimicrobial resistance

Food animals have long been exposed worldwide to antimicrobials to treat or prevent infectious diseases or to promote growth. The administration of antimicrobials to food animals can select for resistance among bacteria which are subsequently transmitted to humans through food or animal contact. Many of these antimicrobials are similar or even identical to the ones used to treat infections in humans. As a consequence of an indiscriminate use of drugs, antimicrobial resistance (AR) has emerged in zoonotic enteropathogens such as *Salmonella* spp. and *Campylobacter* spp. Other factors contributing to increase the number of AR pathogens is the transfer of resistance

genes and bacteria among animals, animal products and the environment (McEwen and Fedorka-Cray, 2002). Thus, the worrying emergence of AR is one of the major public health problems, since it can compromise both human and animal treatment. It is therefore important for public health safeguards the prudent use of antimicrobials, and the use of quinolones and other antimicrobials only in strictly necessary cases.

It is important to know how to deal with AR bacteria that are already in the environment and whether the removal of antimicrobial agents from the environment could have adverse effects (Casewell et al., 2003). In order to address this issue, in 2011the WHO published several instructions and recommendations for the European MS to establish an "intersectorial and multifaceted approach with effective coordination of action and exchange of information among the agricultural, food, veterinary and health sectors" (WHO, 2011).

4.1. *Campylobacter* antimicrobial resistance

In *Campylobacter* infections when the antimicrobial therapy is needed, the first treatment of choice are macrolides (erythromycin, or one of the newer macrolides, such as clarithromycin or azithromycin) followed by (fluoro) quinolones (Aarestrup et al., 2008; Blaser and Engberg, 2008; Guerrant et al., 2001). An alternative to (fluoro) quinolones use are third-generation cephalosporins. However, these drugs have not been proven effective for treating bacteremia (Pacanowski et al., 2008). Tetracycline, doxycycline, and chloramphenicol are other drugs of choice and in serious systemic infections the use of an aminoglycoside such as gentamicin, or a carbapenem such as imipenem is recommended (Skirrow and Blaser, 2000; Okada et al., 2008). However, the emergence of resistance to some of these antimicrobials in recent years may compromise its effectiveness when needed to treat human infections.

In Spain, after the introduction of enrofloxacin for veterinary use in 1990, in order to prevent respiratory and enteric disease in broilers, laying hens, reproductive chicken and pigs, a marked and rapid increase in rates of quinolone resistance among human isolates of *Campylobacter* have been documented (Velazquez et al., 1995). Before

1990, fewer than 3.00 % of human isolates were resistant to quinolones. A marked jump occurred from 1990 to 1991 with an increase of resistant human isolates from 9.00 % to 39.00 %. The percentage continued increasing, with an 83.00 % in 1996.

Macrolides, especially tylosin, were used as feed additives for livestock and poultry. This use might have been responsible of the macrolide resistance in *Campylobacter* isolates from animals and humans (Reina et al., 1994). Also, some studies revealed high rates of resistance to tetracyclines among human isolates of *Campylobacter* (Nachamkin, 1994).

Since 1995, multiple national surveillance programs have been established for the epidemiological monitoring of *Campylobacter* resistance due to the high levels of resistance to ciprofloxacin and tetracycline, and the emerging macrolide resistance in some regions (Moore et al., 2006; Fitzgerald et al., 2008; Luangtongkum et al., 2009). In the most recent EU summary report on AR in zoonotic and indicator bacteria from humans, animals, and food published in 2013, the highest frequency of resistance among human, animals and food isolates was observed for nalidixic acid (47.80 %) and ciprofloxacin (44.40 %), followed by ampicillin (35.30 %) and tetracycline (30.50 %). Low resistance was observed to erythromycin (3.50 %) and gentamicin (0.40 %) (EFSA, 2013b).

4.2. *Salmonella* antimicrobial resistance

Antimicrobial treatment of salmonellosis in humans is rare and only required in cases of generalized and invasive infection with added complications. Nowadays, the antimicrobial of choice for treatment of severe or invasive *Salmonella* infections in humans are nalidixic acid (a first-generation quinolone) or ciprofloxacin (a second-generation fluoroquinolone) (EFSA, 2009). The second most clinically important group of antimicrobials for the treatment are cephalosporins, especially used in children (EFSA, 2009). Resistance in *Salmonella* to these first-line treatments, resulting in infections with AR strains, may cause treatment failure, which in turn can lead to more severe outcomes in patients.

Salmonella resistance to a single antibiotic was first reported in the early 1960s (Montville and Matthews, 2008). Until 1972, *Salmonella* Typhi strains had remained susceptible to antibiotics, including chloramphenicol (the antibiotic most commonly used against typhoid fever). However in 1972, a widespread epidemic in Mexico was caused by a chloramphenicol-resistant strain of *Salmonella* Typhi. Other chloramphenicol-resistant strains have been isolated in India, Thailand, and Vietnam.

Emerging resistance in *Salmonella* Typhi has been described especially in Africa and Asia and the appearance of *Salmonella* Typhimurium DT104 in the late 1980s raised main public health concern, because of its involvement in diseases in animals and humans. Most *Salmonella* Typhimurium DT104 strains are resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines, thereby threatening the lives of infected individuals due to treatment impossibility (Gross et al., 1998; Montville and Matthews, 2008).

According to data from 2011 published by ECDC, of AR surveillance of human non-typhoidal *Salmonella*, the highest level of resistance in all human *Salmonella* isolates was observed for tetracyclines (27.10 %), closely followed by ampicillin (26.60 %). *S*. Enteritidis and *S.* Typhimurium were the first and the second most common *Salmonella* serovars isolated in 2011 from humans. The highest levels of resistance among *S.* Enteritidis isolates were observed for nalidixic acid (23.20 %) and ciprofloxacin (12.70 %) while in *S.* Typhimurium isolates the most frequently antimicrobial resistance detected were to ciprofloxacin (4.80 %) and to cefotaxime (1.00 %)(EFSA, 2013b).

The multi-resistance Salmonella strains observed in human isolates were generally lower than those observed in turkeys, pigs and pig meat. Compared with broilers and laying hens, however, multi-resistance levels observed in humans were generally higher. Antimicrobials widely used for many years in veterinary medicine to treat bacterial diseases such as ampicillin, sulfonamides and tetracyclines showed resistance levels generally moderate to high among isolates from food-producing animals and meat products. For ampicillin, chloramphenicol, sulfonamides and tetracyclines,

resistance levels were highest in isolates from pigs, followed closely by isolates from turkeys, and then cattle. Isolates from Gallus gallus displayed the least resistance to these antimicrobials. Resistance to third-generation cephalosporins (such as cefotaxime) was detected in Salmonella isolates from turkeys, fowl (Gallus gallus), pigs, cattle and the meat derived from broilers and pigs, but at low or very low levels (EFSA,2013b). In wild animals is rare found *Salmonella* antibiotic resistant isolates and when occur could suggest contamination from a human or domestic animal source (Rolland, 1985).

Increasing *Salmonella* resistance to frontline therapies and even the appearance of emergent multidrug resistant *Salmonella* to extended-spectrum cephalosporins such as ceftriaxone, has elicited global concern (Arlet et al., 2006). Ceftriaxone is a third generation cephalosporin which has been used in the last two decades to treat invasive blood infections in children Thus, there is a need of monitoring multidrug resistance (MDR) in *Salmonella enterica*. A continuous surveillance and sharing of antimicrobial susceptibility data for *Salmonella* among countries worldwide would ensure the effectiveness of control programmes.

5. Molecular typing

A wide variety of bacterial typing systems are currently in use, differing to each other in the ability to discriminate between bacterial strains, in their reliability, in effort required and in the cost. No one technique is optimal for all forms of investigation and the typing technique chosen depend both the study design and the aims of the investigation.

Molecular typing methods fall into two broad categories; phenotypic and genotypic methods. Traditional typing systems have been based on phenotype such as serotype, biotype, phage typing or antimicrobial susceptibility profiles. These methods allow discriminating between bacteria from a single species, because they involve gene expression. Phenotype properties have a tendency to vary, based on changes in growth conditions, growth phase and spontaneous mutation (Pfaller, 1999).

Genotypic methods are based on the analysis of the genetic structure including DNA polymorphisms in restriction patterns and the presence or absence of extrachromosomal DNA. Thus, these methods are less subject to natural variation, although they can be affected by random mutations that may create or eliminate restriction endonuclease sites, insertions or deletions of DNA in the chromosome or the gain or loss of extrachromosomal DNA (Tenover et al., 1997). A brief description of several typing methods available is summarized in Table 4.

Typing of microbial pathogens is particularly important for diagnosis, treatment, and epidemiological surveillance of bacterial infections. Strain typing has also applications in studying bacterial population dynamics. The part that the molecular methods have to play in elucidating bacterial diversity is increasingly important.

Investigators in their studies on microbial pathogens have used a variety of DNA-based methods to genotype. These methods use electric fields to separate DNA into unique patterns or fingerprints that are visualized by staining the DNA with ethidium bromide or by nucleic acid hybridization. More recently, techniques based on indirect measures of genetic sequence (such as enterobacterial repetitive intergenic consensus (ERIC)-PCR and pulsed-field gel electrophoresis (PFGE)) and direct measures of genetic sequence (such as multilocus sequence typing (MLST)) have been developed.

Table 4. Comparison of common bacterial typing techniques based on relative discriminatory power, reproducibility, repeatability, time required, cost and whether they give information on dispersed or focal parts of the genome. Adapted from Foxman et al. (2005).

Typing Technique	Relative discriminatory power	Relative repeatability	Relative reproducibility	Dispersed or focal parts of the genome*	Days required post culture	Relative Cost**
Sequencing of entire genome	High	High	High	Entire genome	Months to years	Very high
Comparative hybridization against array containing entire gene sequence	High	Medium to high	Medium to high	Dispersed	Weeks to months	High
Direct sequencing of one or more genetic regions	Moderate to high (depends on gene choice)	High	High	Focal if only one region	2–3	Equipment: Medium to High Labor and Supplies: Medium to High
Multilocus sequence typing (MLST)	Moderate to high (depends on gene choice)	High	High	Dispersed	3+	Equipment: Medium to High Labor and Supplies: High
Binary typing (presence/absence of selected genes or alleles across the genome)	Moderate to high (depends on gene choice)	High	Potentially High	Dispersed (if chose different genes across the genome)	2–3	Equipment: medium Labor and Supplies: Medium

Continued on following page

Table 4. Continued

Typing Technique	Relative discriminatory power	Relative repeatability	Relative reproducibility	Dispersed or focal parts of the genome*	Days required post culture	Relative Cost**
Pulsed-field gel electrophoresis (PFGE)	Moderate to high (depends on number of bands observed)	Medium=> High (depending on species)	Medium =>High	Dispersed	3	Equipment: High Labor and Supplies: High
Restriction fragment length polymorphism (RFLP)	Moderate to High (depends on number of bands observed)	Medium=>High	Medium	Dispersed	1–3	Medium
Amplification of a single target gene specific to a pathogen	Moderate to high (depends on gene choice)	High	Medium=>High	Focal	<1	Equipment: Low to Medium Labor and Supplies: Low
Amplified fragment length polymorphism (AFLP)	Moderate to high	High	Medium=>High	Dispersed	2	Equipment: Low to Medium Labor and Supplies: Low
Automated ribotyping	Moderate	High	High	Focal	1	Equipment: High Labor and Supplies: High
Ribosomal RNA gel electrophoresis	Moderate	High	High	Focal	1	Equipment: Low Labor and Supplies: Medium

Continued on following page

Table 4. Continued

Typing Technique	Relative discriminatory power	Relative repeatability	Relative reproducibility	Dispersed or focal parts of the genome*	Days required post culture	Relative Cost**
Targeting known repetitive gene sequences (ERIC), (REP), (DRE), BOX, (IS), (PGRS)	Low to moderate	Medium	Low	Generally dispersed	1	Equipment: Low to Medium Labor and Supplies: Low
Random primers (randomly amplified polymorphic DNA (RAPD), arbitrary primed PCR (AP-PCR))	Low to moderate	Low	Low	Dispersed	1	Equipment: Low to Medium Labor & Supplies: Low
Restriction endonuclease on a single amplified product	Low to moderate (depends on amplicon)	High	High	Focal	1–2	Equipment: Low to Medium Labor & Supplies: Low
Plasmid profiles	Low	High	Medium	Focal	1	Equipment: Low Labor & Supplies: Low

*Focal corresponds to interrogating a single loci. Dispersed means multiple loci are interrogated.

**Per isolate costs in US dollars in 2005, assuming all equipment are available, and the investigator has access to automatic sequencing, for PCR reactions are ~$5, PFGE~$20, MLST ~$140, comparative hybridization~$1000 to $2000 and total genomic sequencing (assuming a strain has already been sequenced)~$100,000 to $500,000.

Foxman, B., Zhang, L., Koopman, J.S., Manning, S.D., Marrs, C.F., 2005. Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiologic Perspectives and Innovations 2, 10.

5.1. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is one of the DNA banding pattern-based methods, which classify bacteria according to the size of fragments generated by enzymatic digestion of genomic DNA. This method was developed in 1984 and has since then become the "gold standard" of molecular typing methods. The procedural consist in embedding a bacterial suspension of the organisms mixed with a protease and with a detergent (SDS) in molten agarose, making agarose plugs. The protease-SDS mixture denatures the cell membrane proteins forming holes in the cell allowing the release of the chromosomal DNA. The agarose keeps the DNA embedded in its gel matrix. Next, the plug is washed several times to remove cell debris and proteases. A piece of the plug (approximately 1/3) is cut off and added to a restriction endonuclease(s) mixture which cleaves DNA at a specific sequence resulting in 10-30 DNA fragments ranging from 0.5 to 1000 kb. DNA fragments are then separated by size by pulsed-field gel electrophoresis. The smaller DNA fragments move faster through the agarose than the larger fragments and the result is a pattern of DNA fragments.

Migration distances are compared to reference standards of known molecular weight and a profile for each strain/isolate is obtained. The PFGE pattern from one isolate can be compared to other patterns to determine whether the samples may have originated from a common source. The electrophoretic patterns are visualized following staining of the gels with a fluorescent dye such as ethidium bromide. Gel results can be photographed and the data obtained can be analysed using a commercially software package (Tenover et al., 1995; Tenover et al., 1997; Olive and Bean, 1999).

PFGE is one of the most reproducible and highly discriminatory typing techniques available. This method can be easily applied to different species, all the strains can be typed with good reproducibility and restriction profiles are easily read and interpreted. However, this technique demands a high labour-intensive and also high cost, especially for the equipment, both could be important limitations for many laboratories (Matushek et al., 1996).

5.2. Enterobacterial repetitive intergenic concensus (ERIC)-PCR

Repetitive element polymorphism-PCR typing methods are based on the presence of DNA elements that are repeated throughout the genome of different bacterial species (Versalovic and Lupski, 2002). There are three main sets of repetitive DNA elements detected and used in different bacterial genomes: REP, BOX and ERIC (Versalovic et al., 1991). BOX elements were the first repetitive sequences identified in a Gram-positive organism while REP and ERIC-sequences were originally identified in Gram-negative bacteria and then found to be conserved in all related Gram-negative enteric bacteria and in many diverse, unrelated bacteria from multiple phyla (Hulton et al., 1991; Versalovic et al., 1991; Martin et al., 1992; Olive and Bean, 1999) .

These sequences are used to design primers for PCR amplification, so different size amplicons are generated in the same reaction. The amplified fragments can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint (Versalovic et al., 1994). The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Versalovic et al., 1991; Radu et al., 2002).

ERIC-PCR has been shown to have similar or even better strain differentiation power than other methods such a ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (AFLP) (Niemann et al., 1997; Olive and Bean, 1999; Chmielewski et al., 2002). Several studies have shown ERIC-PCR to have good correlation with PFGE results but, in general, with slightly less discriminatory power and less reproducible (Kidd et al., 2011).

ERIC-PCR is a fast, easy and cheap method especially suitable for outbreak studies since it allows the confirmation of the source of infection and the number of strains involved. However, the poor reproducibility and portability of the ERIC-PCR results

makes very difficult to share this information between laboratories (Foxman et al., 2005).

5.3. Multilocus sequence typing (MLST)

DNA sequence analysis has been used to characterize the genetic relationships and phylogeny of a number of bacterial pathogens. MLST is a molecular typing method that compares DNA sequences from portions of housekeeping or virulence genes and/or rRNA sequences (Maiden et al., 1998). Approximately 450 - 500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. Depending on the species of microorganism, different sets of housekeeping genes are selected as targets for MLTS. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type (ST) (Maiden et al., 1998). In MLST the number of nucleotide differences between alleles is ignored and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites. The great advantage of MLST is that sequence data are unambiguous and the allelic profiles of isolates can easily be compared to those in a large central database via Internet. MLST is an especially useful tool for long term investigation of bacterial population structures.

Epidemiological studies have been benefited from the use of this molecular typing technique due to the high number of epidemiologic applications: epidemiological confirmation linkage in outbreak investigations, generate hypotheses about epidemiologic relationships between bacterial strains in the absence of epidemiologic information and describe the distributions of bacterial types and identifying determinants of those distributions, are some of them.

6. Importance of wild birds as reservoirs and vectors for disease

Wild birds can be infected by pathogenic microorganisms that are transmissible to humans. They can act as important reservoirs for several of these microorganisms, such an arboviruses (e. g. West Nile Virus), influenza virus, enteric bacterial pathogens (e.g. *Salmonella*, *Campylobacter*, pathogenic *E. coli*) and drug resistant bacteria (Reed et al., 2003). Also, the behaviour and feeding habits of wild birds can influence the likelihood of their being infected with enteropathogens. These birds could acquire these pathogenic agents after exposure to human contaminated environments or after scavenging on refuse tips and sewage sludge (Cizek et al., 1994).

Salmonella spp, and *Campylobacter* spp. have been found in a wide range of wild bird species such a raptors, gulls, waterfowl, sparrows and pigeons (Chuma et al., 2000; Broman et al., 2002; Waldenstrom et al., 2007; Molina-Lopez et al., 2011). Some of these wild birds migrate across national and intercontinental borders. This behaviour can contribute to the spread of bacteria and even drug resistant organisms that they harbour (Reed et al., 2003; Botti et al., 2013). One example of the contribution of bird migration and movements in the dispersal of antibiotic resistant *Salmonella* spp. are black headed gulls arriving in Sweden during summer (Palmgren et al., 1997).

Each autumn an estimated 5 billion birds migrate from Eastern Europe to Africa, and approximately the same number goes from North America to Central and South America. The migration patterns are complex and variable between species and even different for distinct populations within the same species. For convenience, different movements have been divided into six main types (de Hoyo et al.,2008):

- Routine movements, which consist in movements centralized on the place of residence (on breeding ground or a stop-over site, during migration). It also includes movements to and from roosting or nesting sites to feeding sites. These movements are common in many species of gulls.

- One-way dispersal movements are typical of young birds after becoming independent. They consist in disperse movements in all directions from the place where they were hatched.
- Migration is the regular seasonal movement, often north and south along a flyway between breeding and wintering grounds. Migration occurs mainly in the same time every year where birds are funnelled on to specific routes by natural barriers such as the Mediterranean Sea.
- Dispersive migration includes post-breeding movements in any direction, as in dispersal movements. However, it also involves a return journey, e.g. altitudinal travels in which mountainous bird species move to lower altitudes during winter
- Irruptions are similar to seasonal migration but vary greatly from year to year in the proportion of birds that leave the breeding range and the distances they travel.
- Nomadism includes movements from one area to another where food is available and breeding if possible.

Migration involves long journeys to cover long-distances which results in physiologic stress in the birds. As a consequence, the birds may be immunosuppressed and their susceptibility to infectious diseases increases. This facilitates migrating birds becoming a reservoir of microorganisms. The different ecosystems where the birds stopover during this travel can also increase the risk of exposure to the abundant reservoir of hosts and vectors of zoonotic pathogens. For these reason the migration could be a mechanism to establish a new foci or reservoir of enteric bacterial pathogens and drug resistant bacteria very far from where the bacteria where picked up.

Besides migration, it is important to consider the routine movements of avian species because these movements can also contribute to the maintenance of the bacteria and drug resistant bacteria in the breeding colony and the feeding sites.

6.1. Seagulls: *Larus michahellis* (yellow-legged gull) and *Larus audouinii* (Audouin's gull)

Seagulls are seabirds of the family Laridae in the sub-order Lari. They are in general medium to large birds, typically grey or white, often with black markings on the head or wings. They have stout, longish peaks and webbed feet. Until the twenty-first century most gulls were placed in the genus *Larus* but this arrangement is now known to be polyphyletic.

The genus *Larus* is a large genus of gulls with worldwide cosmopolitan distribution, especially in the Northern Hemisphere. They breed on every continent, including the margins of Antarctica. Many species breed in costal colonies, with a preference for islands.

Eighteen bird species belonging to the genus *Larus* have been recorded in Spain, including its outlying islands. Two of them, *Larus michahellis* (yellow-legged gull) and *Larus audouinii* (Audouin's gull), are sympatric species that differ considerably with regards to population status, movements and feeding ecology. Whereas yellow-legged gull is abundant, Audouin's gull is an endangered species considered rare in the ICBP (International Council for Bird Prevention).

Yellow-legged gull is very common along the Iberian Peninsula coast, can be found in Europe, the Middle East and North Africa. It is resident in much of southern Europe, on the coasts of the Mediterranean, Black Sea and Caspian Sea, on the Azores and Madeira (Portugal), and on the Canary Islands (Spain). Wintering grounds include the coast of south-west Asia (breeders from the steppes), most of the European coast up to Denmark and the coast of Africa from Western Sahara through the eastern Mediterranean (del Hoyo et al., 1996).

More than 90.00 % of the Audouin's gull European breeding population occurs at just four countries (Spain, Algeria, Greece and Italy) and only a single site located in Spain (Ebro Delta) helds 67.00 % of the global breeding numbers in 2007 (Gutiérrez and

Guinart, 2008). There are also small colonies in Portugal, France, Cyprus, Croatia, Turkey, Tunisia and Morocco (Rubinic and Vrezec, 2001; Gutiérrez and Guinart, 2008; Recorbet and Culioli, 2009). It winters on the coast of North and West Africa and there is a small wintering population in the east Mediterranean along the Aegean coast of Turkey (Sanpera et al., 2007).

According to several studies carried in the Mediterranean region, yellow-legged gull behaves as a generalist species, mainly dependent on food from human activities (Fasola et al., 1989; Bosch et al., 1994), while *L. audouinii* is a pelagic species. Audouin's gull is a rather specialized nocturnal predator on shoaling clupeids (Pedrocchi et al., 1996). Nevertheless, both species show great plasticity in their activity patterns and feeding habits (Oro, 1995; Bosch and Sol, 1996). When the resources are overabundant they can easily compete for the resource use.

Certain feeding habits of gulls (feeding in untreated sewage, garbage dumps and manure) facilitates that these wild birds occupy a habitat that substantially overlaps with human activities. Due to this overlapping, gulls can have considerably higher carrier levels of zoonotic bacteria, compared to other wild bird species, (Kapperud and Rosef, 1983; Cizek et al., 1994; Hubalek et al., 1995). The spread of various zoonotic pathogens by gulls is well documented, especially *Salmonella* spp. and *Campylobacter* spp. (Quessy and Messier, 1992; Olsen et al., 1995; Garza et al., 1997; Broman et al., 2002). Therefore, seagulls might play an important role in the epidemiology of both enteropathogens.

7. Importance of poultry reared outdoors as a reservoir for disease

Over the last decade, the occurrence of enteropathogens in conventional broiler flocks has been intensively studied. Although the occurrence of enteropathogens in poultry reared outdoors has received less attention, the consumer interest in free-range and backyard poultry production is growing. Hence, there is a need to gain insight on the occurrence of food-borne enteropathogens and the presence of drug resistant bacteria in this kind of production system.

Alternative poultry production is regulated by the EU (CEE nº 1906/90 of the Council and 1538/91 of the Commission) and has minimum requirements: low density of birds, slow growing breed birds and continuous access outdoors. Alternative poultry production includes free-range and backyard poultry. The former are birds living in a house with continuous access outdoors during the day. Backyard poultry often consists of free indigenous unselected breeds of various ages, with various species mixed in the same flock (Conan et al., 2012).

Poultry reared outdoors closely interact with humans in the same household as well as with wild birds and other livestock. Consequently, they are exposed to multiple sources of contamination and the transmission of pathogens from the environment is easier than in conventional flocks. However, discrepant results regarding the influence of the different production systems in enteropathogens contamination have been reported (Uyttendaele et al., 1999; Heuer et al., 2001; Bailey and Cosby, 2005; Wittwer et al., 2005). In a study carried out in Belgium, poultry products derived from chicken reared outdoors had a significantly lower contamination rate of *Salmonella* spp. than those from enclosed broilers (Uyttendaele et al., 1999). By contrast, a study in USA reported higher *Salmonella* rates in carcasses from free-range poultry compared to carcasses from conventional chickens (Bailey and Cosby, 2005). Similar results were found in a study carried out in Denmark were the 100.00 % of organic broiler flocks were *Campylobacter* spp positive while the prevalence in conventional flocks was 37.60 % (Heuer et al., 2001). Wittwer et al. (2005) reports no significant differences between both production systems in Switzerland.

While some characteristics of alternative production systems can contribute to the low enteropathogen prevalence detected, such as: the higher age of birds at slaughter would enable the birds to develop a mucosal immune response, reducing *Salmonella* infection; the lower bird density and the reduced stress in birds reared outdoors may contribute to a decrease in the shedding rates and the faecal-oral transmission between animals. Other characteristics, such as close contact with the environment, humans and wild animals, can be involved in the high enteropathogen prevalence in poultry reared outdoors.

More studies are needed to clarify the contribution of this alternative poultry production in the epidemiology of *Salmonella* spp. and *Campylobacter* spp. and its implication in the transmission of both enteropathogens to livestock and humans.

Hyphotesis and Objectives

Objectives

The environment is the most likely source of *Campylobacter* spp. and. *Salmonella* spp. to birds. The close contact of wild birds and poultry reared outdoors with the environment and the non-harmful coexistence between these bacteria and the birds host could be important factors in the epidemiology of *Campylobacter* spp. and *Salmonella* spp. Also, despite the importance that seagulls and other wild birds can have as reservoirs of zoonotic bacteria and antimicrobial resistance traits, there is a lack of knowledge on their relevance as reservoirs of thermophilic *Campylobacter* spp and *Salmonella* spp. in southern Europe. Thus, the aim of this thesis is: a) to gain insight into the epidemiology of thermophilic *Campylobacter* spp. and *Salmonella* spp. in poultry reared outdoors and wild birds in the western Mediterranean and eastern Atlantic Ocean, and to a broader extent, in southern Europe; b) to determine the importance of these domestic and wild birds as reservoirs of antimicrobial resistant bacteria.

In order to achieve these goals, the specific objectives of this thesis are:

1. To determine the prevalence, antimicrobial susceptibility and diversity of *Salmonella spp*. and thermophilic *Campylobacter spp*. isolates in backyard and free-range poultry in Spain.

2. To assess the role of waterfowl from Ebro Delta as a reservoir of *Salmonella spp*. and thermophilic *Campylobacter spp*., as well as of resistant isolates.

3. To determine the prevalence and antimicrobial susceptibility of *Salmonella spp*. and thermophilic *Campylobacter spp*. isolates in two seagull species (yellow-legged gull and Audouin's gull) in colonies from the western Mediterranean and eastern Atlantic Ocean.

4. To assess the genetic diversity of *Salmonella* spp. isolates from those two seagull species and poultry reared outdoors from the western Mediterranean and eastern Atlantic Ocean.

Studies

STUDY I

Campylobacter spp. and *Salmonella* spp. in backyard and free-range poultry in Spain: occurrence, antimicrobial resistance and strain diversity

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Abstract

Poultry have been recognized as the main source of transmission of *Campylobacter* and *Salmonella* infections in humans. However, there is limited information on the presence of food-borne pathogens in poultry reared outdoors. In this study we determined the occurrence and the antimicrobial resistance of *Campylobacter* and *Salmonella* in 71 backyard and free-range poultry farms in Spain. *Salmonella* enterica serovar Kottbus 6,8:e,h:1,5 was isolated in two out of 23 (8.70 %) free-range farms analyzed, while *Campylobacter* was detected in 59 out of 71 farms (83.10 %). A significantly higher *C. jejuni* occurrence compared to *C. coli* was found. The 94.81 % of *Campylobacter* isolates were resistant to at least one antimicrobial agent and 16.88 % of them were multiresistant. The main resistances found were to fluoroquinolones. ERIC-PCR and PFGE analyses showed high diversity of strains. These findings suggest that free-range and backyard farms constitute a reservoir of both *Salmonella* and especially *Campylobacter* strains, including resistant and multi-resistant strains which may be of Public Health concern.

Introduction

Campylobacter spp. and *Salmonella* spp. are the leading causes of zoonotic enteric infections worldwide with an increasing incidence even in countries with adequate public health surveillance (EFSA, 2013a). In several Member States of the European Union, the incidence of campylobacteriosis has surpassed that of salmonellosis in recent years and has become the most commonly reported bacterial gastrointestinal disease (EFSA, 2013a). In 2011, the number of notified cases of thermotolerant *Campylobacter* in the EU increased by 2.20 % compared to 2010 and shows a statistically significant increasing trend in the last four years, 2008-2011 (EFSA, 2013a). Although salmonellosis has declined significantly in the last years, in 2011 it was still the second most frequently reported zoonotic disease in humans. The continued decrease in human cases reflects the results of the *Salmonella* control programmes in intensive poultry industry put in place by EU Member States.

Human campylobacteriosis and salmonellosis are usually characterised by the acute onset of fever, abdominal pain, nausea, diarrhoea and sometimes vomiting. Infections are usually self-limiting and the treatment with antimicrobials is therefore usually not required. In cases of severe enteric disease, effective antimicrobials are essential for treatment and can be life-saving. The treatments of choice are fluoroquinolones and third-generation cephalosporins for adults and children, respectively. Resistance to these first-line treatments, resulting in infections with AR strains, may cause treatment failure (Stoycheva and Murdjeva, 2006).

The transmission of *Campylobacter* and *Salmonella* usually occurs when these bacteria are introduced during food preparation or are allowed to multiply in food and also by direct contact with infected animals or humans, or by contact with contaminated environments. The handling or consumption of raw or undercooked poultry meat has been identified as the main source of *Campylobacter* and *Salmonella* infection (Miller and Pegues, 2000; Adzitey and Nurul, 2011; EFSA, 2013a).

Since the great majority of avian production is intensive poultry production, most of the epidemiological studies on *Campylobacter* and *Salmonella* have been focusing on this kind of production system (Franz et al., 2012; Marinou et al., 2012). However, in the last years in the EU, there has been an increase of an alternative farming husbandry where the animals can roam freely for food, rather than being confined in an enclosure (EFSA, 2013a). Limited data exist on food-borne pathogens in these farming systems where poultry is reared outdoors (Wales et al., 2007; Esteban et al., 2008). Hence, to gain insight into the epidemiology of *Campylobacter* and *Salmonella* in alternative production systems, a study was conducted to investigate the occurrence and the antimicrobial resistance of these zoonotic agents in backyard and free-range poultry farms in Spain.

Material and methods

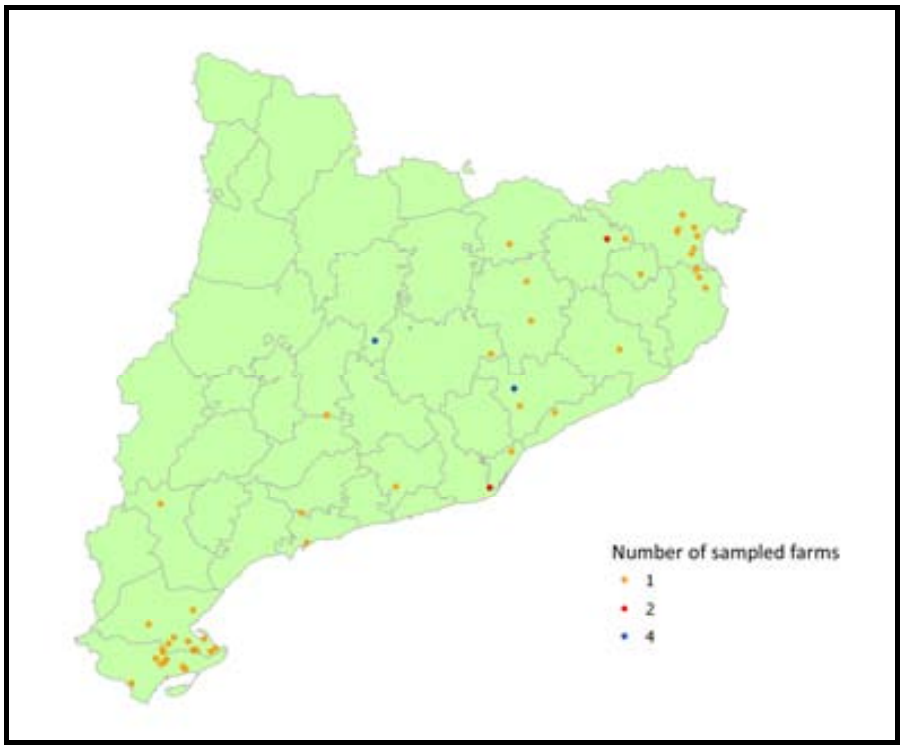
- **Sampling**

A total of 659 birds, including 629 chickens (broilers and laying hens) and 30 ducks, from 71 farms were sampled in Spain between May 2009 and November 2011. Among these, 60 farms were located in north-eastern Spain (Catalonia region) (Figure 1), seven in the south (Málaga, Andalucía region) and four in the north-west (Ons Island, Galicia region). In all the farms, sampled birds had continuous day-time access to open-air runs from the age of four weeks or earlier (weather-permitting). Birds sampled were divided in two groups according to the husbandry: backyard farms (349 chicken from 42 farms) and free-range farms (280 chicken and 30 ducks from 29 farms). Free-range farms where mainly broilers, whereas backyard farms were mainly laying hens and in some cases a mixture of both. Free-reange flocks consisted of 200-500 birds, while in backyard farms were of 4-50 birds. Each farm was sampled at least once and up to 10 birds per farm were sampled (when total number of birds in backyard farms was less than 10, all birds were sampled). Cloacal samples from birds were collected with two sterile swabs and placed in Amies transport medium with charcoal (Deltalab, Barcelona, Spain). Additionally, in free-range farms depending of the size of the farm, up to four samples of fresh droppings were obtained per farm, consisting of a pool of 15 g of fresh faeces. A total of 56 pooled samples were obtained (from both inside the house and in the outside yard). All the samples were kept under refrigeration, transported to the laboratory and processed within 24 h after sample collection. When at least one pool of fresh faeces or a bird was found positive, the farm was considered positive for the pathogen tested.

In order to study the dynamics of *Campylobacter* and *Salmonella* colonization, eight farms (four backyard and four free-range farms) were sampled up to three times from September 2009 to November 2011, with a gap of 2 to 4 months between each sampling. Backyard farms were sampled twice and free-range farms three times. Six farms were located in Catalonia (two backyard and four free-range farms) and two

backyard farms in Galicia (Ons Is.). All the farms had chicken poultry except one free-range from Catalonia with ducks.

Figure 1. Distribution of sampled farms in Catalonia region.



- ***Campylobacter* and *Salmonella* isolation**

For the isolation of *Campylobacter*, pools of 10 g of faecal droppings were enriched in 100 ml of selective Bolton broth (CM0983 with selective supplement SR0183E, Oxoid LTD, Basingstoke, UK), for 48 h (4 h at 37 ºC, followed to further incubation at 42 ºC). Next, one swab was soaked in the culture broth and streaked onto *Campylobacter* blood-free selective agar (mCCDA, modified charcoal cefoperazone desoxycholate agar, CM739 with selective supplement, SR0155E; Oxoid, Basingstoke, UK). Cloacal swabs were directly streaked onto mCCDA. Agar plates were incubated for 48 h at 42 ºC in a microaerobic atmosphere (Anaerocult C, Merck, Darmstadt, Germany). Up to four *Campylobacter*-presumptive colonies per positive bird and fresh faeces samples were subcultured onto blood agar plates (bioMérieux, Marcy l'Etoile, France) and incubated for 48 h at 37 ºC in a microaerobic atmosphere. Isolates with Gram-negative gull-shaped cells, positive reactions in catalase and oxidase tests, and inability to grow

under aerobic conditions at 37 ºC were regarded as *Campylobacter* spp. *Campylobacter* species were identified by PCR with primer pairs specific for *C. jejuni* and *C. coli* (lpxA-Cjejuni: 5'-ACA ACT TGG TGA CGA TGT TGTA-3'; lpxA-Ccoli: 5'-AGA CAA ATA AGA GAG AGA ATC AG-3') and a common reverse primer (lpxARKK2m: 5'-CAC TCA TGD GCD ATA TGA SAA TAH GCC AT-3'). For *C. lari* identification, primer pairs used were: lpxA-Clari (5'-TRC CAA ATG TTA AAA TAG GCG A-3') and lpxARKK2m (Klena et al., 2004).

For *Salmonella* isolation, swabs were enriched in 10 ml of Buffered Peptone Water and 10 g of fresh faeces were enriched in 100 ml of BPW (BPW, Oxoid, Basingstoke, UK) at 37 ºC for 20 h ± 2h. Next, a selective enrichment in Rappaport-Vassiliadis broth (Oxoid, Basingstoke, UK) at 42 °C for 24-48 h was performed, which was then subcultured onto XLT4 (Xylose-Lysine-Tergitol 4, Merck, Darmstadt, Germany) agar; XLT4 plates were incubated at 37 ºC for 24 h. *Salmonell*a-presumptive colonies were subcultured onto MacConkey agar plates and incubated for 24 h at 37 ºC; lactose-negative colonies were confirmed as *Salmonella* spp. with the Mucap (Biolife, Milano, Italy) and indole tests. *Salmonella* serotyping according to the Kauffman-White scheme was carried out at the Departament d'Agricultura, Ramaderia, pesca Alimentació i Medi Natural. Laboratori Agroalimentari (Cabrils, Spain) (Popoff et al., 2001).

- **Antimicrobial susceptibility testing**

Antimicrobial susceptibility of *Campylobacter* and *Salmonella* isolates was performed using the disk diffusion method (Bauer et al., 1966). Briefly, for *Campylobacter* isolates Mueller-Hinton 2 agar with 5.00 % sheep blood (bioMérieux, Marcy l'Etoile, France) was inoculated with a lawn of *Campylobacter* and incubated with antimicrobial disks for 48 h at 37 ºC under microaerobic conditions. *Campylobacter* strains were tested for susceptibility to 7 antimicrobial agents which included three (fluoro) quinolones: nalidixic acid (30µg), ciprofloxacin (10µg) and enrofloxacin (10µg); one aminoglycoside: gentamicin (10µg); one macrolide: erythromycin (15µg); and two other miscellaneus antimicrobials: tetracycline (80µg) and chloramphenicol (60µg).

For *Salmonella* isolates, Mueller-Hinton agar (770305 Difco, Madrid, Spain) was used and plates were incubated for 24 h at 37 ºC. A panel of 18 antimicrobial agents were studied, including three β-lactams: ampicillin (33µg), amoxycillin (30µg) and amoxicillin-clavulanate (30+15µg); one cephalosporin: ceftiofur (30µg); four aminoglycosides: apramycin (40µg), gentamicin (10µg), neomycin (120µg) and streptomycin (100µg); four (fluoro) quinolones: nalidixic acid (30µg); ciprofloxacin (10µg), enrofloxacin (10µg) and norfloxacin (10µg); one polymyxin: colistin (150µg); one phenicol: chloramphenicol (60µg) and four other antimicrobials: tetracycline (80µg), nitrofurantoin (300µg), lincomycin+spectinomycin (15+200µg), and trimethoprim-sulfamethoxazole (5.2+240µg).

The diameter of the bacterial growth inhibition was measured and designated as resistant, intermediate, or susceptible on the basis of Clinical Laboratory Standards (CLSI, 2007).

- **Genotyping**

Genotyping was performed on the subset of isolates obtained from the eight farms which were sampled up to three times, to study the dynamics of *Campylobacter* and *Salmonella* colonization. Both enterobacterial repetitive intergenic consensus (ERIC)-PCR and pulsed field gel electrophoresis (PFGE) was used. ERIC-PCR was used as a screening technique in order to reduce the number of isolates to be tested by PFGE. For each *Campylobacter* species detected, when two or more isolates from the same bird or fresh faeces from a farm showed the same ERIC-PCR band pattern, only one of them was selected for PFGE genotyping.

- **Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR**

ERIC-PCR was performed as previously described (Antilles et al., 2013). DNA was extracted using Instagene Matrix (BioRad, Hercules, CA, USA) and the primers used were ERIC-1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC-2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Versalovic et al., 1991). The Thermal Cycling

System GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) was used for amplification and the annealing temperature was 40ºC.

ERIC-PCR band patterns were normalized, and similarity matrices were calculated using the Dice coefficient with Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Cluster analysis of ERIC-PCR fingerprints was performed by the un-weighted pair group method using average linkages (UPGMA) as previously recommended (Ooyen, 2001). A 2.00 % tolerance level for matching was allowed and the threshold for two isolates to be considered as the same or different strains was set at 90.00 % (Antilles et al., 2013).

- **Pulsed Field Gel Electrophoresis (PFGE)**

PFGE was performed according to the PulseNet standardized protocol "Standardized Laboratory Protocol for Molecular Subtyping of *Campylobacter jejuni* by Pulsed Field Gel Electrophoresis" (www.pulsenetinternational.org). The isolates were analysed using *Sma*I restriction enzyme (Roche Applied Science, Indianapolis, IN) and the resulting PFGE patterns were analysed using the Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Similarity matrices were calculated using the Dice coefficient and cluster analysis was performed by the un-weighted pair group method with arithmetic mean (UPGMA).

- **Statistical analysis**

The associations between isolation results (positive/negative culture to *Campylobacter*) (response variable) and explanatory variables such as species (chicken vs duck) and type of farm (backyard vs free-range farms) were analyzed by means of a Pearson's chi-square test or, when there were less than six observations per category, by the Fisher´s exact test. A Student's t-test for related samples was used to test whether there were differences among *Campylobacter* species occurrence (*C. coli* vs *C. jejuni*). Values with $p < 0.05$ were considered as statistically significant. Statistical analyses were performed using SPSS 15.0 (Statistical Package for Social Sciences (SPSS) Inc., Chicago, IL, USA).

Results

- **Salmonella and Campylobacter occurrence**

Salmonella was isolated only in two out of 23 (8.70 %) free-range farms (chicken and duck farms) from north-eastern Spain, from one bird and one fresh faeces in one farm and only from fresh faeces in the second farm. *Salmonella* isolates were identified as *Salmonella* Kottbus.

Campylobacter was detected in farms throughout the sampling period (from 2009 to 2011), both in backyard farms and in free-range farms (Table 1). It was isolated in 59 out of 71 farms analyzed (83.10 %). The frequency of positive samples within farms ranged from 10.00 to 100.00 %, with a mean occurrence of 39.65 %. *Campylobacter* occurrence was significantly higher in free range compared to backyard farms (p <0.001).

Table 1. Within farm and flock *Campylobacter* occurrence.

	Backyard		Free-range		
	Farms [a]	Birds [b]	Farms	Birds	Fresh faeces
2009	14 /17 (82.35)	36/134 (26.87)	NS[c]	NS	NS
2010	12/16 (75.00)	45/134 (33.58)	14/14 (100.00)	80/140 (57.14)	28/31 (90.32)
2011	7/9 (77.78)	31/81 (38.27)	12/15 (80.00)	82/170 (48.24)	11/25 (44.00)
Total	33/42 (78.57)	112/349 (32.09)	26/29 (89.66)	162/310 (52.26)	39/56 (69.64)

[a] Number of farms sampled: positive/total (%); [b] Number of birds sampled: positive/total (%);[c] NS, Not sampled.

C. jejuni farm occurrence was significantly higher than *C. coli* (p <0.001). In 32 farms (45.07 %; 25 backyard farms and seven free-range farms) only *C. jejuni* was isolated, while in six farms (8.45 %) *C. coli* was the only species found (four backyard farms and two free-range farms). Both *Campylobacter* species were detected in 20 farms (28.17 %; 4 backyard farms and 16 free-range farms) and *C. jejuni*, *C. coli* and *C. lari* were found in one free-range farm (1.41 %) (Table 2). In the majority of backyard farms

(25/42), *C. jejuni* was the species most frequently isolated, while in most of the free-range farms (16/29) both *C. jejuni* and *C. coli* were isolated.

Out of 56 samples of fresh faeces collected from 29 free-range farms, 39 (69.64 %) were *Campylobacter* positive (Table 1). The frequency of *C. coli* and *C. jejuni* were 43.59 % and 35.90 %, respectively. Six (15.38 %) fresh faeces samples were positive to both *C. jejuni* and *C. coli*, while *C. lari* was detected in two fresh faeces from the same farm.

Table 2. *Campylobacter* species distribution within farms.

	Campylobacter species			
	C.jejuni [a]	*C.coli*	*C.jejuni* and *C.coli*	*jejuni, C.coli and C. lari*
Backyard farms	25 (59.52)	(9.52)	4 (9.52)	0 (0.00)
Free-range farms	7 (24.14)	(6.89)	16 (55.17)	1 (3.45)
Total	32 (45.07)	(8.45)	20 (28.17)	1 (1.41)

[a] Number of positive farms (%).

Out of 659 birds sampled, 41.58 % (CI $_{95\%}$: 37.84-45.36 %) (250 chicken and 24 ducks) were *Campylobacter*-positive, with a significant higher occurrence in ducks compared to chickens (80.00 % and 39.75 %, respectively) ($p< 0.001$). In chickens, 185 out of 629 birds sampled were *C. jejuni* positive (29.41 %, CI $_{95\%}$: 25.84-32.96 %), 7.79 % (CI $_{95\%}$: 5.70-9.90 %) were *C. coli* positive and both species were found in 2.54 % (CI $_{95\%}$: 1.28-3.72 %) of the birds. In ducks, 76.67 % were *C. jejuni* and one animal carried two *Campylobacter* species (*C. jejuni* and *C. coli*).

- **Antimicrobial resistance**

The two *Salmonella* Kottbus isolated were resistant to β-lactams (ampicillin, amoxicillin) and tetracycline. One of them was also resistant to nalidixic acid.

A 94.81 % of *Campylobacter* isolates were resistant to at least one antimicrobial. The main resistances detected were to quinolones and fluoroquinolones followed by tetracycline. A 86.04 % of the isolates were nalidixic acid resistant, 77.27 % showed resistance to ciprofloxacin and 19.81 % were enrofloxacin resistant. A 54.47 % of the isolates showed resistance to tetracycline and less than 5.50 % of the isolates were resistant to at least one of the remaining antimicrobials tested. Some isolates (16.88 %) showed multiresistance (resistance to more than 4 antimicrobials), mainly also to quinolones and fluoroquinolones (nalidixic acid, ciprofloxacin and enrofloxacin) and to tetracycline.

- **Longitudinal study**

In order to study the dynamics of *Campylobacter* and *Salmonella* colonization, some farms were sampled up to three times within a period of 12 months (5 months when samplings were performed twice, 8 months when farms were sampled three times). Out of three samplings, only in the second one *Salmonella* Kottbus was isolated in one duck free-range farm from Catalonia. All farms were positive for *Campylobacter* in all samplings, except two farms sampled three times that were negative in the second sampling (Table 3). *C. jejuni* occurrence was higher than that of *C. coli.* Most of the isolates were nalidixic acid and ciprofloxacin resistant (79.75 % and 78.00 %, respectively) (Table 3). In general, there were resistance maintance in all the farms in consecutive samplings, only in the farms 4 and 5 there were a low increase of *Campylobacter* isolates resistant in the second sampling occasion.

Table 3. *Campylobacter* occurrence and AMR in farms with consecutive samplings.

	Farm Type	Sampling	Nº isolates tested	*Campylobacter* species	Antimicrobial agents [c]						
					Ci	En	T	C	E	Gen	Nal
Farm 1[ab]	backyard	1	2	*C. jejuni*	1 (50.00) [d]	0	0	0	0	0	1(50.00)
		2	–	–	–	–	–	–	–	–	–
Farm 2	backyard	1	1	*C. coli*	1 (100.00)	0	1 (100.00)	0	0	0	1 (100.00)
		2	1	*C. jejuni*	1 (100.00)	0	1 (100.00)	0	0	0	1 (100.00)
Farm 3	Free-	1	5	*C. jejuni*	1 (20.00)	0	1 (20.00)	0	0	0	1 (20.00)
	range	2	10	*C. jejuni*	9 (90.00)	1 (10.00)	8 (80.00)	0	0	0	10
		3	6	*C. jejuni*	6 (100.00)	2 (33.33)	5(83.33)	0	1(16.67)	1(16.67)	6 (100.00)
Farm 4	Free-	1	4	*C.coli*	2 (50.00)	0	2 (50.00)	0	0	0	2 (50.00)
	range	2	4	*C.coli / C. jejuni*	3 (75.00)	1 (25.00)	3 (75.00)	0	0	0	4 (100.00)
		3	8	*C.coli / C. jejuni*	8 (100.00)	1(12.50)	8 (100.00)	0	0	0	8 (100.00)
Farm 5	Free-	1	3	*C. jejuni*	3 (100.00)	0	2 (66.67)	0	0	0	3 (100.00)
	range	2	15	*C.coli / C. jejuni*	15	2 (13.33)	13 (86.67)	0	1 (6.67)	1 (6.67)	15
		3	13	*C.coli / C. jejuni*	13	0	9 (69.23)	1	0	0	13
Farm 6	Free-	1	4	*C.coli / C. jejuni*	4 (100.00)	0	4 (100.00)	0	0	0	4 (100.00)
	range	2	11	*C.coli / C. jejuni*	11	1 (9.09)	9 (81.81)	0	0	0	11
		3	9	*C.coli / C. jejuni*	9 (100.00)	0	9 (100.00)	0	0	0	9 (100.00)

Continued on following page

Table 3. Continued

	Farm Type	Sampling	Nº isolates tested	*Campylobacter* species	Antimicrobial agents [c]						
					Ci	En	T	C	E	Gen	Nal
Farm 7	backyard	1	9	*C. jejuni*	9 (100.00)	6 (66.67)	9 (100.00)	0	0	0	9 (100.00)
		2	–	–	–	–	–	–	–	–	–
Farm 8	backyard	1	4	*C. jejuni*	3 (75.00)	1 (25.00)	2 (50.00)	1 (25.00)	1 (25.00)	1 (25.00)	3 (75.00)
		2	4	*C. jejuni*	4 (100.00)	0	2 (50.00)	0	0	0	4 (100.00)
Total					103 (78.00)	15 (9.75)	88 (60.64)	2 (32.69)	2 (48.34)	3 (48.34)	105 (79.75)

[a] Farms 1-6, Catalonia; Farms 7-8, Galicia. [b] Farms 1-3, 5-8 chicken/ hens; Farm 4:ducks. [c] Ci: ciprofloxacin, En: enrofloxacin, T: tetracycline, C: chloramphenicol, E: erytromycin, Gen: gentamicin, Nal: nalidixic acid. [d] Number of positive birds (frecuency).

- **Genotyping**

  - **ERIC-PCR**

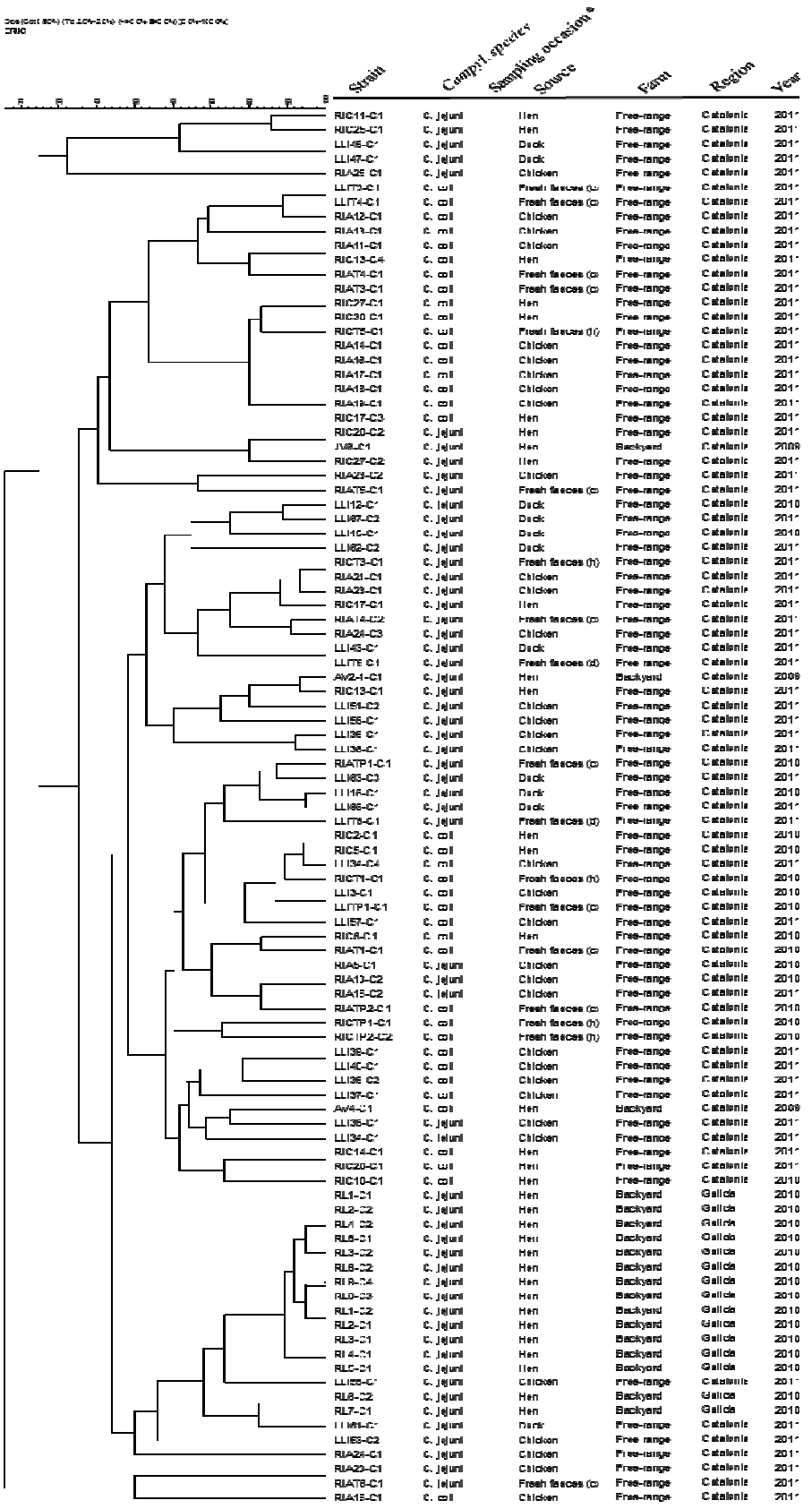
A total of 117 isolates from the eight farms which were sampled 2-3 times were genotyped, one isolate per bird and one isolate per fresh faeces sample. In those cases where more than one *Campylobacter* species was isolated from the birds or the fresh faeces, isolates from the different species were selected for the genotyping studies. A 16.24 % of the 117 isolates were not typeable using ERIC-PCR.

We found 59 different strains, 25 (42.37 %) were *C. coli* and 34 (57.63 %) were *C. jejuni* (similarity 90.00 %) (Figure 2). A total of 38 strains, 24 *C. jejuni* and 14 *C. coli,* were detected more than once: 21 in birds from the same farm and in the same sampling occasion, three in birds from the same farm but different samplings and five strains were found in birds from different farms (two of them were detected in two Catalonia free-range farm with the same owner). The same strains were found not only in fresh faeces and swabs from the same farm but also from different farms.

With a similarity greater than or equal to 50.00 %, eight clusters were obtained. All *C. jejuni* isolates from Galicia clustered together with three *C. jejuni* from Catalonia. Two big clusters encompassed for all *C. coli* from Catalonia, with some exceptions, and most of the *C. jejuni* from Catalonia were included in a single cluster. With regards to bird species, the 13 isolates from ducks were grouped in four different clusters at over 60.00 % similarity, with some of them clustering together with isolates from chickens from Catalonia and in one case with those from Galicia.

Galicia was the location where a lower diversity of strains was found, with 10 out of 15 *C. jejuni* strains showing identical ERIC fingerprint.

Figure 2. Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprints of *C. coli* and *C. jejuni* isolates analyzed by unweighted-pair group method using average linkages (UPGMA) cluster analysis and Dice similarity coefficient. c, fresh faeces from chickens; d, fresh faeces from ducks; h, fresh faeces from hens.



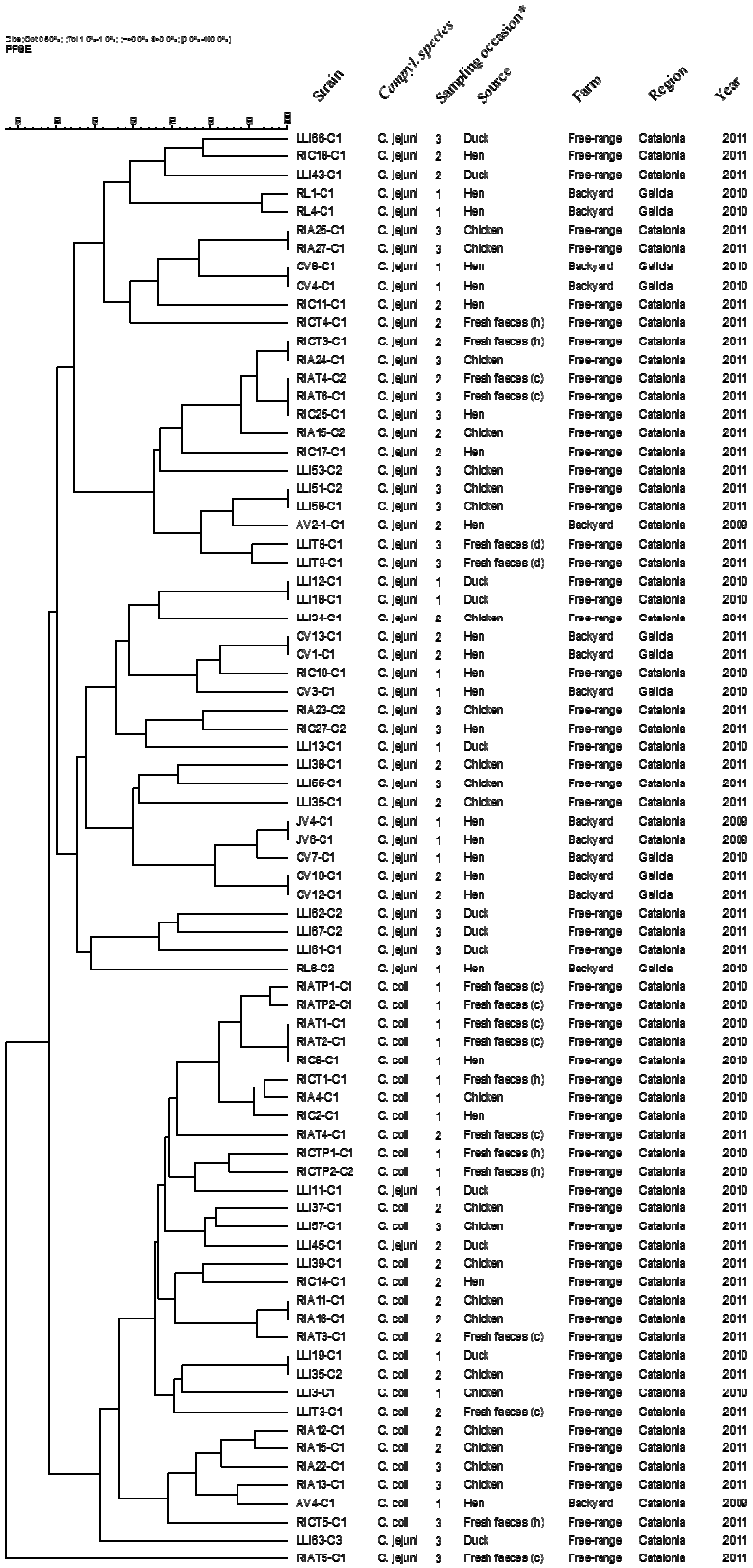
The highest diversity of strains were detected in the second sampling of one free-range farm from Catalonia (RIC, Figure 2) where eight different strains were detected; no common strains to all samplings were found. Some of the RIC strains were also found in other farms (RIA, JV and AV). Farms RIA and RIC belonged to the same owner and some similar strains were detected in both farms.

On the contrary, in the two farms from Lliçà d'Amunt, Catalonia ("LLI" farms: a duck and a chicken farm) which had also the same owner, no common ERIC-PCR fingerprints to both farms were found. Thus, the strains detected in the duck farm were not found in the chicken farm. The same *C. jejuni* strain was found in the first and the third sampling of one farm, In LLI chicken farm no common strains were detected along the different consecutive samplings and the highest diversity of strains was found in the second sampling (seven strains).

- **PFGE**

A total of 78 isolates were genotyped by PFGE (59 different strains detected by ERIC PCR and 19 not typeable by ERIC PCR) (Figure 3). All of the isolates could be typed by this technique and 54 different strains were detected, 35 *C. jejuni* and 19 *C. coli* (similarity 89.00 %). A total of 16 strains were found more than once, eight in birds from the same farm (seven from the same sampling occasion); one strain was detected in two fresh dropping isolates from the same farm, five strains were detected in birds and in fresh faeces (two in the same farm and sampling occasion and three in different farms) and two birds from the same farm and sampling occasion showed the same PFGE pattern than one bird from a different farm.

Figure 3. Pulsed Field Gel Electrophoresis (PFGE) fingerprints of *C. coli* and *C. jejuni* isolates analyzed by unweighted-pair group method using average linkages (UPGMA) cluster analysis and Dice similarity coefficient. * 1st, 2nd and 3rd samling at the same farm; c, fresh faeces from chickens; d, fresh faeces from ducks; h, fresh faeces from hens.



Five clusters were observed with a similarity of 50.00 %. Most of the *C. coli* were grouped in a single cluster, while the *C. jejuni* isolates were distributed in all clusters.

With a similarity of 89.00 % we observed the same PFGE pattern only in farms with the same owner, such as RIA and RIC farms, LLI chicken and duck farms. In the remaining farms, no common strains were detected among farms or even within the same farm during the different sampling occasions. A higher diversity of strains was detected in free-range farms compared to backyard farms. In free-range farms between 12 and 13 different strains per farm were found, while in backyard farms 1 to 7 different strains were detected.

Discussion

Salmonella was only detected in two farms from northeastern Spain. This low occurrence was probably not caused by technical problems. Rather, it might be due to an intermittent shedding of the pathogen, which is reflected by the fact that only two free-range farms (chicken and duck farms) were *Salmonella*-positive. One of them, the duck farm, which was sampled three times, was *Salmonella*-positive only in the second sampling. Low *Salmonella* farm occurrence has also been reported in free-range poultry farms in northern Spain (2.90 %) and in Belgium (1.35 %) (Esteban et al., 2008; Namata et al., 2008). Higher occurrence (10.20 %) has been found in UK (Wales et al., 2007). These findings are opposite to the idea that the risk of contamination with *Salmonella* is thought to be higher in poultry reared outdoors, because of the greater exposure to the environmental contamination (Kinde et al., 1996; EFSA, 2005).

Some characteristics of alternative production systems can also contribute to the low *Salmonella* occurrence detected: the higher age of birds at slaughter would enable the birds to develop a mucosal immune response, reducing *Salmonella* infection; the lower bird density and the reduced stress in birds reared outdoors may contribute to a decrease in the shedding rates and the faecal–oral transmission between animals (Crhanova et al., 2011). The *Salmonella* control programmes implemented in intensive production systems by EU MS and the EC may have also influenced the reduction of

Salmonella prevalence in the alternative production systems, since these systems use chicks/eggs from intensive production.

Salmonella isolated in this study were identified as *Salmonella* Kottbus and to our knowledge; this is the first description of this serotype in birds. *Salmonella* Kottbus has been found in mammals, particularly in foxes and wild boar in other European countries (Handeland et al., 2008; Zottola et al., 2013). It has also been identified as a source of human infection in 2006 causing an outbreak in infants in Gran Canaria (Spain) due to consumption of contaminated bottled water (Palmera-Suarez et al., 2007). It has also been implicated in an outbreak associated with eating alfalfa sprouts in several states in USA (CDC, 2002). *S.* Kottbus should therefore be considered a public health hazard.

Salmonella Kottbus isolates were resistant to amoxicillin, nalidixic acid, tetracycline and ampicillin. This finding is relevant in the context of enteric infections in humans, since multiresistant strains may lead to treatment failure, and thus is of public health concern.

Contrary to *Salmonella*, the lower bird density and the different stresses in birds reared outdoors seems not to have a positive effect in diminishing *Campylobacter* flock contamination, since a high number of *Campylobacter* contaminated farms (83.10 %) were detected. On the other hand, the older the birds, the higher risk of being *Campylobacter* positive, and once birds are positive; there is a continuous shedding of the bacterium (Colles et al., 2009). A high *Campylobacter* occurrence has also been reported in free-range poultry farms within the EU, ranging from 70.00 % to 100.00 % (Heuer et al., 2001; Bull et al., 2006; Esteban et al., 2008). In the present work, *C. jejuni* occurrence was higher than *C. coli* (46.48 vs. 8.45 %). By contrast, a recent report on food-borne pathogens in free-range poultry farms in northern Spain, found that *C. coli* was more prevalent than *C. jejuni* (Esteban et al., 2008). However, higher *C. jejuni* prevalence in free-range farms has been reported in France and Denmark, as well as mixed infections in Denmark (Heuer et al., 2001).

C. jejuni followed by *C. coli* are the most common *Campylobacter* species associated with human enteric infections (EFSA, 2013a). Results indicate that not only intensive poultry production but also free-range farms may be a source of *Campylobacter* infections in humans. *C. lari* has been isolated from intestinal contents of gulls and other animals, river, water fish, shellfish and occasionally human diarrheic faeces. The presence of *C. lari* in caecal content, carcasses and neck skin of broiler flocks is well documented (Hariharan et al., 2009; Di Giannatale et al., 2010; Garin et al., 2012), and few studies describe its isolation from other domestic and wild species (Leatherbarrow et al., 2007). But to our knowledge, this is the first study that reports the presence of *C. lari* in poultry living outdoors in Spain.

Poultry reared outdoors have unrestricted access to the outside environment of the farm. Horizontal transmission from the environment has been pointed out as a likely route of *Campylobacter* infection in birds (Kazwala et al., 1990; Jacobs-Reitsma et al., 1995). Thus, it is likely that in addition to other common sources of *Campylobacter* which are also found in intensive production systems, poultry reared outdoors may be easily colonized by c*ampylobacters* from the external environment. Regardless of the origin of *Campylobacter* colonization in poultry reared outdoors, it is clear that in addition to poultry reared in intensive production systems, those alternative production systems can be also a source of *Campylobacter* infections in humans.

Most of the *Campylobacter* isolates were resistant to at least one antimicrobial agent, and multiresistant strains were also frequently detected. The main resistances found were to fluoroquinolones, which are the antimicrobials of choice to treat severe infections in humans (Stoycheva and Murdjeva, 2006).

Different information is obtained with ERIC-PCR and PFGE techniques. While ERIC-PCR sequences are conserved regions of DNA dispersed throughout the genome of Gram-negative enteric bacteria, PFGE analysis compares the patterns of genomic DNA digested with rare cutting restriction enzymes. It is believed that ERIC-PCR provides a good discrimination power in *Campylobacter* epidemiological studies (Wassenaar and Newell, 2000; Mouwen et al., 2005). However, in this study 16.24 % of the 117 isolates

were not typeable by ERIC-PCR. A selection of the different strains detected by ERIC-PCR and those which were not typeable by this method, were genotyped by PFGE. ERIC-PCR showed the highest diversity of strains. Usually, the same strain was only found in a single farm, but in some occasions the same ERIC-PCR fingerprint was detected in different farms, most of them from the same region. However, in two occasions the same strain was detected in farms from different regions and in different years (RIC20-C2 and JV6-C1, AV2-1-C1 and RIC13-C1), which might indicate a certain circulation of those strains in northeastern Spain. By PFGE, the same strain was detected only within a farm, or between farms having the same owner. This might be due to a cross contamination between farms due to low biosecurity measures, with the farmer facilitating the indirect transmission of *Campylobacter* between farms.

The findings obtained in the present study suggest that poultry reared outdoors are not an important reservoir of *Salmonella* but they are for *Campylobacter*. The diversity of isolates of *Campylobacter* detected in poultry reared outdoors was higher than what can be found in intensive broiler production (unpublish data). The results could be associated to the contact that birds reared outdoors have with the external environment. Poultry reared outdoors harbour antibiotic resistant *Campylobacter*, sometimes with a high prevalence of resistance to certain antimicrobials of common use in human and veterinary medicine. This might be of concern, since it can compromise the effective treatment of bacterial diseases. Thus, monitoring schemes and control strategies are needed in free-range poultry production to reduce the occurrence and carrier levels of *Campylobacter* and consequently the risk of human exposure.

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STUDY II

Free-living waterfowl as a source of zoonotic bacteria in a dense wild bird population area in Northeastern Spain

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Abstract

Salmonella spp. and *Campylobacter* spp. are zoonotic bacteria that represent an economic and public health concern worldwide. Due to the difficulty to collect samples from free-living waterfowl, little is known on their importance as a reservoir of zoonotic agents. Thus, a study was conducted to determine the prevalence, genotypic diversity and antimicrobial susceptibility of *Salmonella* and *Campylobacter* from waterfowl in Ebro Delta (Northeastern Spain), a geographic area with a dense wild bird population. Samples were collected from 318 adult waterfowl belonging to nine fowl species. All the samples were taken during the hunting season from 2008 to 2010. None of the birds were positive for *Salmonella*, while the overall *Campylobacter* prevalence was 12.58 % (40/318). A much higher *Campylobacter coli* prevalence than *Campylobacter jejuni* was found (11.64 % versus 0.94 %). The species *Fulica atra* showed the highest *Campylobacter* prevalence (78.05 %). ERIC-PCR of the isolates showed a high diversity of strains. Antimicrobial susceptibility testing of *Campylobacter* isolates showed that all the isolates were susceptible to the seven antibiotics tested.

Introduction

Salmonella spp. and *Campylobacter* spp. are the leading cause of zoonotic enteric diseases worldwide with an increasing incidence even in countries with adequate public health surveillance (EFSA, 2013a). Both enteropathogens can be transmitted to humans, through the consumption of contaminated food and water, and through the contact with domestic animals. The presence of *Salmonella* and *Campylobacter* in domestic animals is well documented, and particularly, *Campylobacter* is considered part of the normal intestinal microbiota of domestic livestock, including poultry, pigs and cattle (Haruna et al., 2013; Roug et al., 2013). Both enteropathogens are widely distributed in aquatic environments, including sewage and agricultural runoff, and have been isolated from a number of wild animals, especially wild birds (Abulreesh et al., 2006, Waldenström et al., 2007; Andrés et al., 2013). Seagulls in particular, due to

their scavenging feeding habits, are one of the most documented carriers of *Salmonella* and *Campylobacter* (Čížek et al., 1994; Broman et al., 2002).

Both enteropathogens have been isolated from the faeces and/or cloacal swabs of apparently healthy waterfowl worldwide (Luechtefeld et al., 1980; Fallacara et al., 2001), as well as other wild birds (i.e. gulls, pigeons, crows) (Kapperud et al., 1983, Waldenström et al., 2002; Ramos et al., 2010). These observations suggest that there is a non-harmful coexistence between these bacteria and their bird hosts (Luechtefeld et al., 1980; Waldenström et al., 2002). Due to the presence of these two enteropathogens in wild birds, these animals could act as effective spreaders via faecal contamination of pastures and surface waters (Reed et al., 2003). Indeed, an outbreak occurred in Norway caused by untreated drinking water contaminated by pink-footed geese stools (Varslot et al., 1996). Also, a risk of infection may exist when consuming hunted fowl that are carriers of thermophilic campylobacters (Luechtefeld et al., 1980).

The same strains of *Salmonella* and *Campylobacter* have been isolated from humans and wild animals, suggesting that wildlife may serve as a reservoir for gastrointestinal infections in humans (Tauni and Österlund 2000; Colles et al., 2008). However, the occurrence of *Salmonella* and *Campylobacter* in wildlife is probably understated, as few attempts of identifying animal reservoirs have been conducted. Particularly, limited information exists about the occurrence of these zoonotic bacteria in free-living waterfowl. Hence, this study aims to evaluate the prevalence, genetic diversity and antimicrobial susceptibility of *Salmonella* and *Campylobacter* in waterfowl from Ebro Delta (NE Spain).

Material and methods

- S**ampling**

Samples of 318 hunted adult waterfowl were collected at Ebro Delta, a dense wild bird area located in northeast Spain. Sampling was performed during the hunting season (October to February) from end of 2008 to 2011. The following waterfowl species were

sampled: 179 *Anas platyrhynchos*, 54 *Anas crecca*, 41 *Fulica atra*, 26 *Anas clypeata*, five *Anas penelope*, four *Anas ferina*, four *Anas acuta*, three *Netta rufina* and two *Anas strepera*. Faecal samples were collected swabbing twice the cloaca of the birds. Swabs were placed in Amies transport medium with charcoal (Deltalab), kept under refrigeration at 4 ºC and transported to the laboratory, where they were processed within 48 h after sample collection.

- ***Campylobacter* spp. and *Salmonella* spp. isolation and identification**

For the isolation of *Campylobacter*, cloacal swabs were streaked onto *Campylobacter* blood-free selective medium (mCCDA, modified charcoal cefoperazone desoxycholate agar, CM739 with selective supplement, SR0155E; Oxoid, Basingstoke, UK) and incubated for 48 h at 42 ºC in a microaerobic atmosphere (Anaerocult C, Merck, Darmstadt, Germany). *Campylobacter*-presumptive colonies were subcultured onto blood agar plates (bioMérieux, Marcy l'Etoile, France) and incubated for 24 h at 37 ºC in a microaerobic atmosphere. Isolates with Gram-negative gull-shaped cells, giving positive reactions to catalase and oxidase tests, and showing inability to grow under aerobic conditions at 37 ºC were considered as *Campylobacter* spp. *Campylobacter* species were identified by PCR with primer pairs specific for *C. jejuni* (VS-15: 5'-GAA TGA AAT TTT AGA ATG GGG-3' and VS-16: 5'-GAT ATC TAT GAT TTT ATC CTGC-3'), *Campylobacter coli* (CS-F: 5'-ATA TTT CCA AGC GCT ACT CCCC-3' and CS-R: 5'-CAG GCA GTG TGA TAG TCA TGG G-3') and *Campylobacter lari* (CL-55: 5'-ATG GAA GTC GAA CGA TGA AGC GAC-3' and CL-632: 5'-CCA CTC TAG ATT ACC AGT TTC CC-3') (Chuma et al., 2000).

For Salmonella isolation, swabs were pre-enriched in 10 ml of Buffered Peptone Water (BPW, Oxoid) at 37 ºC for 20 h ± 2h. Next, a selective enrichment in Rappaport-Vassiliadis broth (Oxoid) at 42 °C for 24-48 h was performed, and then subcultured onto XLT4 (Xylose-Lysine-Tergitol 4, Merck) agar; plates were incubated at 37 ºC for 24 h. Salmonella-presumptive colonies were subcultured onto MacConkey agar plates and incubated for 24 h at 37 ºC; lactose-negative colonies were confirmed as Salmonella spp. with the Mucap (Biolife, Milano, Italy) and indole tests.

- **Antimicrobial susceptibility testing**

Antimicrobial susceptibility of *Campylobacter* isolates was performed using the disk diffusion method (Bauer et al., 1966). Briefly, Mueller-Hinton 2 agar + 5.00 % sheep blood (bioMérieux) was inoculated with a lawn of Campylobacter and incubated with antimicrobial disks for 48 h at 37 ºC under microaerophilic conditions. The diameter of the bacterial growth inhibition was measured and designated as resistant, intermediate, or susceptible on the basis of Clinical Laboratory Standards (Neo-sensitabs Potency according to CLSI 2006 and Veterinary Practise CLSI 2006). *Campylobacter* strains were tested for susceptibility against seven antimicrobial agents. This panel of antimicrobial agents included three quinolones: ciprofloxacin (10µg), enrofloxacin (10µg) and nalidixic acid (30µg); one aminoglycoside: gentamicin (10µg); one macrolide: erythromycin (15µg); and two other miscellaneus antimicrobials: tetracycline (80µg) and chloramphenicol (60µg).

- **Genotyping of *Campylobacter* spp. Isolates**

To determine the genotypic diversity among strains and the variations in *Campylobacter* populations within an individual host, isolates were genotyped by enterobacterial repetitive intergenic consensus (ERIC)-PCR as previously described (Cerdà-Cuéllar et al., 2010), with minor modifications. Briefly, DNA was extracted using Instagene Matrix (Bio-Rad. Hercules, CA, USA) and the primers used were ERIC-1R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') and ERIC-2 (5'-AAG TAA GTG ACT GGG GTG AGC G-3') (Versalovic et al., 1991). The reaction mix contained 5 µL of 5X Green GoTaq Flexi Buffer (Promega, Madison, WI, USA), 3 µL of 25 mM MgCl2 solution, 1.15 µL of each deoxynucleotide triphosphate (5 mM), 1.5 µL of each primer (20 µM), 0.75 units of Taq DNA polymerase, 100 ng of DNA template and DNA quality water (sufficient to make final volume up to 25 mL). A Thermal Cycling System (GeneAmp PCR System 9700; Applied Biosystems, Foster City, CA, USA) was used for amplification. The cycling conditions used were as follows: one cycle of 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 40 °C for 1 min and 72 °C for 2 min 30 s, with a final extension of 72 °C for 20 min. Amplified products were separated by horizontal electrophoresis through a

2.00 % agarose gel at 60 V for 3 h in Tris-Acetic acid-EDTA buffer. A 2-log DNA ladder (0.1-10.0 kb) (New England Biolabs, Ipswich, UK) was used as a marker. Agarose gel was stained with ethidium bromide (0.05 µg/ml) and amplified products were visualised and photographed under UV light.

Enterobacterial repetitive intergenic consensus-PCR band patterns were normalized, and similarity matrices were calculated using the Dice coefficient with Fingerprinting II v3.0 software (Bio-Rad). A 2.00 % tolerance level for matching was allowed. Cluster analysis of ERIC-PCR fingerprints was performed by the unweighted-pair group method using average linkages (UPGMA) as previously recommended (Ooyen, 2001). Isolates from the same bird showing identical or almost identical profile were considered as the same strain and showed a minimum level of similarity close to 90.00 %. Therefore, the threshold for two isolates to be considered as the same or different strains was set at 90.00 %.

Results

No *Salmonella* was isolated from any of the 318 birds sampled. *Campylobacter* was isolated from five of nine waterfowl species analyzed with an overall prevalence of 12.58 % (40/318). *Campylobacter coli* prevalence was higher than *C. jejuni* (11.64 versus 0.94 %). The frequency of isolation among the different species is shown in Table 1. *Campylobacter jejuni* was only isolated from *A. clypeata*, while *C. coli* was isolated from *F. atra*, *A. platyrhynchos*, *A. crecca* and *A. ferina*. The species *F. atra* showed the highest *Campylobacter* prevalence (78.05 %). All 40 *Campylobacter* isolates tested were susceptible to all of the antimicrobials examined.

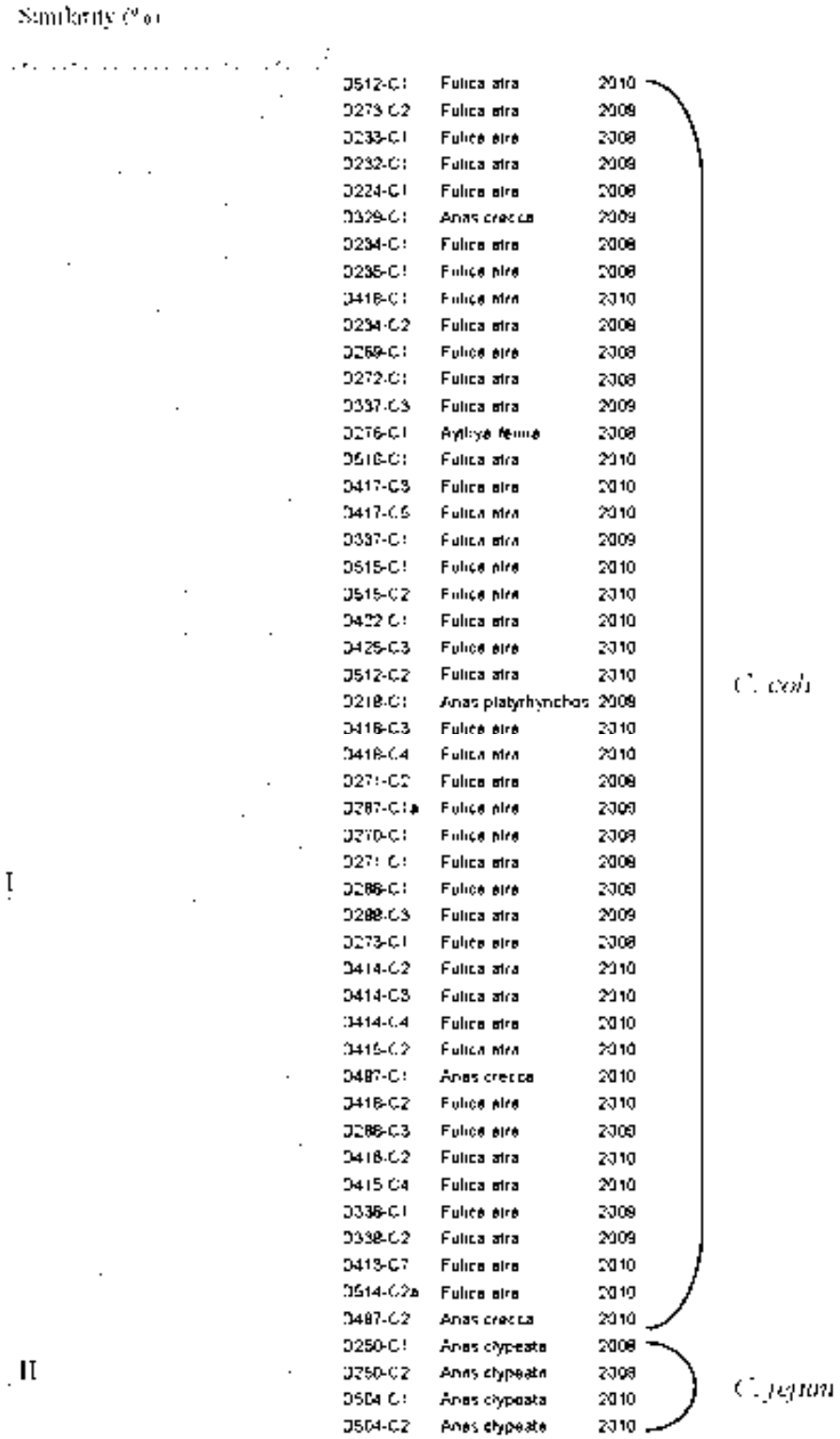
Table 1. *Campylobacter* prevalence in studied waterfowl

Species	N[a]	*C. coli* (%)[b]	*C. jejuni* (%)
Anas platyrhynchos	179	2 (1.12 %)	0
Anas crecca	54	2 (3.70 %)	0
Fulica atra	41	32 (78.05 %)	0
Anas clypeata	26	0	3 (11.54 %)
Anas penelope	5	0	0
Aythya ferina	4	1 (25.00 %)	0
Anas acuta	4	0	0
Netta rufina	3	0	0
Anas strepera	2	0	0
Total	318	37 (11.64 %)	3 (0.94 %)

[a] N, total number of waterfowl sampled. [b] *Campylobacter* positive (prevalence).

Some isolates were not typeable using ERIC-PCR, and therefore could not be included in the study. The ERIC-PCR results revealed two distinct clusters (I and II), one including all *C. jejuni* isolates and the second one comprising all *C. coli* isolates (Figure 1). *Campylobacter jejuni* cluster showed that isolates from the same bird had the same ERIC profile, indicating that the same bird carried a single *C. jejuni* strain. Overall, a higher diversity of strains was obtained in the *C. coli* cluster, although the same individual usually carried a single strain. Also, within the same waterfowl species, same strains were isolated among different individuals sampled during the same year, as well as from birds sampled in different years. Interestingly, the same *C. coli* strain was isolated from different waterfowl species (*F. atra* and *A. crecca*) sampled during the same year.

Figure 1. Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) fingerprints of *Campylobacter coli* (cluster I) and *Campylobacter jejuni* (cluster II) isolates analyzed by unweighted-par group method using average linkages (UPGMA) cluster analysis using Dice similarity coeficient.



Discussion

None of the samples were positive to *Salmonella*. The failure to find *Salmonella* was probably not caused by technical problems. The sampling methods used, with faecal samples from fresh droppings or cloacal swabs, are well established techniques for studying *Salmonella* prevalence in birds (Čížek et al., 1994 and Palmgren et al., 1997). Also, the laboratory methods used, with an enrichment step in Rappaport-Vassiliadis broth and subsequent culturing on XLT4-agar, are extremely sensitive for detecting *Salmonella*, even for samples highly contaminated with other Enterobacteriaceae (Isenberg et al., 1998). The same method of *Salmonella* isolation from other wild birds, such as seagulls, is used in our laboratory, and a prevalence of 11.00 % has been found in the same area (Ebro Delta) (Ramos et al., 2010). Thus, the negative results for *Salmonella* might be due either to an intermittent shedding of the pathogen or its real absence in waterfowl from Ebro Delta. Other studies have also reported a low prevalence (2.00 %) of *Salmonella* from free-living waterfowl (Fallacara et al., 2004).

Among the few studies where *Campylobacter* spp. has been reported in waterfowl, a high variable prevalence has been found, ranging from 0.00 % to 44.00 %. *Campylobacter* prevalence (12.58 %) in waterfowl in the present study was similar or slightly higher to that reported in northern Europe (11.00 % UK, 5.00 % Sweden) (Abulreesh, 2005 and Waldenström et al., 2007). However, in the USA and Taiwan, higher *Campylobacter* prevalence has been reported (41.00 % and 44.00 %, respectively) (Fallacara et al., 2004 and Tsai and Hsiang, 2005). With regard to the *Campylobacter* species detected in this study, *C. coli* was the most prevalent (11.64 %), similarly to other studies on waterfowl in Europe (Waldenström et al., 2007). However, *C. jejuni* was the most common *Campylobacter* species isolated from waterfowl in the USA, with prevalences ranging from 12.90 % to 41.30 % (Luechtefeld et al., 1980, Yogasundram et al., 1989 and Fallacara et al., 2004).

In Ebro Delta, waterfowl share habitat with other wild birds, including several seagull species. However, it seems that *Campylobacter* species carriage by these wild birds show a different pattern: while *C. coli* is mainly isolated from waterfowl, *C. jejuni* is the

most prevalent *Campylobacter* species isolated from Audouin's gull and yellow-legged gull (*Larus audouinii* and *Larus michahellis*, respectively) (unpublished data).

The variation noted in bacterial isolation rates in waterfowl species may be related to differences in feeding habits. The lowest carriage rates were found in species which feed almost exclusively on vegetable matter. In contrast, *F. atra* which has been reported to have coprophagic habits (Vogrin, 1997), had a 78.05 % prevalence of *C. coli*. To our knowledge, this is the first study reporting such a high *Campylobacter* prevalence in this waterfowl species.

The sampling area, Ebro Delta, is the most important agricultural region of Catalonia (NE Spain), with rice being the main crop and a source of feed for wild birds from April to September. It seems likely that coprophagy in *F. atra* most probably occurs only during hard winters and possibly also when access to food is difficult. This correlates with the sampling period (October to February) of this study and may explain the high *Campylobacter* carriage found in this waterfowl species. Differences in *Campylobacter* prevalence related to different feeding habits have also been reported in other wild birds, with insectivores and granivores showing a very low prevalence, while a high prevalence was found in raptors and opportunistic feeders (Waldenström et al., 2002).

Enterobacterial repetitive intergenic consensus-PCR is a useful genotypic method to compare large numbers of *Campylobacter* isolates, as it is not particularly time-consuming, it is relatively easy to perform compared with other DNA techniques and gives a good discrimination between *Campylobacter* isolates. The high strain diversity found among Campylobacter isolates may reflect infection with various *Campylobacter* types from a number of sources. It can also be due to the ease with which *C. coli* and *C. jejuni* can take up DNA from *Campylobacter* species from the environment and integrates the DNA into the genome (Wang and Taylor, 1990); these changes can occur within the intestinal tract of the infected animal.

All *Campylobacter* isolates were susceptible to all of the antimicrobials examined. This is the result expected for wild birds because they do not naturally come into contact

with antibiotics. Similar results were reported by Fallacara et al. (2004), indicating that the majority of the isolates from waterfowl were susceptible to the antibiotics tested.

It is well established that wild birds, including waterfowl, can act as carriers of zoonotic bacteria and contribute to their dispersal in the environment (e.g. water sources, recreational waters and pastures) through faecal shedding. As a consequence, waterfowl in the studied area may play a role in disease transmission to sympatric livestock (mostly avian species reared outdoors), with which they share habitat. Therefore, the high *Campylobacter* prevalence in certain waterfowl species studied and the fact that waterfowl is abundant in the studied area is of prime concern. Also, as waterfowl species are hunted every year at Ebro Delta for home consumption, eating such meat may lead to health related issues. Game meat aimed for human consumption should be examined, and it is strongly recommended that hunters manipulate animals and carcasses under maximal hygienic conditions to avoid environmental contamination and human contagion. Moreover, consumers should follow strict hygiene and food safety practices to avoid potential health hazards associated with the handling, preparation or consumption of waterfowl meat.

Data provided in this study emphasize on the importance of certain waterfowl species as contributors of *Campylobacter* to the environment and as a source of infections for domestic animals and humans. This research also highlights the importance of practicing good hygiene when manipulating hunted waterfowl to avoid transmission of zoonotic bacteria to humans and among animal populations.

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STUDY III

Occurrence, risk factors and antimicrobial resistance of Salmonella spp and Campylobacter spp in seagull colonies of the Western Mediterranean and Eastern Atlantic coasts

In preparation

Abstract

Campylobacter spp. and *Salmonella* spp. are the two most frequent zoonotic bacteria involved in human enteric infections in the UE. Both enteropathogens have been isolated from a great number of wild birds in Northern Europe, including seagulls, but information from southern Europe is scarce. This study was conducted to determine the *Campylobacter* and *Salmonella* prevalence and the antimicrobial resistance pattern in two species of seagulls, yellow-legged gulls (*Larus michahellis*) and Audouin's gulls (*Larus audouinii*), in breeding colonies of Spain and Tunisia. Moreover, risk factors that may be associated with the frequency of occurrence of these organisms were studied. A total of 1785 seagull's fledglings (1,222 *L. michahellis* and 563 *L. audouinii*) from nine colonies were sampled during the late chick-rearing period between 2009 and 2011. Overall, *Salmonella* spp. and *Campylobacter* spp. occurrences were 20.84 % and 5.21 %, respectively. A high diversity of *Salmonella* serotypes was isolated, being the most frequent serotypes those also reported in human outbreaks. *S.* Typhimurium was the most prevalent serotype. The most predominant *Campylobacter* species was *C. jejuni* (94.60 %). A 51.50 % of *Salmonella* isolates were resistant to at least one antimicrobial agent and 21.12 % were multiresistant. A 20.20 % of *Campylobacter* isolates showed resistance to at least one antimicrobial agent. Seagulls may contribute to spread *Salmonella* and *Campylobacter* resistant strains that could be a risk for the public health.

Introduction

The most frequent zoonoses in developed countries are foodborne infections caused by species of *Salmonella* and *Campylobacter*. The incidence of *Campylobacter* infections in humans is increasing, even exceeding *Salmonella* infections (EFSA, 2013a). These infections are often self-limiting and antimicrobial treatment usually is not required. However, in those cases of severe enteric disease, the use of effective antimicrobials is essential. Fluoroquinolones and third-generation cephalosporins are the treatment of choice (EFSA, 2009).

Despite the health impact of these enteropathogenic bacteria, their full epidemiological pathways leading to infection in humans have not been yet elucidated. Both *Salmonella* and *Campylobacter* can be transmitted to humans through the consumption of contaminated food and water, and through the contact with infected domestic animals. Fresh chicken meat, minced chicken meat and chicken meat preparations are the foodstuffs in which both enteropathogens are most frequently reported in EU (EFSA, 2013a). Even though poultry is considered to be a major source of these foodborne pathogens, it is evident that other reservoirs also exist (Sacks et al., 1986; Tomar et al., 2006). Wild birds have been considered important reservoirs of human infectious agents. Particularly, migrating birds are species that cyclically cross one or more national boundaries and use a variety of habitats including wetlands, marshes and other water bodies. Given their ability to fly freely and cover long distances during annual migrations, migratory birds can potentially play a relevant role in the dissemination of these enteropathogenic bacteria (Hubalek, 2004; Sensale et al., 2006; Waldenström et al., 2007).

Compared with other migratory wild bird species, seagulls can carry numerous zoonotic bacteria, probably due to their scavenging feeding habits (Kapperud and Rosef, 1983; Cizek et al., 1994; Hubalek et al., 1995). Laridae are marine birds which occupy habitats that often overlaps with human activities and are reported to spread various animal pathogens (Olsen et al., 1995; Garza et al., 1997). During the last decades, a dramatic increase of populations of seagulls species has occurred throughout Australia, North America, and Europe. This fact has led to an increasing number of studies concerning seagulls and environmental public health risks (Smith and Carlile, 1993; Vidal et al., 1998). However, the information on zoonotic bacteria in seagull colonies in Southern Europe or in the Mediterranean Basin is very limited.

On the Mediterranean coast, there are important colonies of *Larus michahellis* (yellow-legged gulls) and *Larus audouinii* (Audouin's gulls). yellow-legged gulls can also be found in the rest of Europe, in the Middle East and North Africa. The yellow-legged gull is considered a generalist species mainly feeding on fish and marine invertebrates, but also on a number of terrestrial vertebrates and invertebrates as well as on resources

derived from human activities, such as waste from refuse dumps (Olsen and Larsson, 2004). Audoun's gull was historically thought to feed far out to sea, but more recent observations show that it feeds regularly along the coast. The diet consists mostly of epipelagic fish, especially clupeiformes, some aquatic and terrestrial invertebrates, small birds and plants (Mañosa et al., 2004). However, Audouin's gulls may also occasionally feed on food discarded at tourist beaches (Cramp and Simmons, 1983; Christel et al., 2012).

This work aims to study the frequency of occurrence of *Salmonella* spp. and thermophilic *Campylobacter*, in seagulls at several breeding colonies, their antimicrobial susceptibility and the influence of some factors such as species, location or presence of other enterobacteria on *Salmonella* occurrence.

Material and methods

- **Study area**

The study was carried out in nine seagull colonies along the western Mediterranean and in the eastern Atlantic Ocean: Medes Is., Ebro Delta, Columbretes Is., Dragonera Is., Alboran Is., Ons Is, Lanzarote Is., Tenerife Is. and Zembra Is. All of them are considered important reserves for breeding and migratory seagulls. The location and the number of breeding pairs of each gull species in each colony are described in Figure 1.

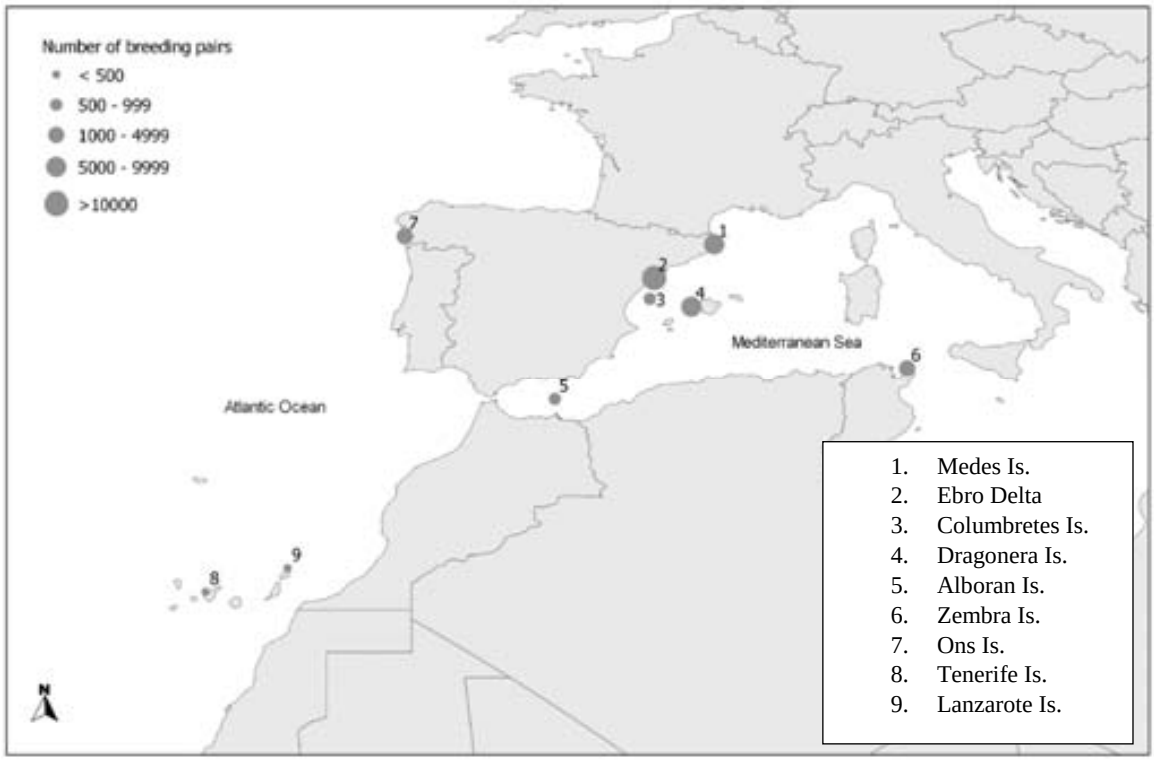
- **Sampling**

A total of 1,785 fledglings from yellow-legged gulls (N=1222) and Audouin's gulls (N=563) were sampled during the late chick-rearing period in 2009, 2010 and 2011 at the nine colonies along the western Mediterranean and in the eastern Atlantic Ocean. Ebro Delta was the only site where both seagull species were sampled. Audouin's gulls were also sampled in Alboran Is. Yellow-legged gull fledglings were sampled in all sites

but Alboran Is. Nests in each colony were randomly sampled. A single fledgling from each brood was captured, sampled and marked.

Duplicate cloacal swabs from each chick were taken and placed in Amies transport medium with charcoal (Deltalab, Barcelona, Spain). All the samples were kept under refrigeration and transported to the laboratory where they were processed.

Figure 1. Map locations of the studied seagull colonies along the western Mediterranean and the eastern Atlantic ocean.



- ***Campylobacter* and *Salmonella* isolation**

Isolation was performed by directly streaking cloacal swabs onto *Campylobacter* blood-free selective agar (mCCDA, modified charcoal cefoperazone desoxycholate agar, CM739 with selective supplement, SR0155E; Oxoid, Basingstoke, UK). Plates were incubated for 48 h at 42 ºC in a microaerobic atmosphere (Anaerocult C, Merck, Darmstadt, Germany). *Campylobacter*-presumptive colonies were subcultured onto duplicated blood agar plates (bioMérieux, Marcy l'Etoile, France) and incubated for 24 h at 37 ºC in a microaerobic atmosphere and in aerobiosis. Those isolates that

presented Gram-negative gull-shaped cells, positive reactions to catalase and oxidase tests, and inability to grow under aerobic conditions at 37 ºC were regarded as *Campylobacter* spp. *Campylobacter* species were identified by PCR with primer pairs specific for *C.jejuni* (VS-15: 5'-GAA TGA AAT TTT AGA ATG GGG- 3' and VS-16: 5'- GAT ATC TAT GAT TTT ATC CTGC- 3'), *C. coli* (CS-F: 5' - ATA TTT CCA AGC GTC ACT CCCC- 3' and CS-R: 5' - CAG GCA GTG TGA TAG TCA TGGG- 3') and *C. lari* (CL-55: 5-ATG GAA GTC GAA CGA TGA AGC GAC-3and CL-632: 5-CCA CTC TAG ATT ACC AGT TTC CC-3) (Chuma et al., 2000).

For *Salmonella* isolation, swabs were enriched in 10 ml of Buffered Peptone Water (BPW, Oxoid, Basingstoke, UK) at 37 ºC for 20 h ± 2h. Next, a selective enrichment in Rappaport-Vassiliadis broth (Oxoid Basingstoke, UK) at 42 °C for 24-48 h was performed, which was then subcultured onto XLT4 (Xylose-Lysine-Tergitol 4, Merck, Darmstadt, Germany) agar; XLT4 plates were incubated at 37 ºC for 24 h. *Salmonell*a-presumptive colonies were subcultured onto MacConkey agar plates and incubated for 24 h at 37 ºC; lactose-negative colonies were confirmed as *Salmonella* spp. with the Mucap (Biolife, Milano, Italy) and indole tests. *Salmonella* serotyping was carried out at the Departament d'Agricultura, Ramaderia, pesca Alimentació i Medi Natural; Laboratori Agroalimentari (Cabrils, Spain). *Salmonella* serovar was assigned based on the scheme of Kauffmann-White (Grimont and Weill, 2007).

For the prevalence determination of *Campylobacter* and *Salmonella* in the different colonies sampled, all the confidence limits for the proportions obtained were calculated with a score with continuity correction (Fleiss Quadratic) using the open source OpenEpi (Dean at al., 2011).

- **Antimicrobial susceptibility testing**

Antimicrobial susceptibility of *Campylobacter* and *Salmonella* isolates was performed using the disk diffusion method (Bauer et al., 1966). Briefly, for *Campylobacter* isolates Mueller-Hinton 2 agar with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) was inoculated with a lawn of *Campylobacter* and incubated with antimicrobial disks for 48

h at 37 ºC under microaerophilic conditions. *Campylobacter* strains were tested for susceptibility against 7 antimicrobial agents which included three quinolones/fluoroquinolones: nalidixic acid (30 μg), ciprofloxacin (10 μg) and enrofloxacin (10 μg); one aminoglycoside: gentamicin (10 μg); one macrolide: erythromycin (15 μg); and two other miscellaneus antimicrobials: tetracycline (80 μg) and chloramphenicol (60 μg).

For *Salmonella* isolates, Mueller-Hinton agar (770305 Difco, Madrid, Spain) was used and plates were incubated for 24 h at 37 ºC. A panel of 18 antimicrobial agents were studied, including three β-lactams: ampicillin (33 μg), amoxycillin (30 μg) and amoxycillin-clavulanate (30 + 15 μg); one cephalosporin: ceftiofur (30 μg); four aminoglycosides: apramycin (40 μg), gentamicin (10 μg), neomycin (120 μg) and streptomycin (100 μg); four quinolones/fluoroquinolones: nalidixic acid (30 μg); ciprofloxacin (10 μg), enrofloxacin (10 μg) and norfloxacin (10 μg); one polymyxin: colistin (150 μg); one phenicol: chloramphenicol (60 μg) and four other antimicrobials: tetracycline (80 μg), nitrofurantoin (300 μg), lincomycin + spectinomycin (15 + 200 μg), and trimethoprim-sulfamethoxazole (5.2 + 240 μg).

The diameter of the bacterial growth inhibition was measured and designated as resistant, intermediate, or susceptible on the basis of Clinical Laboratory Standards (Neo-sensitabs Potency according to CLSI 2007).

- **Study of factors that may influence the frequency of *Salmonella* occurrence**

With the aim of assessing the influence that some factors, such as species, location or year, could have on the occurrence of *Salmonella*, a statistical analysis was performed using generalized linear modelling. Due to the unbalanced nature of the samplings, outcomes were divided in three logistic regression models. Firstly, to assess the association between the species and the frequency of *Salmonella* in different years and the interactions with the presence of *Campylobacter,* a model was performed uniquely from the samples collected in the Ebro Delta, where both species were present (first regression model). In this model, the species, the year and the presence

of *Campylobacter* and their possible interactions were included as independent variables and the presence of *Salmonella* as dependent variable. Then, the frequency of *Salmonella* was analysed separately in yellow-legged gulls and in Audouin's gulls in different colonies over the time (second and third regression model respectively). In these two models the independent variables were the location, the year and the presence of *Campylobacter*, and the dependent variable was the presence of *Salmonella*.

The analyses were performed with R (R Development Core Team 2013) using mainly the "stats" and "vcd" packages.

Results

- ***Campylobacter* and *Salmonella* occurrence**

Overall, we sampled 1,222 and 563 fledglings from yellow-legged and Audouin's gulls, respectively. *Salmonella* positive proportion was higher in yellow-legged than in Audouin's gulls, 26.27 % (321/1222) and 9.24 % (52/563), respectively. *Campylobacter* and *Salmonella* occurrence in both seagull species per year and sampling sites are shown in Table 1. It is noteworthy that when a bird was *Campylobacter*-positive, it usually was *Salmonella*-negative and vice versa. Only in a very few cases fledglings were positive to both pathogens.

All seagull colonies were *Salmonella*-positive during the three year sampling, except Audouin's gulls breeding at Ebro Delta in 2009. Among yellow-legged gull colonies, Medes Is. was the sampling site with the highest *Salmonella* occurrence, with 111 out of 270 gulls positive for *Salmonella* (41.11 %, CI $_{95\%}$: 35.23 - 46.97 %). Other colonies with a high *Salmonella* occurrence were Zembra Is. (38.89 %, CI $_{95\%}$: 23.63 - 56.47%), Tenerife Is. (34.21 %, CI $_{95\%}$: 20.14 - 51.42%) and Lanzarote Is. (31.25 %, CI $_{95\%}$: 20.57 - 44.20%). In Medes Is., occurrence increased over the three-years sampling (7.25 %, (CI $_{95\%}$: 2.70 - 16.79 %); 35.65 % (CI $_{95\%}$: 27.09 - 45.18 %) and 75.58 %, (CI$_{95\%}$: 64.91 –

83.93%)). The same phenomenon was observed in Dragonera Is. and Columbretes Is., whereas Ons Is. showed the opposite trend. Among Audouin's gull colonies, Alboran Is. was the locality with an upward *Salmonella* prevalence trend, whereas Ebro Delta showed an increasing prevalence from 2010 to 2011 (1.14 %, (CI $_{95\%}$: 0.06 – 7.05 %) vs 24.11 % (CI $_{95\%}$: 16.75 – 33.28 %)). In general, the highest *Salmonella* prevalence for both seagull species was detected in 2011, due to a progressive increase year after year.

Concerning *Campylobacter* occurrence, Audouin's gulls (14.74 %, CI $_{95\%:}$ 11.77 - 17.63%) showed higher values than yellow-legged gulls (0.82 %, CI $_{95\%}$: 0.30 - 1.30 %). The mean *Campylobacter* occurrence in sampling sites with at least one positive gull was 14.74 % (CI $_{95\%}$: 11.97 – 18.00 %). In yellow-legged gull colonies, *Campylobacter* occurrence ranged from 1.12% to 5.56 %, and both values correspond to Ons Is. (Table 1). *Campylobacter* was not detected in Zembra Is., Medes Is. and Columbretes Is. In the Ebro Delta *Campylobacter* was only detected in 2009. In Dragonera Is. *Campylobacter*-positive birds were found in 2010 but not in 2011, opposite to Canary Is., where it was only detected in Lanzarote in 2011. In Audouin's gull colonies, *Campylobacter* was isolated, from the two sampled colonies, with prevalences ranging from 2.02 % to 31.82 % and with an overall prevalence higher in Ebro Delta than in Alboran Is. (21.83 % (CI $_{95\%}$: 16.99 – 27.54 %) vs 9.00% (CI $_{95\%}$: 5.97 – 12.5 %). An increase of *Campylobacter* prevalence was found in the second year in both Audouin's gulls colonies.

- ***Campylobacter* species**

Among the 93 *Campylobacter*-positive seagulls (10 yellow-legged gulls and 83 Audouin's gulls), *C. jejuni* was the most frequently isolated species (94.60 % of birds, CI $_{95\%:}$ 90.01 - 99.19 %). *C. coli* was only detected in two Audouin's gulls from Ebro Delta in 2010 (2.15%, CI $_{95\%:}$ 0.37 – 8.29 %). In that same colony, one bird was positive to two *Campylobacter* species, *C. jejuni* and *C. coli*. *C. lari* was found in two yellow-legged gulls in 2010, one at Dragonera Is. and another one at Ons Is.

Table 1. Positive proportions of *Salmonella* and *Campylobacter* in yellow-legged gulls (*L. michahellis*) and Audouin's gulls (*L. audouinii*) according to the sampling site.

		2009		2010		2011	
		S [1]	C	S	C	S	C
Yellow-legged gulls	Zembra	14/36 (23.63-56.47%)[2]	0/36 (0-12,01%)	NS [3]	NS	NS	NS
	Medes	5/69 (2.69-16.79%)	0/69 (0-6.57%)	41/115 (27.09-45.18%)	0/115 (0-4.03%)	65/86 (64.91-83.93%)	0/86 (0-5.33%)
	Columbretes	7/86 (3.61-16.58%)	0/86 (0-5.33%)	17/80 (13.21-32.11%)	0/80 (0-5.71%)	37/80 (35.16-57.70%)	0/80 (0-5.71%)
	Ebro Delta	5/84 (21-13.96%)	2/84 (0.40-8.94%)	25/100 (17.12-34.84%)	0/100 (0-4.61%)	13/86 (8.61-24.83%)	0/86 (0-5.33%)
	Lanzarote	NS	NS	14/45 (18.63-46.80%)	0/45 (0-9.80%)	6/19 (13.56-56.5%)	1/19 (0.28-28.11%)
	Tenerife	NS	NS	13/38 (20.14-51.42%)	NS	0/38 (0-11.43%)	NS
	Dragonera	NS	NS	8/53 (7.20-28.14%)	1/53 (0.10-11.38%)	24/66 (25.14-49.18%)	0/66 (0-6.85%)
	Ons	NS	NS	15/89 (10.05-26.59%)	1/89 (0.06-6.98%)	12/90 (7.38-22.52%)	5/90 (2.06-13.08%)
Audouin's gulls	Ebro Delta	0/52 (0-8.57%)	12/52 (12.98-37.17%)	1/88 (0.06-7.05%)	28/88 [4] (22.52-42.72%)	27/112 (16.75-33.28%)	15/112 (7.94-21.44%)
	Alboran	6/101 (2.44-12.99%)	11/101 (5.83-19.04%)	8/111 (3.39-14.14%)	15/111 (8.01-21.62%)	9/99 (4.50-16.99%)	2/99 (0.35-7.81%)

[1] S: *Salmonella* spp., C: *Campylobacter* spp.; [2] nº positive samples / total of samples (95 % confidence interval, Fleiss Quadratic correction); [3] NS: not sampled; [4] one animal carried two *Campylobacter* species (*C. jejuni* and *C. coli*).

- ***Salmonella* serotypes**

Among the 373 *Salmonella*-positive birds (321 yellow-legged gulls and 52 Audouin's gulls), 412 isolates were serotyped (356 from yellow-legged gulls and 56 from Audouin's gulls). A great diversity of serotypes was found, with 69 different serotypes in yellow-legged gulls and 21 in Audouin's gulls (Table 2). In some cases the same individual carried more than one *Salmonella* serotype, with up to three serotypes per bird detected. Although serotype diversity was lower in Audouin's gulls, there were five serotypes which were found only in this seagull species (*S.* Montevideo, S. Liverpool, *S.* Blockey, *S.* Isangi and *S.* Stanley).

Regardless of the seagull species, the serotypes most frequently isolated were *S.* Typhimurium (including monophasic variants) (27.67 %, 114/412), *S.* Agona, *S.* Kentucky*, S.* Hadar and *S.* Derby with a 6.07 % (25/412), 4.85 % (20/412), 4.37 % (18/412) and 4.37 % (18/412) occurrence, respectively. In yellow-legged gulls, *S.* Typhimurium was by far the most frequent serotype (27.81 %, 99/356), followed by *S.* Agona (6.74 %, 24/356), *S.* Derby (5.06 %, 18/356) and *S.* Senftenberg (4.78 %, 17/356). In Audouin's gulls*, S.* Typhimurium was also the most frequent serotype detected (26.79%, 15/56), while *S.* Kentucky and *S.* Montevideo were the second and the third most common serotypes found (16.07 % (9/56) and 10.71 % (6/56), respectively).

S. Enteritidis was found in all but two localities (frequencies ranging from 0.82 % to 20.00 %, Medes Is. and Zembra Is., respectively) and *S.* Typhimurium was detected in all colonies, except in Alboran Is., with frequencies ranging from 15.38 % (Tenerife Is.) to 43.66 % (Columbretes Is.).

Medes Is. was the locality with the highest diversity of serotypes especially in 2010 and 2011. *S.* Agona and *S.* Typhimurium were the only serotypes isolated throughout the three sampling years. Also, together with Lanzarote Is and Ons Is, Medes Is was one of the sites where Paratyphi B serotype was detected. On the other hand, Zembra Is was the locality with the lowest serovar diversity; about half of the strains serotyped were *S.* Typhimurium.

More than 50.00 % of the serotypes were only detected in one of the localities sampled, such as *S.* Montevideo in Alboran Is, *S.* Senftenberg in Medes Is, or *S.* Muenchen in Dragonera Is, among others. However, the 44.59 % of the *Salmonella* serotypes were found in more than one locality and even some of them were found in at least five localities (e.g. *S.* Typhimurium, *S.* Enteritidis, *S.* Hadar, *S.* Agona, *S.* Cerro, *S.* Derby and *S.* Kentucky) (Table 2).

In Alboran Is. and Ebro Delta, the serotype diversity was higher in 2010 than in 2009 and 2011. However, in 2011 new serotypes not previously detected in Ebro Delta were isolated. The serotype diversity in Ons Is. and in Lanzarote Is. in 2011 was slightly lower than in 2010, while the greatest diversity of serotypes in Dragonera and Columbretes Is was detected in 2011.

- **Antimicrobial resistance**

- ***Salmonella* antimicrobial resistance**

Antimicrobial susceptibility testing was performed in 412 *Salmonella* isolates (356 from yellow-legged gulls and 56 from Audouin's gulls). More than the 50.00 % of the isolates were resistant to at least one antimicrobial agent (179 from yellow-legged gulls and 33 Audouin's gulls). Among them, 87 (41.04 %) were multiresistant (showed resistance to four or more antimicrobial agents), 69 (38.55 %) isolates from yellow-legged gulls and 18 (55.55 %) isolates from Audouin's gulls. The antimicrobial resistance pattern of these multiresistant strains is shown in Table 3.

S. Typhimurium (including monophasic variants) accounted for the majority of the 87 multiresistant isolates (N=54; 60.67 %), followed by *S.* Kentucky (N=16; 18.39 %), *S.* Hadar (N=5; 5.75 %), and *S.* Rissen and *S.* Wien (2 isolates each; 3.30 %). Serotypes with a single multiresistant isolate included *S.* Agona, *S.* Bredeney, *S.* Goldcoast, *S.* Grumpensis, *S.* Havana, *S.* Infantis, *S.* Stanley and non typeable *Salmonella*. One of these multiresistant isolates showed resistance to 9 antibiotics (*S.* Kentucky from Columbretes Is.) and 6 *Salmonella* isolates were resistant to eight antimicrobials (Table

3). There were also 13 isolates resistant to 7 antimicrobials and 7, 17 and 43 isolates were resistant to 6, 5 and 4 antimicrobials, respectively (Table 3). Multiresistant strains were detected in all the localities sampled, all along the three sampling years and also in both seagull species.

The antimicrobial resistances more frequently detected in both seagull species were to tetracycline, streptomycin, amoxicillin, ampicillin and nalidixic acid (Figure 2). In Audouin's gulls, the most frequent resistance detected was to nalidixic acid (35.71 %). Resistance to fluoroquinolones (enrofloxacin, ciprofloxacin and norfloxacin) was relatively high in Audouin's compared to yellow-legged gulls. A high proportion of resistant isolates was detected in Audouin's gulls in the two localities sampled, Ebro Delta and Alboran Is. (59.38 % and 58.33 %, respectively). Multiresistant isolates were also found in both colonies, with a higher frequency in Ebro Delta (34.38 %) than in Alboran (29.17 %).

In yellow-legged gulls the highest number of resistant isolates was found in Ebro Delta (74.47 %), followed by Zembra Is. and Columbretes Is (66.67 % and 66.20 %, respectively), where the highest number of multiresistant isolates was also detected (Columbretes Is, 35.21 %; Zembra, 26.67 %) (Figure 3). The proportion of resistant isolates from yellow-legged gulls in Ons Is. and Dragonera Is. was also high (56.25 % and 5143 %, respectively). In Ebro Delta, where yellow-legged gulls and Audouin's gulls are sharing habitat, the percentage of isolates resistant to at least one antimicrobial was higher in Audouin's gulls compared to yellow-legged gulls (58.98 % vs 50.28 %). Also, the frequency of multiresistant strains from Audouin's gulls was higher (32.14 % vs 19.66 %).

In Medes Is and Columbretes Is, around 75.00 % of the multiresistant strains were *S.* Typhimurium serotype, and most of them had the same antimicrobial pattern (AmSTA). Ons Is, Lanzarote Is. and Medes Is. were the three localities where S. Paratyphi B was found, and only the strain detected in Ons Is. showed resistance to two antimicrobial agents (amoxicillin and ampicillin).

Table 2. Number of strains of the different *Salmonella* serotypes detected in each sampling site

Serotypes	Alboran LA [1]	Ebro Delta LA	Ebro Delta LM	Medes LM	Columbretes LM	Dragonera LM	Ons LM	Tenerife LM	Lanzarote LM	Zembra LM	N (%)[2]
Agona		1		19	2	1	2				25 (6.07)
Altona			1		1			2			4 (0.97)
Amsterdam				12							12 (2.91)
Anatum							2			1	3 (0.73)
Brandenburg			2		2	2					6 (1.46)
Bredeney			2		2				1		5 (1.21)
Cerro			1	1	1			1	1		5 (1.21)
Coeln		1		1	1						3 (0.73)
Corvallis		1				2	2		1		6 (1.46)
Derby			1	9	3	4	1				18 (4.37)
Enteritidis	2		1	1	3		2		1	3	13 (3.16)
Goldcoast			3			2	1				6 (1.46)
Hadar	1	1	6	4	1	4				1	18 (4.37)
Infantis	1				1		3				5 (1.21)
Kentucky	7	2			7	1				3	20 (4.85)
Kottbus		3			3						6 (1.46)
London		1		12	1				2		16 (3.88)
Manhattan			1	2							3 (0.73)
Montevideo	6										6 (1.46)
Muenchen						3					3 (0.73)
Muenster				1		1		1			3 (0.73)

Continued on following page

Table 2. Continued

Serotypes	Alboran LA [1]	Ebro Delta LA	Ebro Delta LM	Medes LM	Columbretes LM	Dragonera LM	Ons LM	Tenerife LM	Lanzarote LM	Zembra LM	N (%)[2]
Newport	1	1			2		1		3		8 (1.94)
Ohio					1				2		3 (0.73)
Paratyphy B				1			1		1		3 (0.73)
Rissen			1	2	1						4 (0.97)
Salmonella spp.		1	3	2			1				7 (1.70)
Schwarzengrund	1				1			1		1	4 (0.97)
Senftenberg				17							17 (4.13)
Stanley		3									3 (0.73)
Thompson	1				2						3 (0.73)
Typhimurium		13	10	17	26	9	11	2	4	6	98 (23.79)
Typhimurium monophasic		2	2	5	5	1	1				16 (3.88)
Virchow			2		1				1		4 (0.97)
Wien		2	2	1							5 (1.21)

[1] LA: *L. audouinii* (Audouin's gull), LM: *L. michahellis* (yellow-legged gull); [2] N: Nº of strains of each serovar.

Serotypes with 1 or 2 strains: Annedal, Abony, Bareilly, Berta, Blegdam, Blockey, Bovismorbificans, Bradford, Clackamas, Dublin, Fyris, Give, Grumpensis, Havana, Isangi, IV(Argentina), Kaapstad, Kapemba, Litchfield, Liverpool, Mbandaka, Mikawasima, Oakey, Okatie, Oranienburg, Orion, Oslo, Pomona, Poona, Saintpaul, Singapore, Sinstorf, Stanleyville, Suberu, subespecie II (Sofia), Tilburg, Toulon, Urbana, Vejle, Westhampton and Wippra.

Table 3. Antimicrobial resistance patterns of multiresistant *Salmonella* strains isolated from seagulls.

AMR pattern [1]	N [2]	Serotype	Gull species[3]	Locality	Year
AmAmcNalCiEnTClANor	1	Kentucky	LM	Columbretes	2011
AmAmcSNalTClLnA	1	Typhimurium	LM	Medes	2011
AmAmcSTClLnTmA	2	Typhimurium	LM	Ons	2011
AmGenSNalCiEnTA	1	Typhimurium	LA	Ebro Delta	2011
AmNalCiEnTTmANor	1	Kentucky	LM	Zembra	2009
AmSAprNalTClLnA	1	Typhimurium	LM	Medes	2010
AmAmcCeSTTmA	1	Typhimurium monophasic	LM	Medes	2011
AmAmcSTClNitA	1	Typhimurium	LM	Medes	2010
AmCeSTLnTmA	1	Agona	LA	Ebro Delta	2011
AmNalCiEnTTmA	1	Typhimurium	LA	Ebro Delta	2011
AmSTClLnNitA	1	Typhimurium	LM	Tenerife	2010
AmSNalCiEnTA	2	Kentucky	LA	Ebro Delta	2011
AmSNalTClLnA	2	Typhimurium	LM	Columbretes, Ebro Delta	2010 / 2011
AmNalCiEnTANor	4	Kentucky	LM	Columbretes	2010 / 2011
AmNalCiEnANor	2	Kentucky	LM	Zembra	2009
AmAmcTClLnA	2	Typhimurium	LM	Dragonera, Medes	2009 / 2011
AmSTClLnA	3	Typhimurium (2), Infantis (1)	LM	Ebro Delta, Ons	2009 / 2010
AmCeSLnA	1	Grumpensis	LM	Ebro Delta	2009
AmNeNalTA	1	Hadar	LM	Medes	2010
AmSNalTA	1	Hadar	LA	Alboran	2010
AmSNeoTA	1	Hadar	LM	Zembra	2009

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Table 3. Continued

AMR pattern [1]	N [2]	Serotype	Gull species[3]	Locality	Year
AmSTANor	1	Typhimurium	LA	Ebro Delta	2011
AprNalCiEnNor	1	Kentucky	LA	Alboran	2011
GenSNalTA	1	Hadar	LM	Ebro Delta	2010
NalCiEnClNor	1	Kentucky	LM	Dragonera	2010
SNalCiEnNor	1	Kentucky	LA	Alboran	2011
AmTClLnA	2	Typhimurium	LM	Columbretes	2010
AmSTTmA	6	Goldcoast (1), Wien (2), Typhimurium (1), Typhimurium monophasic (1), Havana (1)	LM, LA	Alboran, Columbretes, Ebro Delta, Medes	2009 / 2010 / 2011
AmAmcClLn	1	Typhimurium monophasic	LM	Dragonera	2011
CeSNalT	1	Hadar	LM	Medes	2011
NalTNitTm	1	*Salmonella* spp 6,7:r:-	LA	Ebro Delta	2011
SNeTCl	1	Stanley	LA	Ebro Delta	2011
STLnTm	1	Rissen	LM	Medes	2011
AmTTmA	2	Typhimurium, Bredeney	LM	Columbretes	2009 / 2011
NalCiEnNor	3	Kentucky	LA	Alboran	2011
AmSTA	33	Rissen (1), Typhimurium (6), Typhimurium monophasic (26)	LM, LA	Columbretes, Dragonera, Ebro Delta, Lanzarote, Medes	2009 / 2010 / 2011

[1] AMR: antimicrobial resistance pattern.
A: ampicillin (33µg), Am: amoxycillin (30µg), Amc: amoxycillin-clavulanate (30+15µg), Ce: ceftiofur (30µg), Apr: apramycin (40µg), Gen: gentamicin (10µg), Ne: neomycin (120µg), S: streptomycin (100µg), Nal: nalidixic acid (30µg), Ci: ciprofloxacin (10µg), En: enrofloxacin (10µg), Nor: norfloxacin (10µg), Cl: chloramphenicol (60µg), T: tetracycline (80µg), Nit: nitrofurantoin (300µg), Ln: lincomycin+spectinomycin (15+200µg), Tm: trimethoprim-sulfamethoxazole (5.2+240µg).
[2] N: number of *Salmonella* strains per antimicrobial resistance pattern and per serotype

[3] LA: *L. audouinii* (Audouin's gull), LM: *L. michahellis* (yellow-legged gull).

- ***Campylobacter* antimicrobial resistance**

Nineteen out of 94 *Campylobacter* isolates tested (10 from yellow-legged gulls and 84 from Audouin's gulls) were resistant to at least one antimicrobial agent and two of them showed multiresistance (both isolated in Alboran Is. in 2009 and 2010). The most frequent antimicrobial resistances detected were to tetracycline (16.00 %) and nalidixic acid, (6.40 %), while a low frequency of resistance to fluoroquinolones (ciprofloxacin, 2.10%; enrofloxacin, 1.10 %), and to gentamicin (1.10 %) was found.

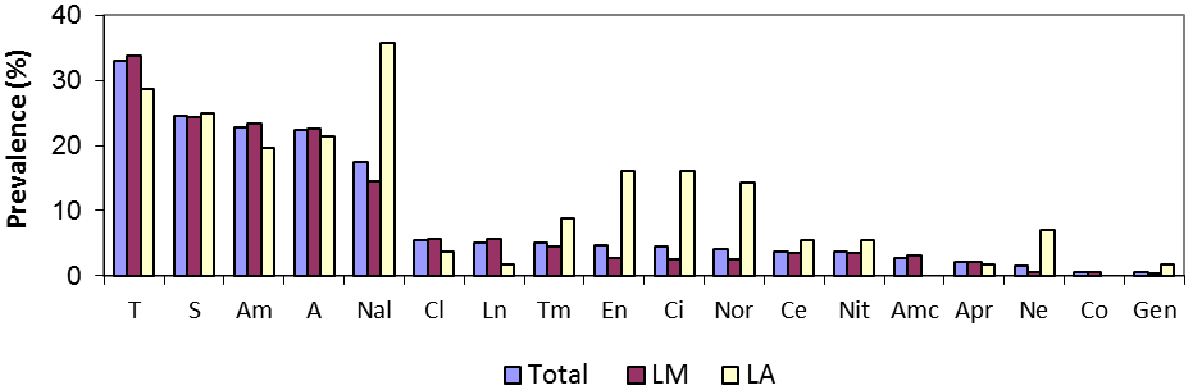
The frequency of *Campylobacter* resistant isolates in yellow-legged gulls was higher than in Audouin's gulls, (60.00 % vs 15.70 %). The two multiresistant *C. jejuni* isolates found were isolated from Audouin's gulls from Alboran in 2009 and 2010.

In Ebro Delta, three *C. coli* identified in 2010 from Audouin's gulls showed susceptibility to all of the antimicrobial agents tested. The same occurred with the two *C. jejuni* detected in 2009 from yellow-legged gulls. Ten *C. jejuni* out of 58 *Campylobacter* isolates from this colony showed resistance to at least one antimicrobial agent and the main resistances were to tetracycline and nalidixic acid (15.50 % and 1.70 %, respectively).

C. lari from Dragonera Is. and *C. jejuni* from Tenerife Is. were susceptible to all of the antimicrobials tested and *C. jejuni* from Lanzarote Is. was nalidixic acid resistant. In Ons Is. 5 out of 6 isolates showed antimicrobial resistance: one *C. lari* and one *C. jejuni* were resistant to nalidixic acid and three *C. jejuni* were tetracycline resistant.

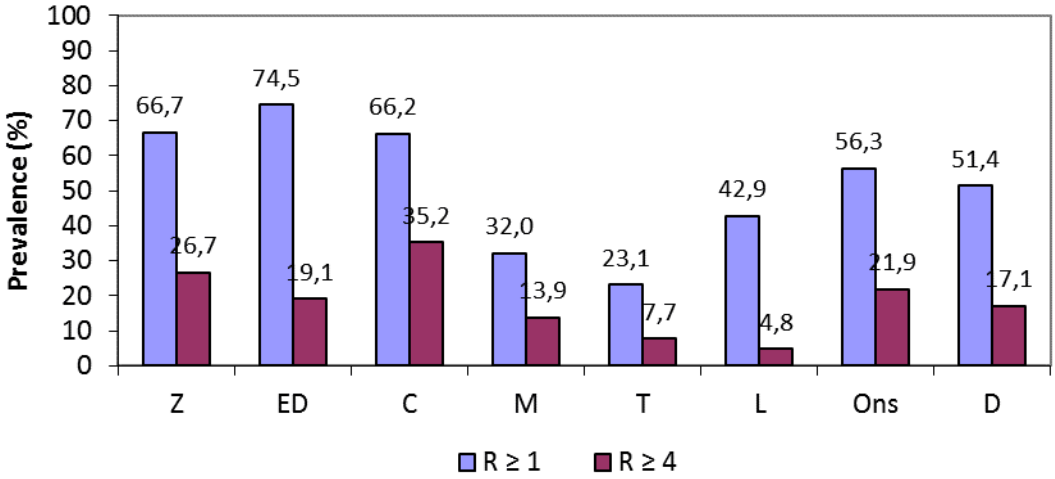
At Alboran Is, 10.71 % (3 out of 28) of the *C. jejuni* isolates were resistant to at least one antimicrobial agent and two of them were multiresistant (NalCiTGen and NalCiTEn, respectively).

Figure 2. Antimicrobial resistance of *Salmonella* isolates from Audouin's gull and yellow-legged gull



A : ampicillin (33µg), Am: amoxycillin (30µg), Amc: amoxycillin-clavulanate (30+15µg), Ce: ceftiofur (30µg), Apr: apramycin (40µg), Gen: gentamicin (10µg), Ne: neomycin (120µg), S: streptomycin (100µg), Nal: nalidixic acid (30µg), Ci: ciprofloxacin (10µg), En: enrofloxacin (10µg), Nor: norfloxacin (10µg), Co: colistin (150µg), Cl: chloramphenicol (60µg), T: tetracycline (80µg), Nit: nitrofurantoin (300µg), Ln: lincomycin+spectinomycin (15+200µg), Tm: trimethoprim-sulfamethoxazole (5.2+240µg).
LA: *L. audouinii* (Audouin's gull), LM: *L. michahellis* (yellow-legged gull).

Figure 3. Proportion of *Salmonella* antimicrobial resistant strains from yellow-legged gulls according to the sampling sites.



Z: Zembra Is., ED: Ebro Delta, C: Columbretes Is., M: Medes Is., T: Tenerife Is., L: Lanzarote Is, Ons: Ons Is, D: Dragonera Is.
R≥1: resistance to at least one antimicrobial agent, R≥4: resistance to at least four antimicrobial agents

- **Study of factors that may influence the frequency of occurrence of *Salmonella***

In the first logistic regression model, which included samples collected from both seagull species in the Ebro Delta, a significant interaction of the presence of *Salmonella* in Audouin's gull in 2010 was found (Table 4). There were not significant associations with the remaining factors included in the model. Logistic regression analysis of samples collected from yellow legged gulls in those colonies sampled along the three consecutive years (Columbretes Is., Medes Is. and Ebro Delta), both the locality and the year were variables associated with the presence of *Salmonella* (p values < 0.05), while the presence of *Campylobacter* was not significant. However, when the same model was applied to the two Audouin's gull colonies (Alboran Is, and Ebro Delta) which were sampled during all three years, none of the dependent variables or their respective interactions were statistically significant (p values > 0.05).

Table 4. Regression models results

First regression model in Ebro Delta in Audouin's gulls (SPECIES 0) and yellow-legged gulls (SPECIES 1)
SALMONELLA ~ CAMPYLOBACTER + SPECIES + CAMPYLOBACTER:SPECIES + as.factor(YEAR) + as.factor(YEAR:SPECIES), family = binomial(link = "logit")

AIC: 373.8

Variables	Estimate	z value	Pr
(Intercept)	-18.5719	-0.02	0.98362
CAMPYLOBACTER1	0.0253	0.04	0.96742
SPECIES1	-0.5753	-1.50	0.13274
as.factor(YEAR)2010	18.0486	0.02	0.98408
as.factor(YEAR)2011	17.4217	0.02	0.98463
as.factor(YEAR:SPECIES)2009:1	16.4129	0.02	0.98552
as.factor(YEAR:SPECIES)2010:0	-3.9507	-3.58	0.00034 ***
as.factor(YEAR:SPECIES)2010:1	NA	NA	NA
as.factor(YEAR:SPECIES)2011:0	NA	NA	NA
as.factor(YEAR:SPECIES)2011:1	NA	NA	NA
CAMPYLOBACTER1:SPECIES1	-15.8570	0.00	0.99726

Second regression model in yellow-legged gulls from Columbretes Is. (LOCATION 1), Ebro Delta (LOCATION 2) and Medes Is. (LOCATION 3)
SALMONELLA ~ LOCATION + as.factor(YEAR), family = binomial(link = "logit")

AIC: 785.6

Variables	Estimate	z value	Pr
(Intercept)	-2.696	-9.45	< 2e-16 ***
LOCATION2	-0.651	-2.77	0.00566 **
LOCATION3	0.762	3.64	0.00027 ***
as.factor(YEAR)2010	1.603	5.57	2.5e-08 ***
as.factor(YEAR)2011	2.470	8.58	< 2e-16 ***

Third regression model in Audouin's gulls from Alboran Is. (LOCATION 0) and Ebro Delta (LOCATION 1)
SALMONELLA ~ CAMPYLOBACTER + LOCATION + as.factor(YEAR) + LOCATION:YEAR + CAMPYLOBACTER:YEAR + CAMPYLOBACTER:LOCATION, family = binomial(link = "logit")

AiC: 312.9

Variables	Estimate	z value	Pr
(Intercept)	-2.639	-6.25	4.2e-10 ***
CAMPYLOBACTER1	-16.451	-0.01	0.99
LOCATION1	-16.778	-0.01	0.99
as.factor(YEAR)2010	-0.352	0.00	1.00
as.factor(YEAR)2011	18.304	0.01	0.99
LOCATION0:YEAR2010	0.283	0.00	1.00
LOCATION1:YEAR2010	NA	NA	NA
LOCATION0:YEAR2011	-17.946	-0.01	0.99
LOCATION1:YEAR2011	NA	NA	NA
CAMPYLOBACTER1:YEAR2010	17.287	0.01	0.99
CAMPYLOBACTER1:YEAR2011	0.540	0.00	1.00
CAMPYLOBACTER1:LOCATION1	15.637	0.01	0.99

Discussion

- **Campylobacter and Salmonella occurrence**

In this study, focussed in two seagull species, a wide sampling was performed throughout three years in nine colonies along the western Mediterranean and in the eastern Atlantic Ocean. Overall, 1,785 seagull fledglings of yellow-legged gulls and Audouin's gulls were sampled, and a higher *Salmonella* spp. occurrence (20.84 %) was found, compared with that of *Campylobacter* spp. (5.21 %). Infections with *Salmonella* spp. and *Campylobacter* spp. in seagulls is probably influenced by feeding habits of these birds. Seagulls can harbour both bacteria in the normal microbiota of their gastrointestinal tract and can also acquire these pathogenic bacteria after exposure to human contaminated environments, or after scavenging on refuse tips and sewage sludge. Several reports point out the relation between the presence of pathogenic bacteria in seagull faeces and the proximity of the breeding colonies to a garbage dump (Kapperud and Rosef, 1983; Fricker, 1984; Ferns and Mudge, 2000).

While the highest *Salmonella* spp. occurrence was found in yellow-legged gulls, almost all thermophilic *Campylobacter* were isolated from Audouin's gulls. An explanation for this could be the different origin of infection with the bacteria or a certain host specificity of *Campylobacter* in Audouin's gulls. However, it is striking both *Salmonella* prevalence in Audouin's gull colony from Ebro Delta in 2011 and *Campylobacter* prevalence in the two Audouin's gull colonies studied. This seagull species is supposed to have "clean" feeding habits, compared to yellow-legged gulls. The latter species are well known scavengers and forage more frequently in refuse tips and sewage than Audouin's gulls, particularly when colonies are close to human settings such as Ebro Delta or Medes Is. Therefore, Audouin's gull colonies would be expected to have a lower carriage levels of zoonotic bacteria than yellow-legged gulls. Thus, *Campylobacter* and *Salmonella* prevalence in Audouin's gulls from Ebro Delta might be indicative of a change in feeding habits in this seagull species over the last two decades. This is probably associated to the increase of the exploitation of trawler discards, which propitiated an exponential population increase of this species in the

Ebro Delta to the current levels, coupled with an artificial fluctuation of this resource in relation to trawler moratoriums. During trawler moratorium food availability drops sharply and Audouin's gulls may need to search for alternative food resources such as refuse tips. The peak of *Salmonella* prevalence in Audouin's gulls in Ebro Delta in 2011 can be due to a drop of food availability which might have forced gulls to search for alternative feed in refuse tips.

The increase of *Salmonella* prevalence year after year was remarkable, especially in yellow- legged gulls in some localities. Statistically significant differences of *Salmonella* prevalence and sampling year were found among the three localities where this seagull species was sampled (Medes Is., Columbretes Is. and Ebro Delta). A possible explanation for the presence of *Salmonella* in these colonies could be the contact with contaminated water. The presence of *Salmonella* in both sea and river water is well documented (Polo et al., 1999). Also, seagulls foraging during autum-winter in contaminated areas may get intected and became *Salmonella*-persistent asintomatic carriers that will subsequently infect both adults and offsprings during the breeding season.

By contrast with the high *Salmonella* prevalence, the overall *Campylobacter* prevalence was low (5.21 %). These differences may be due to differential ecological behaviour between *Salmonella* and *Campylobacter*. *Salmonella* can persist in the environment for a long time, even between breeding periods, which allows a continuous infection of birds in the colony (Literák et al., 1996; Sinton et al., 2007). On the contrary, *Campylobacter* infection may be restricted to direct transmission, since some abiotic variables, particularly dehydration, negatively affect the survival of *Campylobacter* in the environment (Murphy et al., 2006).

A higher *Campylobacter* prevalence was observed in Audouin's gulls compared to yellow-legged gulls, especially in 2010 in the Ebro Delta. Ebro Delta is a locality where diverse trophic resources are available (Oro and Ruiz, 1997; Navarro et al., 2010). Thus, this increase of *Campylobacter* occurrence detected in 2010 in this colony may be due to a marked opportunistic behaviour of Audouin's gull that year.

Hence, the management of food from human origin would be an effective, and even definitive, way for controlling the source of *Salmonella* and *Campylobacter* infection of gulls, especially in yellow-legged gulls. In the near future the intention is that refuse tips will be progressively closed or properly managed and fishery waste will be reduced, following the implementation of European Union environmental policies (Gewin, 2004), which should help to improve the control of these zoonotic bacteria.

By far, the most predominant *Campylobacter* species isolated from gulls was *C. jejuni* (94.6%), followed by *C. coli* and *C. lari*, which were detected only in two individuals. *C. jejuni* is the most important thermophilic *Campylobacter* responsible of food-borne and water-borne bacterial enteritis in humans worldwide (Tauxe, 2001). *C. coli* and *C. lari* account for the majority of the remaining human cases of infection (Kapperud and Rosef, 1983; Moore et al., 2005; Lastovica, 2006). Since the occurrence of thermophilic *Campylobacter* spp. in seagull faeces was significant, especially in Audouin's gulls, these marine birds may contribute to the environmental contamination with *Campylobacter* spp. By contaminating the environment, including surface waters, beach sands and pastures, seagulls (particularly Audouin's gulls) may be involved in the epidemiology of human-associated campylobacteriosis in the studied areas. In Ebro Delta, both species of seagulls share habitat with other wild birds, including waterfowl. In this locality, the *Campylobacter* species carriage by wild birds shows a different pattern: while *C. jejuni* is mainly isolated from seagulls, *C. coli* was the most prevalent in waterfowl (Antilles at al., 2013). Hence, this data also suggest a host specificity of *Campylobacter* species in wild birds.

- ***Salmonella* serotypes**

A high diversity of *Salmonella* serotypes was found in seagulls. The two most important serotypes causing human food-borne disease, *S.* Enteriditis and *S.* Typhimurium, were isolated in most of the studied colonies. It is particularly remarkable the fact that overall, *S.* Typhimurium was the most prevalent serotype isolated (27.67 %). Other studies carried out in Europe have pointed to gulls as the most important wild bird *Salmonella* reservoir in Europe (Hubalek et al., 1995;

Hernandez et al., 2003), and to *S*. Typhimurium as the most common serotype found in wild birds (Palmgren et al., 1997). Other serotypes isolated from gulls in one or several localities studied have also been increasingly reported in human food-borne diseases during the last years, such as Infantis, Agona, Hadar and Virchow (Lenglet, 2005; Toyofuku et al., 2006; de Jong et al., 2007; EFSA, 2013a; Graziani et al., 2013). Of relevance is also the finding of the public health important serotype *S.* Paratyphi B, which is able to cause both enteric fever and gastroenteritis and is mainly recovered from humans (Martínez-Urtaza et al., 2006); this serotype has been previously isolated from yellow-legged gulls in Medes Is. (Ramos et al., 2010).

Several serotypes frequently reported in food animals in the EU, including *S.* Enteritidis and *S.* Hadar (poultry), *S.* Typhimurium, *S.* Derby, *S.* Infantis, and *S.* London (swine), *S.* Typhimurium and *S.* Dublin (bovine) (EFSA, 2013a), are also among the most frequently isolated serotypes in seagulls in this study. This suggests food animals as a source of infection of seagulls and vice versa.

Salmonella serotypes with public health implications have also been reported in studies carried out in seagulls in southern Europe (Duarte et al., 2002). In Sweden and in Czech Republic, the Black-headed gull (*Larus ridibundus*) is the wild bird where more often *Salmonella* spp. has been detected and also a high diversity of serotypes has been found (Hubalek et al., 1995; Palmgren et al., 2006).

Salmonella spp. has also been isolated from other wild birds such a waterfowl, pigeons, sparrows and vultures (Chuma et al., 2000; Waldenström et al., 2007; Molina-Lopez et al., 2011). However, in most of them only the serotype Typhimuirium was detected while a great diversity of serotypes is usually observed in seagulls (Hubálek *et al* 1995; Palmgren *et al* 2006). The higher diversity of *Salmonella* serotypes found in seagulls could be due to the close contact of the birds with the environment and with humans garbage, two places where most likely these birds can become infected with *Salmonella*.

- **Antimicrobial resistance**

Although minimal exposure to antibiotics is expected in wildlife species, *Salmonella* and *Campylobacter* strains isolated in the present study from seagulls showed resistance to several antimicrobial agents. Most of the multiresistant *Salmonella* strains belonged to the serotypes Typhimurium, Kentucky and non typeable *Salmonella* spp. Also, a high resistance of *Campylobacter* isolates was also found. These high antimicrobial resistances found both in *Salmonella* and *Campylobacter* isolates are of concern, particularly taking into account that important resistances to antimicrobials commonly used in human infections were detected. These include cephalosporins and fluoroquinolones, the antimicrobials of choice to treat severe salmonellosis and campylobacteriosis in humans.

These results suggest that the isolated strains are not specific to seagulls, and more likely originate from human or animal sources where antimicrobial usage is high. The extended use of antimicrobial agents in animal husbandry and the inappropriate use in humans play an important role in the emergence or persistence of resistant strains. The presence of these resistant and multidrug-resistant strains in seagulls could be due to the scavenging feeding habits of these birds. Seagulls might acquire resistant strains from the environment and/or when feeding in refuse dumps where human and animal wastes accumulate.

The World Health Organization (WHO) and health authorities recognize the increase in the number of resistant and multiresistant strains of bacteria as one of the major problems in public health (Helmuth, 2001). The careful prescription of antimicrobial agents in veterinary practice and responsible use in human medicine can contribute to reduce this public health problem.

Data provided in the present study highlights on the importance of seagulls as a reservoir of *Salmonella*, *Campylobacter* and antimicrobial resistance, and thus as an important source of infection for humans and domestic animals. Also, seagulls can serve as a sentinel for antibiotic pressure from the surrounding farms and urban

settings. Therefore, to better understand the overall problem of antibiotic resistance, monitoring wild birds may be a useful parameter to evaluate the impact of anthropic pressure in a specific location. On the other hand, to gain insight into the epidemiology of *Salmonella* spp. and *Campylobacter* spp. in the wild in southern Europe, it would be of interest molecular epidemiological studies. Such studies in the seagull colonies studied are granted.

Acknowledgements

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STUDY IV

Genetic diversity of *Salmonella* spp isolated from two seagull species (*Larus michahellis* and *Larus Audouinii*) in southern Europe

In preparation

Abstract

Gulls (*Laridae*) are amongst the most important wild birds in *Salmonella* epidemiology. However, there is scarce information on the molecular epidemiology of *Salmonella* spp. from these marine birds in southern Europe. Thus, 1115 *Salmonella* spp. isolates of 56 serotypes from gull fledglings of *Larus michahellis* (yellow-legged gull) and *Larus audouinii* (Audouin's gull) from nine colonies in the western Mediterranean and eastern Atlantic Ocean, sampled during 2009, 2010 and 2011, was investigated. Genotyping of the isolates was carried out by means of enterobacterial repetitive intergenic consensus (ERIC)-PCR and pulsed-field gel electrophoresis (PFGE). Isolates from gulls were also compared with those from poultry reared outdoors in farms sited in an area of influence of seagulls. Birds usually carried a single strain, but overall a high diversity of profiles within certain *Salmonella* serotypes was obtained with both typing methods. *S*. Typhimurium, *S*. Agona, *S*. Derby and *S*. Newport showed a notable diversity of strains while a low clonality was observed in *S.* Enteritidis, *S.* Hadar and *S.* Amsterdam serotypes. Common PFGE patterns were found among gulls from different colonies regardless of its proximity. However, the same patterns were more often detected in close colonies (Medes Is., Ebro Delta and Columbretes Is.). The same S. Kottbus macrorestriction profile was found in seagulls and free range poultry, suggesting that gulls may constitute a source of infection of domestic birds or vice versa. Gulls contribute to the maintenance of *Salmonella* spp. in a colony and also play an important role in the dissemination of these pathogenic bacteria to other geographic areas.

Introduction

Salmonella spp. is the second most important bacteria involved in foodborne human infections in Europe. Its incidence in humans is only exceeded by *Campylobacter* infections (EFSA, 2013a). *Salmonella* infections are usually self-limiting and the treatment with antimicrobials is therefore only required in cases of severe enteric

disease. However, the economic impact of these infections in the European Union has been estimated at approximately 3 billion euros per year (EFSA, 2013a). Because of this economic impact, there is an interest in lowering *Salmonella* infections.

Wild birds are well known as important carriers of pathogens, and especially migrating birds can represent an important public health threat due to their ability to fly freely crossing one or more national boundaries. Thus, wild birds can play an important role in the dissemination of pathogenic bacteria (Botti et al., 2013; Hubalek, 2004; Reed et al., 2003). Among wild birds, gulls (*Laridae*) are marine migrating birds frequently described as carriers of *Salmonella* spp. in high levels (Duarte et al., 2002). This is probably due to their scavenging feeding habits, which sometimes consist in the use of refuse dumps and sewage outlets to feed on (Cizek et al., 1994; Hubalek et al., 1995; Wilson and Moore, 1996). Several studies have addressed the occurrence of zoonotic bacteria, including *Salmonella*, in wild birds in northern Europe and other continents, and particular interest has been posed on seagulls (Cizek et al., 1994; Waldenström et al., 2002). However, scarce information is available on the epidemiology of *Salmonella* in those marine birds in southern Europe.

In a previous study carried out by our research group in order to determine the role of seagulls in the epidemiology of *Salmonella* spp., nine breeding colonies of *Larus michahellis* (yellow-legged gulls) and *Larus audouinii* (Audouin's gulls) along the western Mediterranean and in the eastern Atlantic Ocean were sampled during the chick-rearing period of 2009 to 2011. Sampled colonies included Medes Islands, Ebro Delta, Columbretes Islands, Ons Island, Lanzarote Island, Tenerife Island, Dragonera Island, Alboran Island and Zembra Island. High *Salmonella* prevalence and serotype diversity in seagulls was found.

Pulsed-field gel electrophoresis (PFGE), has proven to be highly discriminatory and useful in *Salmonella* epidemiological investigations (Hansen et al., 2002; Refsum et al., 2002). Also, Enterobacterial repetitive intergenic consensus (ERIC)-PCR is an efficient fingerprinting method for the differentiation of *Salmonella* spp, and is particularly useful when there is a high number of isolates to be typed (Burr et al., 1998; Lim et al.,

2005). Thus, to improve our understanding on the epidemiology of *Salmonella* spp, and to gain insight into *Salmonella* dynamics and diversity in seagulls and the environment, DNA typing of *Salmonella* spp. isolates recovered from those nine seagull colonies was performed by means of ERIC-PCR and PFGE. *Salmonella* isolates recovered from two free-range poultry farms sited in an area of influence of seagulls were also included in the study.

Material and methods

- ***Salmonella* isolates**

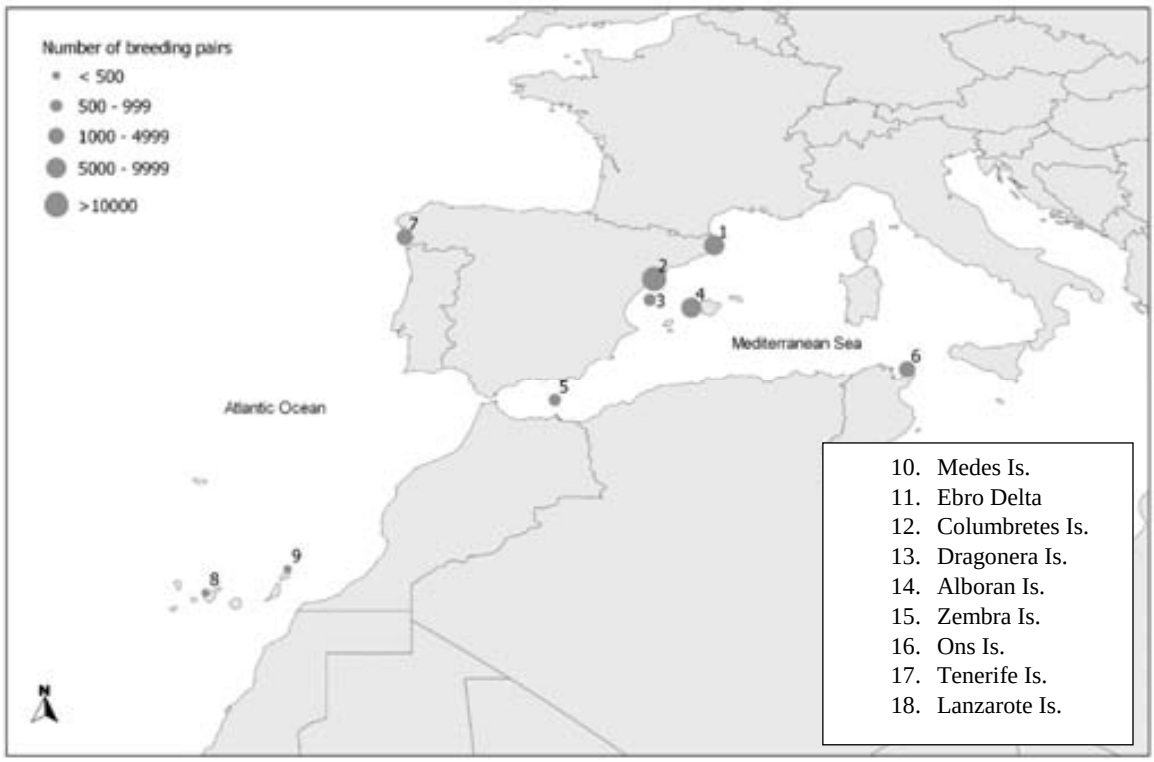
All *Salmonella* isolates from seagulls were recovered from cloacal swabs of *L. michahellis* (yellow-legged gulls) and *L. audouinii* (Audouin's gulls) fledglings sampled during the late chick-rearing period of 2009 to 2011. Overall, nine colonies along the western Mediterranean and the eastern Atlantic Ocean were sampled (Figure 1): Medes Is., Ebro Delta, Columbretes Is., Ons Is., Lanzarote Is., Tenerife Is., Dragonera Is, Alboran Is and Zembra Is. Ebro Delta was the only site where both seagull species were sampled. Audouin's gulls were also sampled in Alboran Is. yellow-legged gull fledglings were sampled in all sites but Alboran Is.

Overall, 1115 *Salmonella* spp. isolates from seagulls (936 from yellow-legged gulls and 179 from Audouin's gulls) were typed. Also, four *Salmonella* Kottbus isolates from free-range poultry farms from Catalonia region (Spain) were included for comparison purposes.

The 1115 *Salmonella* isolates analyzed included 56 serotypes (number of isolates per serotype in parentheses): Typhimurium (348), Kentucky (70), Agona (68), Hadar (59), Senftenberg (50), London (48), Derby (41), Enteritidis (36), Amsterdam (23), Goldcoast (20), Montevideo (20), Wien (19), Branderup (18), Newport (18), Infantis (17), Bredeney (16), Kottbus (16), Corvallis (13), Stanley (13), Altona (10), Muenchen (10), Virchow (10), Anatum (9), Cerro (8), Paratyphi B (8), Bovismorbificans (7), Coeln (7), Litchfield (7), Mikawasima (7), Muenster (7), Oakey (7), Rissen (7), Schwarzengrund (7),

subs. II (Sofia) (7), Thompson (7), Toulon (6), Abony (5), Manhattan (5), *Salmonella* spp. (5), Berta (4), Fyris (4), Give (4), Isangi (4), Kapemba (4), Stanleyville (4), Urbana (4), Vejle (4), Wippra (4), Havana (3), Liverpool (3), Ohio (3), Westhampton (3), Blegdam (2), Blockey (2), Bradford (2) and Tilburg (2).

Figure 1. Map locations of the studied seagull colonies along the western Mediterranean and the eastern Atlantic ocean.



- **Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR)**

All isolates were genotyped by ERIC-PCR as previously described (Antilles et al., 2013). ERIC-PCR was used as a screening tool and allowed to determine the genotypic diversity among *Salmonella* isolates within an individual host and within a gull colony. Isolates from the same bird showing identical ERIC-PCR profile were considered as the same strain and only one of them was selected for PFGE typing. Thus, representative isolates from the different ERIC-PCR patterns identified per bird, which included all the different genotypes identified were analyzed by PFGE.

- **Pulsed-field gel electrophoresis (PFGE)**

Overall, 315 *Salmonella* isolates (313 from seagulls and two from two free-range poultry farms) were investigated by PFGE. PFGE was performed according to the PulseNet standardized protocol "Standard Operating Procedure for PulseNet PFGE of *Escherichia* coli O157:H7, *Escherichia* coli non-O157 (STEC), *Salmonella* serotypes, *Shigella* sonnei and *Shigella* flexneri" (www.pulsenetinternational.org). The isolates were analyzed using *Xbal* restriction enzyme (Roche Applied Science, Indianapolis, IN) and the resulting PFGE patterns were analyzed using the Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Similarity matrices were calculated using the Dice coefficient and cluster analysis was performed by the unweighted-pair group method with arithmetic mean (UPGMA). Isolates with a minimum level of similarity of 89% were considered genetically similar or identical. Isolates from the same colony and the same sampling period showed this level of similarity and epidemiologically were considered to represent the same strain.

Results

- **ERIC-PCR**

Overall, a high diversity of ERIC-PCR profiles was obtained. Usually, isolates from the same bird showed the same ERIC-PCR profile, indicating that birds usually carried a single *Salmonella* strain. However, 23.8 % of the fledglings showed more than one ERIC-PCR profile. The same ERIC-PCR profiles were found in different birds from the same colony, but also among birds from different localities. In a few cases, the same profile was found in the same colony during different breeding seasons.

- **PFGE**

Molecular typing of 102 *S.* Typhimurium isolates showed 41 different PFGE profiles. When one PFGE profile was found more than once, it was designated with a letter in

alphabetic order (from A to O). A subset of *S.* Typhimurium isolates (26 out of 102) showed unique profiles and were included in a group designated as "other profiles" (Figure 2). The number of isolates belonging to each profile, according to the locality, is shown in Table 1. Ebro Delta was the sampling site with a higher diversity of *S.* Typhimurium PFGE profiles (A, B, D, E, F, G, K, M, N and 6 unique profiles). Of these, six were also found in Columbretes Is. and five in Medes Is., either in the same year or in different years (Table 1). In Ebro Delta, the only locality where both seagull species (yellow-legged gulls and Audouin's gulls) were sampled, profile B was the only PFGE pattern common to both seagull species. Four macrorestriction profiles (A, B, K and N) were found in Audouin's gulls from Ebro Delta, while six patterns (B, D, E, F, G and M) were detected in yellow-legged gulls in that locality. Certain *S.* Typhimurium macrorestriction profiles were only found in colonies from the Mediterranean (A, K, L, N, O profiles), with A profile being widespread in all five Mediterranean colonies, while the other 4 profiles were common to the four colonies more close to each other (Ebro Delta, Medes Is., Columbretes Is., Dragonera Is.) (Table 1). On the other hand, profile F was widespread among most of the Mediterranean and Atlantic colonies, while profile B was specific of Ebro Delta but common to both gull species breeding in this area. M profile was common to several Mediterranean colonies and Ons Is., sited in the Atlantic Ocean. Patterns C and I were specific of Zembra Is. and Dragonera Is., respectively.

All but one of the 16 *S.* Enteritidis isolates from both seagull's species clustered together at a 92 % similarity. Within this cluster, 11 isolates had an undistinguishable PFGE pattern, including isolates from five different localizations (Alboran Is., Columbrets Is., Lanzarote Is., Ons Is. and Zembra Is.) from different years and both seagull's species. (Figure 3). PFGE typing of the 29 *S.* Agona isolates from 5 different colonies and both seagull species showed ten different profiles (similarity 90 %) (Figure 4). Profiles G and H were the most abundant and included only isolates from Medes Is. Profiles A and C included isolates from two different colonies (Columbretes Is. and Medes Is., Dragonera Is. and Ons Is., respectively).

Table 1. *Salmonella* Typhimurium PFGE profiles found in the different sampling sites.

Locality	PFGE pattern	Number of Isolates	Year	Gull species
Ebro Delta	A	6	2011	La*
	B	5	2011	Lm*, La
	D	2	2010	Lm
	E	1	2010	Lm
	F	1	2009	Lm
	G	1	2010	Lm
	K	1	2011	La
	M	1	2010	Lm
	N	1	2011	La
	Other	6	2010,2011	Lm, La
Medes	A	3	2009,2010	Lm
	E	1	2010	Lm
	F	3	2009,2010	Lm
	J	1	2009	Lm
	K	1	2011	Lm
	M	1	2011	Lm
	O	2	2011	Lm
	Other	8	2010,2011	Lm
Columbretes	A	1	2011	Lm
	D	1	2011	Lm
	G	1	2010	Lm
	H	1	2009	Lm
	J	1	2011	Lm
	K	3	2011	Lm
	L	1	2011	Lm
	M	4	2010,2011	Lm
	N	9	2011	Lm
	O	2	2011	Lm
	Other	4	2009,2010,2011	Lm
Dragonera	A	2	2010	Lm
	I	2	2011	Lm
	L	2	2011	Lm
	Other	3	2011	Lm
Tenerife	F	1	2010	Lm
	Other	1	2010	Lm
Lanzarote	F	1	2010	Lm
	Other	1	2010	Lm
Ons	E	1	2011	Lm
	F	3	2010,2011	Lm
	H	1	2011	Lm
	M	3	2010,2011	Lm
	Other	3	2010,2011	Lm
Zembra	A	1	2009	Lm
	C	4	2009	Lm

*La= Audouin's gull (Larus adouinii); Lm= yellow-legged gull (Larus michahellis)

PFGE typing of 25 *S.* Hadar isolates showed a low diversity of strains. Isolates were grouped in two clusters (I, II) and a unique profile (Figure 5). At a 92 % of similarity, cluster I included 21 isolates from both seagull's species, from 6 different colonies and all three sampling years. Cluster II included three isolates from yellow-legged gulls, two from the same gull sampled in Ebro Delta on 2009 and one isolate from Medes Is. from 2010. Eight different macrorestriction profiles (89 % of similarity) were observed among the 19 *S.* Derby isolates (Figure 6). The same profile was found the same year in two different colonies (A, C and E profiles) and in the same colony during different years (G). Four different PFGE profiles were observed among the 18 *S.* Senftenberg isolates; most of them belonged to B profile (11 out of 18 isolates) (Figure 7). All these isolates were recovered from yellow-legged gulls from Medes Is. in 2011.

The *XbaI* digest of 17 S. Kentucky isolates produced five different macrorestriction profiles that differed from each other by three or four bands (Figure 8). A cluster at a similarity level of 92% grouped the highest number of isolates (ten isolates, profile A). This cluster included isolates from both yellow-legged gulls and Audouin's gulls, as well as from different colonies and year of isolation. Profile C included four isolates, all from Audouin's gulls from the two studied colonies. Three *S.* Kentucky isolates with unique profile were also detected. With regards to the 17 *S.* London isolates analyzed, five different PFGE profiles with *XbaI* digest were generated, two of them being unique profiles (Figure 9). Isolates showing 100 % similarity originated from the same seagull colony and the same sampling year.

All *S.* Amsterdam isolates were recovered from yellow-legged gulls from Medes Is. in 2010-2011 and showed a low diversity of strains. (Figure 10). Twelve out of 14 isolates grouped in a single cluster with a 89% similarity and the remaining two isolates showed unique profiles. On the contrary, a higher diversity was found among the 12 *S.* Newport isolates, with three clusters at a 96% similarity level and 4 unique profiles (Figure 11). Isolates from the same bird showed a 100 % of similarity, although one Audouin's gull (AA172) from Alboran carried two different strains. On the other hand, isolates from both seagull's species and different locality clustered together.

Three different macrorestriction profiles were detected among the eight *S.* Braenderup isolates (Figure 12). Profiles A and B included only isolates from the same colony and sampling occasion, and profile C included isolates from Ebro Delta and Columbretes Is. from 2010-2011. The eight *S.* Corvallis isolates showed a 92,8 % of similarity and those isolates from the same bird or from the same colony were genetically indistinguishable (100 % of similarity) (Figure 13).

All eight *S.* Kottbus, which included isolates from free-range poultry farms and from both seagull's species, were genetically related, showing over 94 % of similarity (Figure 14). Particularly, those isolates from free-range poultry farms showed a 100% similarity to one *Salmonella* isolate from a seagull from Ebro Delta.

Cluster analysis of macrorestriction profiles of the seven *S*. Infantis isolates from seagulls grouped them in two different clusters at a 84% similarity. Pattern A included the isolates from yellow-legged gulls from Ons Is. and Columbretes Is., while profile B included the isolates from an Audouin's gull from Alboran (Figure 15).

The six *S.* Stanley isolates were recovered from Audouin's gulls from Ebro Delta in 2011. All of them showed a similarity of 95.8 % (Figure 16). *S.* Virchow isolates from yellow-legged gulls from Ebro Delta and Lanzarote Is. clustered together in a single cluster (similarity 91 %) (Figure 17). *S.* Paratyphi B isolates were recovered in 2011 in three different colonies (Lanzarote Is., Ons Is. and Medes Is.) and the same strain (100 % similarity) was isolated in two of these localizations (Lanzarote Is. and Ons Is.) (Figure 18).

Figure 2. Distribution of *Salmonella* Typhimurium PFGE profiles according to sampling sites.

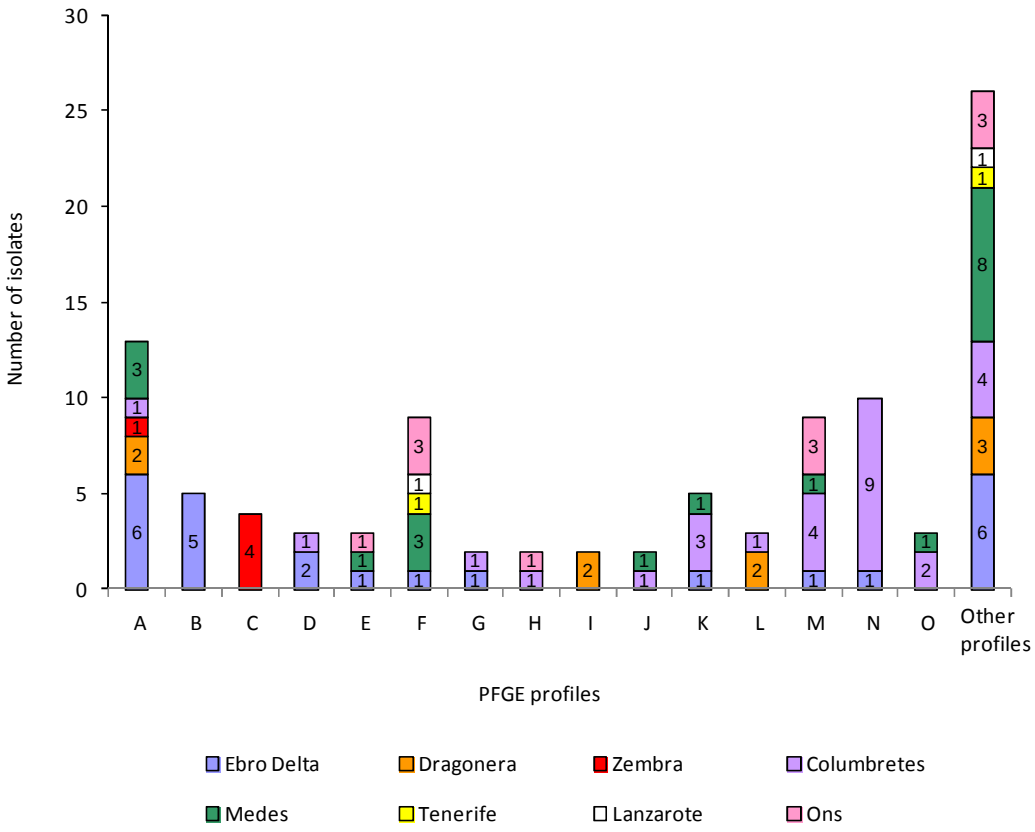
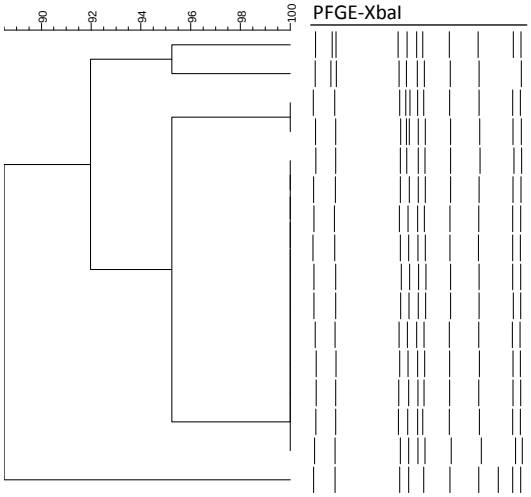
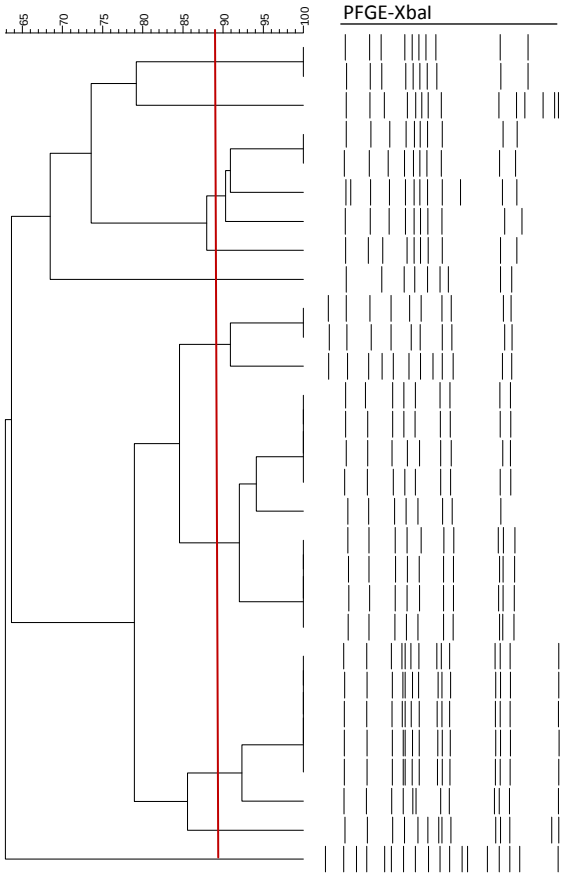


Figure 3. **PFGE dendrogram of XbaI patterns of *S.* Enteritidis isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method.



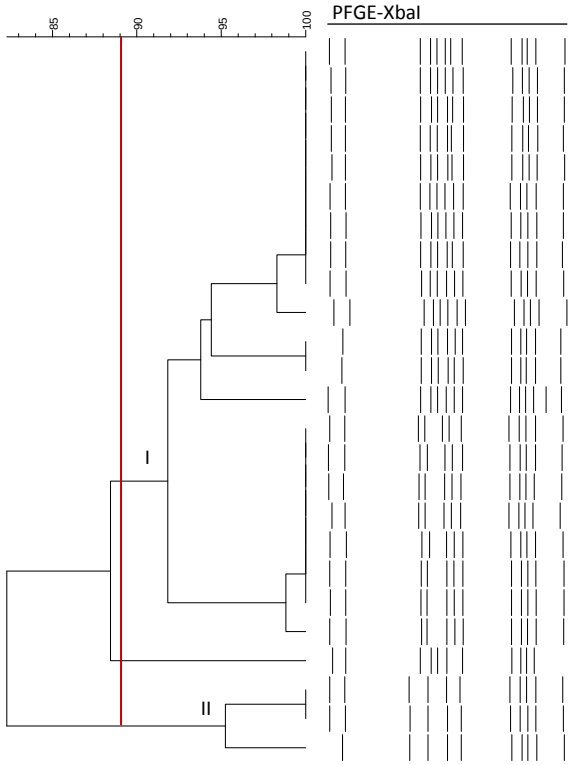
Strain	Serotype	Gull species	Sampling location	Year
AA145-S1	S.Enteritidis	L. audouinii	Alboran	2010
AA192-S1	S.Enteritidis	L. audouinii	Alboran	2010
DM199-S1	S.Enteritidis	L. michahellis	Ebro Delta	2011
CM236-S1	S.Enteritidis	L. michahellis	Columbretes	2011
AA145-S2	S.Enteritidis	L. audouinii	Alboran	2010
CM206-S1	S.Enteritidis	L. michahellis	Columbretes	2011
CM208-S1	S.Enteritidis	L. michahellis	Columbretes	2011
MCM58-S2	S.Enteritidis	L. michahellis	Lanzarote	2011
MCM58-S3	S.Enteritidis	L. michahellis	Lanzarote	2011
MCM58-S4	S.Enteritidis	L. michahellis	Lanzarote	2011
ZM26-S1	S.Enteritidis	L. michahellis	Zembra	2009
ZM32-S1	S.Enteritidis	L. michahellis	Zembra	2009
ZM11-S1	S.Enteritidis	L. michahellis	Zembra	2009
ZM11-S3	S.Enteritidis	L. michahellis	Zembra	2009
GAM148-S1	S.Enteritidis	L. michahellis	Ons	2011
MM212-S1	S.Enteritidis	L. michahellis	Medes	2011

Figure 4. **PFGE dendrogram of XbaI patterns of *S.* Agona isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



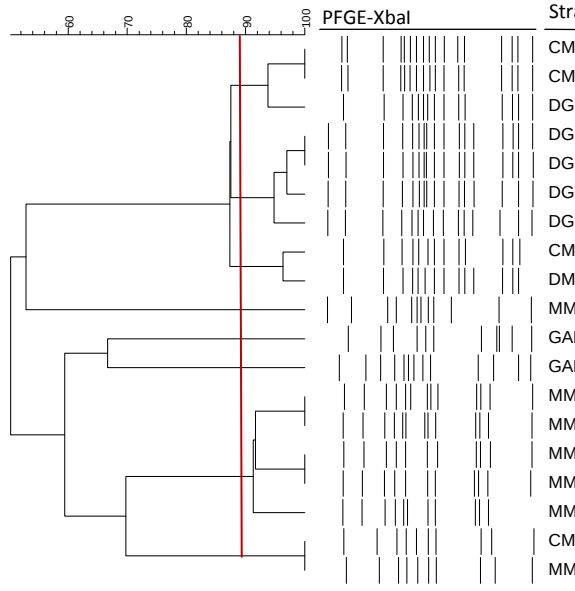
Strain	*	Serotype	Gull species	Sampling location	Year
CM63-S1	A	S.Agona	L. michahellis	Columbretes	2009
MM63-S2	A	S.Agona	L. michahellis	Medes	2009
DA230-S1	B	S.Agona	L.audouinii	Ebro Delta	2011
DGM96-S1	C	S.Agona	L. michahellis	Dragonera	2011
GAM109-S3	C	S.Agona	L. michahellis	Ons	2011
DGM96-S4	C	S.Agona	L. michahellis	Dragonera	2011
GAM158-S1	C	S.Agona	L. michahellis	Ons	2011
DA230-S2	D	S.Agona	L.audouinii	Ebro Delta	2011
CM95-S3	E	S.Agona	L. michahellis	Columbretes	2010
MM206-S1	F	S.Agona	L. michahellis	Medes	2011
MM218-S1	F	S.Agona	L. michahellis	Medes	2011
MM189-S1	F	S.Agona	L. michahellis	Medes	2011
MM122-S1	G	S.Agona	L. michahellis	Medes	2010
MM161-S1	G	S.Agona	L. michahellis	Medes	2010
MM190-S1	G	S.Agona	L. michahellis	Medes	2011
MM195-S4	G	S.Agona	L. michahellis	Medes	2011
MM249-S1	G	S.Agona	L. michahellis	Medes	2011
MM225-S1	G	S.Agona	L. michahellis	Medes	2011
MM238-S1	G	S.Agona	L. michahellis	Medes	2011
MM244-S2	G	S.Agona	L. michahellis	Medes	2011
MM255-S1	G	S.Agona	L. michahellis	Medes	2011
MM267-S2	H	S.Agona	L. michahellis	Medes	2011
MM268-S2	H	S.Agona	L. michahellis	Medes	2011
MM268-S4	H	S.Agona	L. michahellis	Medes	2011
MM270-S1	H	S.Agona	L. michahellis	Medes	2011
MM270-S3	H	S.Agona	L. michahellis	Medes	2011
MM206-S4	H	S.Agona	L. michahellis	Medes	2011
MM215-S1	I	S.Agona	L. michahellis	Medes	2011
MM189-S3	I	S.Agona	L. michahellis	Medes	2011

Figure 5. **PFGE dendrogram of XbaI patterns of *S.* Hadar isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method.



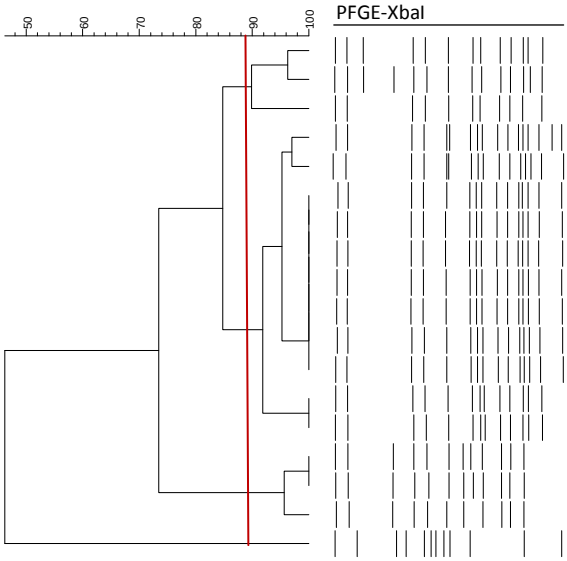
Strain	Serotype	Gull species	Sampling location	Year
DM169-S2	S.Hadar	L. michahellis	Ebro Delta	2010
DM170-S1	S.Hadar	L. michahellis	Ebro Delta	2010
DM170-S2	S.Hadar	L. michahellis	Ebro Delta	2010
AA151-S1	S.Hadar	L. audouinii	Alboran	2010
AA151-S2	S.Hadar	L. audouinii	Alboran	2010
CM104-S1	S.Hadar	L. michahellis	Columbretes	2010
DM128-S1	S.Hadar	L. michahellis	Ebro Delta	2010
AA151-S3	S.Hadar	L. audouinii	Alboran	2010
DGM91-S2	S.Hadar	L. michahellis	Dragonera	2011
DGM91-S3	S.Hadar	L. michahellis	Dragonera	2011
ZM17-S1	S.Hadar	L. michahellis	Zembra	2009
ZM17-S3	S.Hadar	L. michahellis	Zembra	2009
DA187-S2	S.Hadar	L. audouinii	Ebro Delta	2011
DM113-S1	S Hadar	L. michahellis	Ebro Delta	2010
MM233-S1	S.Hadar	L. michahellis	Medes	2011
MM198-S1	S.Hadar	L. michahellis	Medes	2011
MM233-S3	S.Hadar	L. michahellis	Medes	2011
DGM71-S2	S.Hadar	L. michahellis	Dragonera	2011
DGM101-S1	S.Hadar	L. michahellis	Dragonera	2011
DGM101-S2	S.Hadar	L. michahellis	Dragonera	2011
DGM94-S1	S.Hadar	L. michahellis	Dragonera	2011
DM95-S3	S.Hadar	L. michahellis	Ebro Delta	2010
DM23-S1	S.Hadar	L. michahellis	Ebro Delta	2009
DM23-S2	S.Hadar	L. michahellis	Ebro Delta	2009
MM141-S1	S.Hadar	L. michahellis	Medes	2010

Figure 6. **PFGE dendrogram of XbaI patterns of *S.* Derby isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



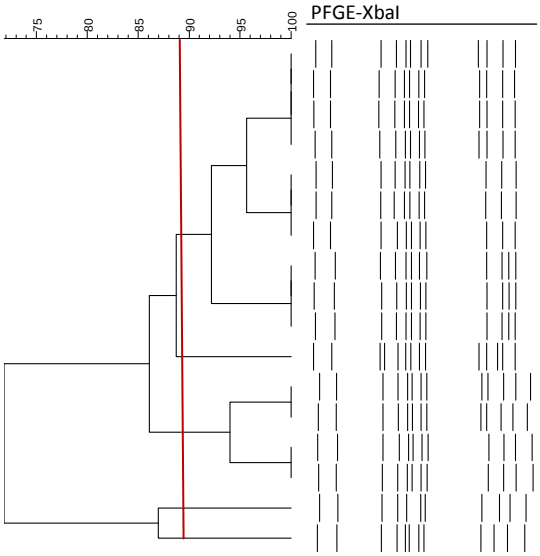
Strain	*	Serotype	Gull species	Sampling location	
CM167-S1	A	S.Derby	L. michahellis	Columbretes	2011
CM167-S3	A	S.Derby	L. michahellis	Columbretes	2011
DGM79-S1	A	S.Derby	L. michahellis	Dragonera	2011
DGM113-S1	B	S.Derby	L. michahellis	Dragonera	2011
DGM113-S2	B	S.Derby	L. michahellis	Dragonera	2011
DGM119-S2	B	S.Derby	L. michahellis	Dragonera	2011
DGM64-S1	B	S.Derby	L. michahellis	Dragonera	2011
CM122-S1	C	S.Derby	L. michahellis	Columbretes	2010
DM139-S1	C	S.Derby	L. michahellis	Ebro Delta	2010
MM178-S1	D	S.Derby	L. michahellis	Medes	2010
GAM8-S1	E	S.Derby	L. michahellis	Ons	2010
GAM8-S3	F	S.Derby	L. michahellis	Ons	2010
MM113-S1	G	S.Derby	L. michahellis	Medes	2010
MM215-S3	G	S.Derby	L. michahellis	Medes	2011
MM106-S1	G	S.Derby	L. michahellis	Medes	2010
MM255-S3	G	S.Derby	L. michahellis	Medes	2011
MM228-S1	G	S.Derby	L. michahellis	Medes	2011
CM95-S1	H	S.Derby	L. michahellis	Columbretes	2010
MM137-S1	H	S.Derby	L. michahellis	Medes	2010

Figure 7. **PFGE dendrogram of XbaI patterns of *S.* Senftenberg isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



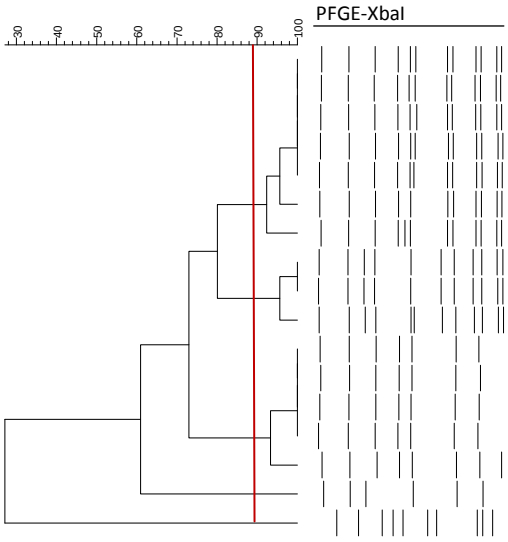
Strain	*	Serotype	Gull species	Sampling location
MM193-S1	A	S.Senftenberg	L. michahellis	Medes
MM200-S1	A	S.Senftenberg	L. michahellis	Medes
MM193-S2	A	S.Senftenberg	L. michahellis	Medes
MM242-S3	B	S.Senftenberg	L. michahellis	Medes
MM269-S1	B	S.Senftenberg	L. michahellis	Medes
MM235-S1	B	S.Senftenberg	L. michahellis	Medes
MM242-S1	B	S.Senftenberg	L. michahellis	Medes
MM253-S1	B	S.Senftenberg	L. michahellis	Medes
MM250-S4	B	S.Senftenberg	L. michahellis	Medes
MM253-S2	B	S.Senftenberg	L. michahellis	Medes
MM253-S3	B	S.Senftenberg	L. michahellis	Medes
MM259-S2	B	S.Senftenberg	L. michahellis	Medes
MM185-S1	B	S.Senftenberg	L. michahellis	Medes
MM195-S1	B	S.Senftenberg	L. michahellis	Medes
MM204-S1	C	S.Senftenberg	L. michahellis	Medes
MM223-S1	C	S.Senftenberg	L. michahellis	Medes
MM222-S1	C	S.Senftenberg	L. michahellis	Medes
MM249-S4	D	S.Senftenberg	L. michahellis	Medes

Figure 8. **PFGE dendrogram of XbaI patterns of *S.* Kentucky isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



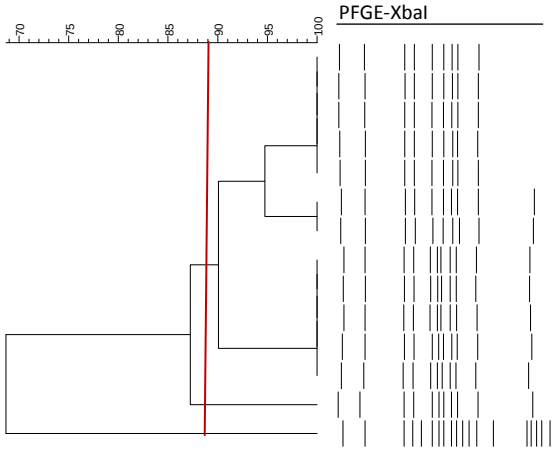
Strain	*	Serotype	Gull species	Sampling location	Year
AA280-S2	A	S.Kentucky	L. audouinii	Alboran	2011
ZM28-S1	A	S.Kentucky	L. michahellis	Zembra	2009
ZM28-S4	A	S.Kentucky	L. michahellis	Zembra	2009
ZM1-S1	A	S.Kentucky	L. michahellis	Zembra	2009
CM103-S1	A	S.Kentucky	L. michahellis	Columbretes	2010
CM103-S2	A	S.Kentucky	L. michahellis	Columbretes	2010
DA216-S3	A	S.Kentucky	L. audouinii	Ebro Delta	2011
CM174-S1	A	S.Kentucky	L. michahellis	Columbretes	2011
CM223-S1	A	S.Kentucky	L. michahellis	Columbretes	2011
CM235-S1	A	S.Kentucky	L. michahellis	Columbretes	2011
ZM15-S1	B	S.Kentucky	L. michahellis	Zembra	2009
AA257-S1	C	S.Kentucky	L. audouinii	Alboran	2011
AA309-S1	C	S.Kentucky	L. audouinii	Alboran	2011
DA187-S1	C	S.Kentucky	L. audouinii	Ebro Delta	2011
DA126-S2	C	S.Kentucky	L. audouinii	Ebro Delta	2010
CM103-S1	D	S.Kentucky	L. michahellis	Columbretes	2010
CM103-S2	E	S.Kentucky	L. michahellis	Columbretes	2010

Figure 9. **PFGE dendrogram of XbaI patterns of *S.* London isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



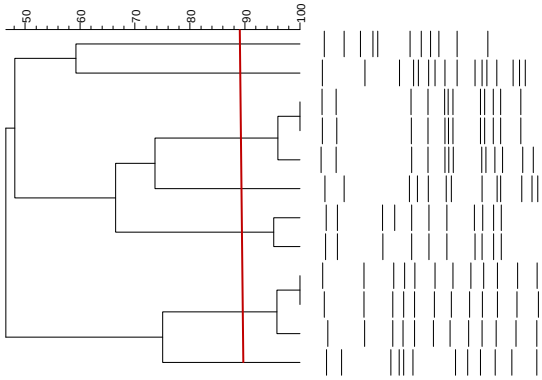
Strain	*	Serotype	Gull species	Sampling location	Year
MM220-S2	A	S.London	L. michahellis	Medes	2011
MM221-S4	A	S.London	L. michahellis	Medes	2011
MM258-S1	A	S.London	L. michahellis	Medes	2011
MM261-S1	A	S.London	L. michahellis	Medes	2011
MM261-S4	A	S.London	L. michahellis	Medes	2011
MM232-S3	A	S.London	L. michahellis	Medes	2011
MM209-S2	A	S.London	L. michahellis	Medes	2011
MCM52-S1	B	S.London	L. michahellis	Lanzarote	2011
MCM55-S1	B	S.London	L. michahellis	Lanzarote	2011
DA155-S3	B	S.London	L. audouinii	Ebro Delta	2011
MM220-S1	C	S.London	L. michahellis	Medes	2011
MM221-S1	C	S.London	L. michahellis	Medes	2011
MM224-S1	C	S.London	L. michahellis	Medes	2011
MM232-S3	C	S.London	L. michahellis	Medes	2011
MM192-S1	C	S.London	L. michahellis	Medes	2011
CM69-S1	D	S.London	L. michahellis	Columbretes	2009
MM241-S2	E	S.London	L. michahellis	Medes	2011

Figure 10. **PFGE dendrogram of XbaI patterns of *S.* Amsterdam isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



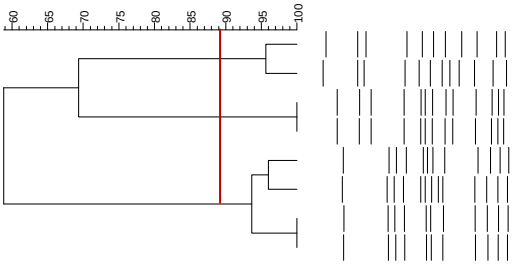
Strain	*	Serotype	Gull species	Sampling location	Year
MM216-S3	A	S.Amsterdam	L. michahellis	Medes	2011
MM227-S1	A	S.Amsterdam	L. michahellis	Medes	2011
MM251-S1	A	S.Amsterdam	L. michahellis	Medes	2011
MM105-S1	A	S.Amsterdam	L. michahellis	Medes	2010
MM217-S1	A	S.Amsterdam	L. michahellis	Medes	2011
MM150-S1	A	S.Amsterdam	L. michahellis	Medes	2010
MM209-S1	A	S.Amsterdam	L. michahellis	Medes	2011
MM89-S1	A	S.Amsterdam	L. michahellis	Medes	2010
MM97-S1	A	S.Amsterdam	L. michahellis	Medes	2010
MM99-S1	A	S.Amsterdam	L. michahellis	Medes	2010
MM217-S2	A	S.Amsterdam	L. michahellis	Medes	2011
MM227-S2	A	S.Amsterdam	L. michahellis	Medes	2011
MM210-S1	B	S.Amsterdam	L. michahellis	Medes	2011
MM251-S2	C	S.Amsterdam	L. michahellis	Medes	2011

Figure 11. **PFGE dendrogram of XbaI patterns of *S.* Newport isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



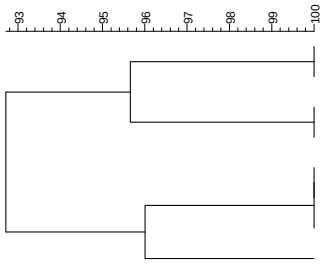
PFGE-XbaI	Strain	*	Serotype	Gull species	Sampling location	Year
	MCM11-S1	A	S.Newport	L. michahellis	Lanzarote	2010
	MCM24-S1	B	S.Newport	L. michahellis	Lanzarote	2010
	DA157-S3	C	S.Newport	L. audouinii	Ebro Delta	2011
	DA157-S4	C	S.Newport	L. audouinii	Ebro Delta	2011
	MCM57-S2	C	S.Newport	L. michahellis	Lanzarote	2011
	CM124-S2	D	S.Newport	L. michahellis	Columbretes	2010
	AA172-S3	E	S.Newport	L. audouinii	Alboran	2010
	AA172-S4	E	S.Newport	L. audouinii	Alboran	2010
	AA172-S2	F	S.Newport	L. audouinii	Alboran	2010
	AA172-S1	F	S.Newport	L. audouinii	Alboran	2010
	GAM62-S3	F	S.Newport	L. michahellis	Ons	2010
	CM129-S1	G	S.Newport	L. michahellis	Columbretes	2010

Figure 12. **PFGE dendrogram of XbaI patterns of *S.* Braenderup isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



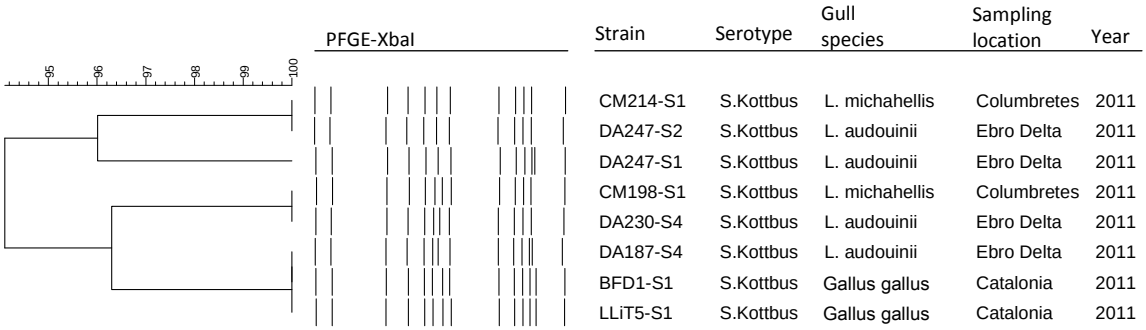
PFGE-XbaI	Strain	*	Serotype	Gull species	Sampling location	Year
	CM93-S4	A	S.Braenderup	L. michahellis	Columbretes	2010
	CM93-S3	A	S.Braenderup	L. michahellis	Columbretes	2010
	DGM82-S1	B	S.Braenderup	L. michahellis	Dragonera	2011
	DGM85-S1	B	S.Braenderup	L. michahellis	Dragonera	2011
	DM157-S2	C	S.Braenderup	L. michahellis	Ebro Delta	2010
	CM212-S1	C	S.Braenderup	L. michahellis	Columbretes	2011
	DM82-S1	C	S.Braenderup	L. michahellis	Ebro Delta	2010
	DM157-S1	C	S.Braenderup	L. michahellis	Ebro Delta	2010

Figure 13. **PFGE dendrogram of XbaI patterns of *S.* Corvallis isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method.



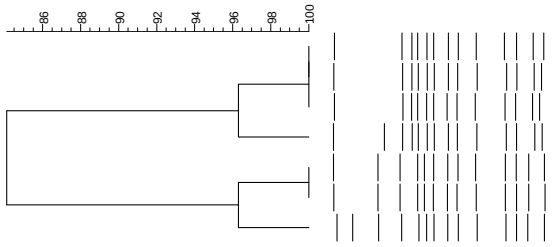
PFGE-XbaI	Strain	Serotype	Gull species	Sampling location	Year
	MCM57-S3	S.Corvallis	L. michahellis	Lanzarote	2011
	MCM57-S4	S.Corvallis	L. michahellis	Lanzarote	2011
	GAM83-S3	S.Corvallis	L. michahellis	Ons	2010
	GAM83-S4	S.Corvallis	L. michahellis	Ons	2010
	DGM96-S3	S.Corvallis	L. michahellis	Dragonera	2011
	DGM104-S1	S.Corvallis	L. michahellis	Dragonera	2011
	DGM104-S2	S.Corvallis	L. michahellis	Dragonera	2011
	DA157-S1	S.Corvallis	L. audouinii	Ebro Delta	2011

Figure 14. **PFGE dendrogram of XbaI patterns of *S.* Kottbus isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method.



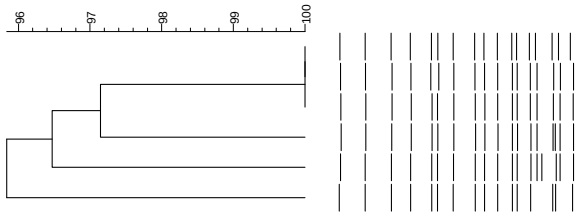
Strain	Serotype	Gull species	Sampling location	Year
CM214-S1	S.Kottbus	L. michahellis	Columbretes	2011
DA247-S2	S.Kottbus	L. audouinii	Ebro Delta	2011
DA247-S1	S.Kottbus	L. audouinii	Ebro Delta	2011
CM198-S1	S.Kottbus	L. michahellis	Columbretes	2011
DA230-S4	S.Kottbus	L. audouinii	Ebro Delta	2011
DA187-S4	S.Kottbus	L. audouinii	Ebro Delta	2011
BFD1-S1	S.Kottbus	Gallus gallus	Catalonia	2011
LLiT5-S1	S.Kottbus	Gallus gallus	Catalonia	2011

Figure 15. **PFGE dendrogram of XbaI patterns of *S.* Infantis isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



Strain	*	Serotype	Gull species	Sampling location	Year
CM42-S1	A	S.Infantis	L. michahellis	Columbretes	2009
GAM13-S1	A	S.Infantis	L. michahellis	Ons	2010
GAM1-S3	A	S.Infantis	L. michahellis	Ons	2010
GAM19-S1	A	S.Infantis	L. michahellis	Ons	2010
AA114-S1	B	S.Infantis	L. audouinii	Alboran	2010
AA114-S2	B	S.Infantis	L. audouinii	Alboran	2010
AA114-S3	B	S.Infantis	L. audouinii	Alboran	2010

Figure 16. **PFGE dendrogram of XbaI patterns of *S.* Stanley isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method.



Strain	Serotype	Gull species	Sampling location	Year
DA239-S1	S.Stanley	L. audouinii	Ebro Delta	2011
DA239-S2	S.Stanley	L. audouinii	Ebro Delta	2011
DA239-S3	S.Stanley	L. audouinii	Ebro Delta	2011
DA245-S2	S.Stanley	L. audouinii	Ebro Delta	2011
DA245-S3	S.Stanley	L. audouinii	Ebro Delta	2011
DA244-S3	S.Stanley	L. audouinii	Ebro Delta	2011

Figure 17. **PFGE dendrogram of XbaI patterns of *S.* Virchow isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method.

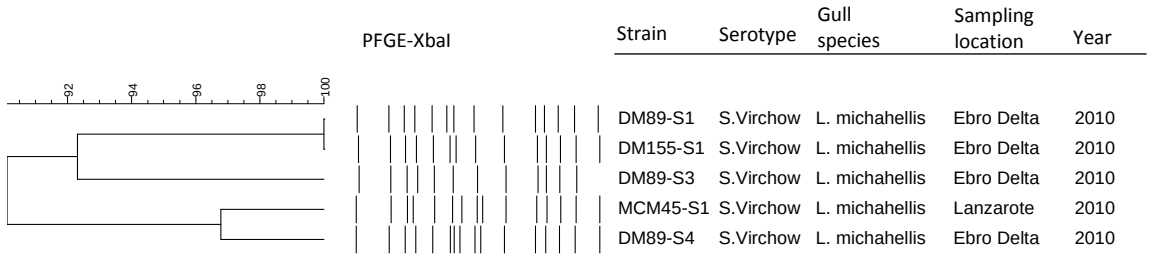
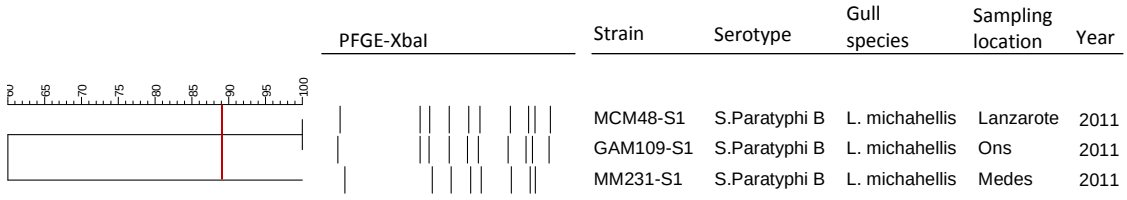


Figure 18. **PFGE dendrogram of XbaI patterns of *S.* Paratyphi B isolates.** The similarities between strains were evaluated using the Dice coefficient and the UPGMA clustering method; (*) PFGE profile.



Discussion

The main reservoirs of *Salmonella* spp. are the alimentary tract of wild and domesticated birds and mammals. Seagulls are carriers of a great diversity of serotypes. Most of the serotypes isolated from seagulls in this study were frequently reported in Spain and in the EU in human outbreaks associated with contaminated food and in food animals (EFSA, 2013a). This includes Typhimurium and Enteritidis serotypes, which are the ones most commonly reported in human salmonellosis in the EU (EFSA, 2013). This coincidence of serotypes in humans, food animals and seagulls suggests a role of seagulls in contributing to the dispersal and maintenance of public health important serotypes in the environment.

Overall, genotyping analysis (ERIC-PCR, PFGE) of *Salmonella* spp. isolates showed a notable diversity of strains within certain serotypes (Typhimurium, Agona, Derby, Newport) and a low clonality within other serotypes (*S.* Enteritidis, *S.* Hadar and *S.* Amsterdam). Birds usually were carriers of a single *Salmonella* strain as demonstrated by ERIC-PCR (data not shown).

S. Typhimurium was the serotype with the highest number of profiles detected by ERIC-PCR and PFGE. This might be due to the higher number of isolates analyzed within this serotype, but may also reflect the diversity of strains circulating in the environment. Most *S.* Typhimurium PFGE profiles were detected in different colonies, both in the same and in different years. Also, the same profile was detected in the same colony during different years (e.g. Medes Is., 2009-2010; Columbretes Is., 2010-2011; Ons Is., 2010-2011). Two different PFGE profiles were detected in different sampling sites during the three-years sampling. This is indicative that there are some *S.* Typhimurium resident strains that survive for long periods of time within a colony, but also that seagull foraging or migrating movements contribute to the introduction of new strains in these colonies every year.

With regards to other *Salmonella* serotypes, common PFGE profiles were detected in closer localities but also in distant sites, (e.g. *S.* Kentucky, *S.* Infantis *S.* Hadar, *S*. Enteritidis, *S.* Corvallis, *S.* Paratyphi and *S*. Agona). It is noteworthy the high dispersal of a strain of *S.* Enteritidis (isolated in Alboran Is., Zembra Is., Columbretes Is., Lanzarote Is. and Ons Is.) and of *S.* Hadar (detected in Ebro Delta, Columbretes Is., Zembra Is., Dragonera Is. and Alboran Is). These findings suggest the contribution of the migratory behaviour of seagulls in the dispersal of *Salmonella* strains of public health importance over very large distances. However, for certain gull colonies, a common source of infection of the seagulls cannot be ruled out.

Food availability influences the foraging movements of seagulls, and therefore the potential introduction of new strains and serotypes in a colony. In Columbretes Is. (about 50 Km from the coast of Valencia), seagulls frequently feed on fisheries discards. However, from November to December trawler boats cannot work out at sea and consequently the access of seagulls to fisheries discards becomes difficult and gulls have to move to other sites in search of food, usually to the nearest coast of Valencia (Arcos, 2001). This change in gull's behaviour along the year can contribute to the introduction of new strains and serotypes to the island every year. On the contrary, in Medes Is. there has been an increasing abundance of alternative food from

urban dumps nearby the coast. This increase of food resources causes a reduction in the migratory trend of gulls and promotes a sedentary behaviour of these birds (Kilpi and Saurola, 1985). Usually, seagulls get infected when feeding at those refuse tips and subsequently can transmit *Salmonella* to their fledglings, other adults of the colony or contaminate the water through the droppings (Durrant and Beatson, 1981). Also, most of these gulls stay in the colony throughout the year, contributing to the high *Salmonella* prevalence and the maintenance of resident *Salmonella* strains (e.g, some Agona and Amsterdam strains) throughout the year. Hence, chicks can easily get infected by those resident strains, since it has been reported that *Salmonella* strains can survive in the environment of a breeding colony between reproductive periods (Literák et al. 1996).

Gulls are considered as obligate partial migrating birds. When food resources are scarce, the competition for food increases and some gulls might be forced to forage far away from the colony, especially if they are unable to find alternative food. One of the most important alternative food used by seagulls originates from human activities, which are used by gulls of all ages, although adults use them more frequently than immature gulls (Duhem et al., 2003; Ramos et al., 2009). This is because of learned experience by adults. Thus, since immature gulls are inferior in foraging efficiency, they usually leave their natal area more frequently than adults (Burger and Gochfeld, 1981). Dispersal and migrating movements of immature gulls in search of food, and the trend of adults to stay in the colony could explain the genotypic diversity of *Salmonella* spp. strains found in several colonies and the maintenance of resident strains in a colony (Carrera et al., 1981; Galarza et al., 2012; Martínez-Abrain et al., 2002).

While adult gulls are important in the maintenance of strains in a colony, sub-adults but also some adults are essential in the strains dispersal when migrating to other sites. Thus, while some colonies had exclusive PFGE profiles of certain serotypes, probably due to a sedentary behaviour of adult gulls, others showed PFGE profiles in common to other sites. These common PFGE profiles were found in closer colonies (< 500 Km between colonies). This might reflect migrating movements of some adults

and sub-adults among close colonies (Medes Is., Ebro Delta and Columbretes Is.), resulting in strains dispersal within those colonies.

Ons Is. is an island far away from the rest of the studied colonies and it may be expected to find exclusive PFGE profiles not found in the other sampling sites. However, all of the *S.* Typhimurium profiles found in this colony were also detected in other localizations (Ebro Delta, Medes Is., Columbretes Is., Lanzarote Is. and Tenerife Is.). This situation also occurred in Tenerife Is. and Lanzarote Is.; these two islands are very close to each other and common *S.* Typhimurium PFGE profiles to gulls from both colonies were detected, but also with gulls from other distant colonies. Only Zembra Is. and Dragonera Is. had some exclusive *S.* Typhimurium PFGE patterns. These common PFGE profiles among distant colonies might be due to the widespread distribution of certain clones within this serotype.

Wild birds are thought to be an important source of farms infection if they gain direct access to poultry reared outdoors, or by contaminating surface water or soil that is readily accessible to free-range birds. As such, wild birds and free-range poultry could be expected to have several *Salmonella* genotypes in common. In the present study, *S.* Kottbus isolated from both seagulls and free range poultry showed the same macrorestriction profile. This finding could suggest a common source of infection through food or from contaminated environment, or could be due to a direct transmission between wild and domestic birds. Infected food animals that will be consumed by humans became a major public health hazard.

It can be concluded that seagulls from western Mediterranean and eastern Atlantic Ocean are important carriers of a high diversity of *Salmonella* genotypes. Their maintenance in the colonies and its spread beyond the colony and surrounding area by migrating seagulls is an important issue to be considered in order to better understand the epidemiology of this zoonotic agent. These results provide new insights into the relevance that migrations and dispersal movements of seagulls have in the global epidemiology of *Salmonella*. Further studies are granted to determine the contribution

of seagulls on the human and on-farm epidemiology of *Salmonella* spp. infections in southern Europe.

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General Discussion

Salmonella spp. and thermophilic *Campylobacter* spp. are the most important zoonotic pathogens causing foodborne diseases worldwide (EFSA, 2013a). Based on economic impact and statistics of the infections caused by both bacteria, there is a need of further research to better understanding the epidemiology of both bacteria. This will allow establishing effective surveillance programs and controlling measures focused to reduce the risk of human infections.

Most human infections by these enteropathogens are believed to result from consumption of poultry meat which is thought to be contaminated primarily by faeces (Shane, 1992; Kramer et al. 2000). Poultry is the main source of these foodborne pathogens, but it is clear that other carriers also exist (Sacks et al., 1986; Tomar et al., 2006). The epidemiology of *Salmonella* spp. and *Campylobacter* spp. cannot be explained solely by food-borne exposure; both bacteria are widely distributed in the environment and have been isolated from a range of environmental samples, including soil, water, sand, and the faeces of a number of wildlife species, including wild birds (Cizek et al., 1994; Molina-Lopez et al., 2011; Refsum et al., 2002). Both bacteria have usually been isolated from the faeces and/or cloacal swabs of apparently healthy wild birds (i.e. gulls, pigeons, crows) (Kapperud et al., 1983, Waldenström et al., 2002; Ramos et al., 2010). Particularly, *Campylobacter* spp. is considered part of the normal intestinal microbiota of domestic animals (Haruna et al., 2013; Roug et al., 2013).

The non-harmful coexistence between *Salmonella* spp. and *Campylobacter* spp. and their birds host is a disturbing factor in the control of both bacteria (Luechtefeld et al., 1980; Waldenström et al., 2002). Hence, it is of concern the important risk that those wild birds may represent, since they may act as effective spreaders of both enteropathogens via faecal contamination of pastures and water (Reed et al., 2003). Particularly, migrating birds are species that can cyclically cross one or more national boundaries and use a variety of habitats (wetlands, marshes and other water bodies). Given their ability to fly freely and cover long distances during annual migrations, migratory birds can play a crucial role in the dissemination of these enteropathogenic bacteria to the environment (Hubalek, 2004; Sensale et al., 2006; Waldenström et al., 2007).

Seagulls, compared with other migratory wild bird species, can have considerably higher carrier levels of zoonotic bacteria (Cizek et al., 1994; Hubalek et al., 1995; Kapperud and Rosef, 1983). During the past several decades, populations of several species of gulls (*Larus* spp.) have increased throughout Europe, Australia and North America. These marine birds are occupying a habitat that substantially overlaps with human activities and are reported to spread various animal pathogens (Garza et al., 1997; Olsen et al., 1995). This situation has led to an increasing number of studies concerning seagulls and environmental public health (Smith and Carlile, 1993; Vidal et al., 1998). Nevertheless, limited data exist about the occurrence of *Salmonella* spp. and *Campylobacter* spp. in seagulls in Southern Europe. Also, the existing information about the presence of these zoonotic bacteria in free-living waterfowl is scarce. Waterfowl species are hunted every year for home consumption and can represent an important risk of infection to humans if consumed hunted fowl is infected with *Salmonella* or *Campylobacter* (Luechtefeld et al., 1980). Therefore, studies focussed to gain insight into the epidemiology of zoonotic bacteria in these wild birds are useful to develop control measures to reduce the risk of exposure to humans.

Most of the epidemiological studies on *Campylobacter* spp. and *Salmonella* spp. carried out to date have been focussed in intensive poultry production, and little attention has been posed to the alternative farming husbandry, despite its remarkable increase in the EU in recent years (Franz et al., 2012; Marinou et al., 2012). In the alternative farming husbandry, animals can roam freely for food, rather than being confined in an enclosure. These animals have a close contact with the environment and consequently are exposed to multiple sources of contamination by pathogens.

The same *Salmonella* spp. and *Campylobacter* spp. strains have been isolated from farm birds, wild birds and humans. These findings suggest a circulation of both bacteria between farm and wild birds (Colles et al., 2008). Whether wild birds are a source of infection for humans or domestic livestock or are mainly recipients of domestic animal or environmental strains is not fully understood. To contribute to understand these

issues, there is a need to gain insight into the carriage and the antimicrobial resistance of both bacteria in wild and domestic birds in Southern Europe.

The transfer of resistance genes and bacteria among animals, animal products and the environment, and the indiscriminate use of drugs in animals and humans are factors contributing to increase the number of antimicrobial resistant *Salmonella* spp. and *Campylobacter* spp. (McEwen and Fedorka-Cray, 2002; Luangtongkum et al., 2009; EFSA, 2013b). Nowadays, antimicrobial resistance is one of the major public health problems, since it can compromise both human and animal treatment. It is therefore important the prudent use of antimicrobials and to know how to deal with the antimicrobial resistant bacteria that is already in the environment (Casewell et al., 2003).

In order to answer some of these questions, several studies were conducted to determine the contribution of domestic birds (poultry reared outdoors) and wild birds (waterfowl and seagulls) in the epidemiology and antimicrobial resistance of *Salmonella* spp. and *Campylobacter* spp. in Southern Europe.

Campylobacter spp. was isolated in both domestic and wild birds. The highest occurrence was found in poultry reared outdoors (83.10 %) followed by waterfowl (12.58 %) and finally seagulls, where *Campylobacter* was mainly detected in Audouin's gull colonies, with a mean occurrence of 14.74 %. There were great differences of *Campylobacter* carriage among the different waterfowl species studied, which could be due to differences in feeding habits. Eurasian coot (*Fulica atra*), which has coprophagic habits (Vogrin et al., 1997), showed the highest occurrence of *Campylobacter*, while the lowest occurrence was detected in waterfowl species which feed almost exclusively on vegetable matter.

Most of the *Campylobacter* positive birds detected in these studies share habitat in Ebro Delta, one of the largest wetland areas in the western Mediterranean, where *Campylobacter* species carriage in these birds showed a different pattern. Poultry reared outdoors demonstrated to be an important reservoir of *Campylobacter* spp.,

especially *C. jejuni.* This species was also the most predominant in seagulls, while in waterfowl *C. coli* was the main species isolated. *C. jejuni* and *C. coli* are the main *Campylobacter* species associated with human enteric infections worldwide, and are therefore of public health importance (Tauxe, 2001; EFSA, 2013a).

The high occurrence and diversity of thermophilic *Campylobacter* found in poultry reared outdoors and certain wild birds, might be due to their great exposure to multiple environmental sources of contamination and to the horizontal transmission of this zoonotic agent (Kazwala et al., 1990; Jacobs-Reitsma et al., 1995; Newell and Wagenaar, 2000).

Salmonella was not isolated from waterfowl, which might be due to an intermittent shedding of the pathogen or its real absence in waterfowl from Ebro Delta. Also, the low *Salmonella* occurrence detected in poultry reared outdoors (only two *Salmonella*-positive free-range farms, chicken and duck farms), may be explained by the intermittent shedding of *Salmonella* spp. and by some characteristics of the alternative production systems (e.g. low bird density and reduced stress in bird reared outdoors) (Crhanova et al., 2011). All *Salmonella* isolates found in poultry reared outdoors were identified as *S.* Kottbus, a serotype considered a public health hazard due to its implication in human outbreaks (CDC, 2002; Palmera-Suarez et al., 2007).

There were also differences between *Salmonella* and *Campylobacter* carriage in seagulls. *Salmonella* spp. was found more often in yellow-legged gulls (17.98 %), while *Campylobacter* spp. was more frequently isolated from Audouin's gulls (14.74 %.). The feeding habits of yellow-legged gulls, which are well known scavengers, particularly when colonies are close to human activities such as Ebro Delta or Medes Is., can explain the *Salmonella* carriage levels of this seagull species. On the other hand, Audouin's gull is supposed to have "clean" feeding habits and therefore, it would have been expected to have a low carriage of zoonotic bacteria. However, this gull species shows greater foraging plasticity than expected, linked to marine food resources availability and the exploitation of terrestrial resources (Christel et al., 2012), which

may influence its zoonotic bacterial carriage. *Campylobacter* might be part of the normal gut microbiota of gulls, which can explain its presence in both gull species.

The two most important serotypes causing human food-borne disease, *S.* Enteriditis and *S.* Typhimurium, were isolated in most of the studied seagull colonies. Other serotypes which have been increasingly reported in human food-borne diseases in the UE during the last years, such as Infantis, Agona, Hadar and Virchow (EFSA, 2013a), were also found in these birds. The high diversity of *Salmonella* serotypes found in seagulls could be due to the close contact of the birds with the environment and with refuse tips and sewage, where most likely these birds can become infected with Salmonella of human or domestic animal origin.

All *Campylobacter* isolates from waterfowl were susceptible to all of the antimicrobials examined. Since wild birds do not naturally come into contact with antimicrobials, this full susceptibility would be expected. However, *Salmonella* and *Campylobacter* strains isolated from seagulls showed resistance to several antimicrobial agents. The main resistances found in *Salmonella* isolates were to tetracycline, streptomycin, amoxicillin, ampicillin and nalidixic acid. *Campylobacter* isolates showed resistance to tetracycline, quinolones and fluoroquinolones. Also, *Campylobacter* spp. and *Salmonella* spp. resistant strains were isolated from poultry reared outdoors. A 94.81 % of *Campylobacter* isolates from poultry were resistant to at least one antimicrobial; the main resistances were to quinolones and fluoroquinolones, followed by tetracycline. *Salmonella* Kottbus strains were resistant to β-lactams and tetracycline. The high antimicrobial resistances found both in *Salmonella* and *Campylobacter* isolates are of concern, particularly taking into account that important resistances to antimicrobials commonly used in human infections were detected. These include cephalosporins and fluoroquinolones, the antimicrobials of choice to treat severe salmonellosis and campylobacteriosis in humans. The emergence of resistance to some of these antimicrobials in recent years may compromise the effectiveness of treatment in enteric human infections which in turn can lead to more severe outcomes in patients.

A high diversity of *Campylobacter* strains was found in poultry reared outdoors and waterfowl, as demonstrated by ERIC-PCR and PFGE techniques. This can be due to the close contact of these birds with the environment and may reflect a variety of infection sources with various *Campylobacter* types. A great diversity was also detected in *Salmonella* isolates from seagulls. However, some genotypic *Salmonella* patterns were detected more than once in different seagull colonies and in different seagull species (yellow-legged gull and Audouin's gull), which can suggest a common origin of infection or a spread of *Salmonella* strains by the seagull annual migration or dispersal movements. On the other hand, the detection of the same *Salmonella* Kottbus strain in poultry reared outdoors and seagulls might be due to a common origin of *Salmonella* infection between farm and wild birds or a direct transmission between both bird species. Nevertheless, more studies are needed in order to assert this hypothesis.

In summary, the data provided in this thesis highlights the importance of poultry reared outdoors, seagulls and certain waterfowl species as contributors of *Campylobacter* spp. to the environment and as a possible source of infection for humans in the study area. Also, while waterfowl and poultry reared outdoors seem not to be an important reservoir of *Salmonella* spp., seagulls are important carriers of a variety of *Salmonella* serotypes, some of them of important public health concern. The presence of *Salmonella* and *Campylobacter* resistant strains in seagulls and poultry reared outdoors, especially with a high prevalence of resistance to certain antimicrobials of common use in human and veterinary medicine, are of concern, since it can compromise the effective treatment of bacterial diseases. Monitoring schemes, improved surveillance efforts and development of appropriate control strategies are needed in poultry reared outdoors and certain wild birds, in order to reduce the occurrence and carrier levels of *Salmonella* and *Campylobacter* in those birds and consequently the risk of human exposure.

Conclusions

1. Poultry reared outdoors constitutes a reservoir for *Campylobacter* spp. in Spain with *C. jejuni* being the dominating species.

2. Waterfowl, especially those species with coprophagic habits, are carriers of thermophilic *Campylobacter* species, mainly *C. coli*, in Ebro Delta.

3. The strain diversity of *C. jejuni* and *C. coli* from poultry reared outdoors and waterfowl may be the result of a great exposure of these birds to different environmental sources.

4. Yellow-legged gulls are an important reservoir of *Salmonella* serotypes in Southern Europe and Audouin's gulls are important carriers of thermophilic *Campylobacter* spp. in Ebro Delta and Alboran Island.

5. The most important *Salmonella* serotypes causing food-borne diseases in humans, as well as those more frequently isolated from food animals are present in gulls in Southern Europe. This indicates that humans and production animals are a source for *Salmonella* transmission to these marine birds.

6. Seagulls and poultry reared outdoors, but not waterfowl, are reservoirs of antimicrobial resistant and multiresistant strains of *Campylobacter* spp. and *Salmonella* spp., which may have public health implications..

7. ERIC-PCR and PFGE typing of *Salmonella* isolates from gulls suggest that these wild birds factor in the persistance of *Salmonella* strains in different localities between breeding seasons, and play a role in their dissemination in the environment.

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