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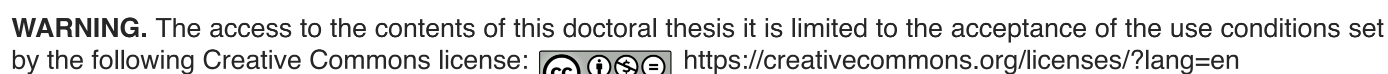
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en

A One Health approach into the epidemiology of *Campylobacter* and *Salmonella*: The continuum seabirds - humans

Elisabet Moré Mir

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Bellaterra, 2018

Universitat Autònoma de Barcelona

A One Health approach into the epidemiology of *Campylobacter* and *Salmonella*: the continuum seabirds - humans

Tesi doctoral presentada per na **Elisabet Moré Mir** per optar al grau de Doctora en el marc del programa de Doctorat de Medicina i Sanitat Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. **Marta Cerdà Cuéllar** i la tutoria del Dr. **Joaquim Segalés Coma**.

Bellaterra, 2018

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La Dra. **Marta Cerdà Cuéllar**, investigadora del Centre de Recerca en Sanitat Animal de l'Institut de Recerca i Tecnologia Agroalimentàries (CReSA-IRTA), i el Dr. **Joaquim Segalés Coma**, professor titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigador adscrit al CReSA-IRTA,

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Al meu avi,

l’home més bo que he conegut mai

Agraïments

Miro enrere després d'aquest llarg camí que m'ha portat fins aquí i me n'adono que hi ha moltíssimes persones que m'han acompanyat i que gràcies a la seva ajuda han fet possible aquesta tesis. És per això, que vull agrair:

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List of abbreviations

AMR	Antimicrobial resistance
API	Analytical Profile Index
CC	Clonal complex
CFU	Colony-forming units
cgMLST	Core-genome multilocus sequence typing
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic acid
DR	Direct repeats
eBG	eBurst group
ECDC	European Centre for Disease Prevention and Control
EEA	European Economic Area
EFSA	European Food Safety Authority
ERIC	Enterobacterial repetitive intergenic consensus
EU	European Union
FISH	Fluorescence *in situ* hybridization
GALT	Gut-associated lymphoid tissue
GBS	Guillain-Barré syndrome
HIV	Human immunodeficiency virus
iNTS	Invasive nontyphoidal *Salmonella*
IS	Insertion sequences
ISO	International Organization for Standardization
LPS	Lipopolysaccharide
LPSN	List of prokaryotic names with standing in nomenclature
MCP	Methyl-accepting chemotaxis protein
MDR	Multidrug resistance
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing

MPS	Mononuclear phagocyte system
MUCAP	4-methylumbelliferyl caprylate
NGS	Next-generation sequencing
NTS	Nontyphoidal *Salmonella*
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PMNL	Polymorphonuclear leukocytes
qPCR	Quantitative polymerase chain reaction
RFLP	Restriction fragment length polymorphism
rMLST	Ribosomal MLST
rRNA	Ribosomal ribonucleic acid
SCV	*Salmonella*-containing vacuole
SNP	Single nucleotide polymorphism
SPI	*Salmonella* pathogenicity island
ST	Sequence type
SVR	Short variable region
TCRS	Two-component regulatory system
T3SS	Type III secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system
UK	United Kingdom
USA	United States of America
VBNC	Viable but non-culturable
WHO	World Health Organization
WHOCC-Salm	WHO Collaborating Centre for Reference and Research on *Salmonella*
wgMLST	Whole genome multilocus sequence typing
WGS	Whole genome sequencing

Summary

Zoonotic thermophilic *Campylobacter* spp. and nontyphoidal *Salmonella enterica* are a major cause of foodborne human gastroenteritis worldwide. Both bacteria are able to infect a broad range of domestic and wild animals. A wide variety of wild birds, especially gulls, have been reported as asymptomatic carriers of these zoonotic agents in Europe, America and Australia. However, there is scarce information about these reservoirs in Africa and remote regions of the Southern Ocean, and the role of wild birds in the epidemiology of these pathogens is not fully understood. Thus, within the framework of this PhD thesis we have investigated the occurrence, antimicrobial susceptibility, virulence potential and population structure or genetic diversity of *Campylobacter* and *Salmonella* spp. in seabird species along the western coast of South Africa (near the Benguela Upwelling Region) and across the Antarctic and Subantarctic region. We have also analysed the genetic relation and virulence potential of isolates of *Salmonella* serovars from seabirds, poultry and humans, to assess whether common strains are circulating among different niches in Southwestern Europe.

In Western Cape (South Africa), we detected thermophilic *Campylobacter* spp., mainly *C. jejuni*, in kelp gulls and greater crested terns with similar prevalences. Most *C. jejuni* sequence types (ST)s belonged to the clonal complex (CC)-1275, which is mainly related to aquatic environments and wild birds. On the contrary, a higher occurrence of *Salmonella* was observed in kelp gulls than in greater crested terns, which seems to be related to the scavenging feeding habits of the former. Anatum, Enteritidis and Hadar were the most frequent *Salmonella* serovars, although a great diversity of other zoonotic serovars were found, especially in gull colonies near urban areas. The same or highly similar pulsed-field gel electrophoresis genotypes (pulsotypes) were detected in some *Salmonella* isolates from seabirds and humans presenting with salmonellosis in Cape Town hospitals. Most *S*. Enteritidis and *S*. Typhimurium isolates belonged to ST-11 and ST-34, respectively, which are genotypes globally distributed in a broad range of

hosts. In addition to virulence potential, both *Campylobacter* and *Salmonella* isolates exhibited antimicrobial resistance to several agents, including critically important antimicrobials (quinolones, tetracyclines and β-lactams), and multidrug resistance in *Salmonella* serovars from kelp gulls.

Thermophilic *Campylobacter* spp. were also found in all sampled Antarctic and Subantarctic islands, mainly *C. lari*, but also *C. jejuni*, specially in brown skuas, one of the main opportunistic seabird species in the Southern Ocean region. It is noteworthy that *C. jejuni* CC-21, CC-45 and CC-206, associated to domestic animals and human infections, were isolated. However, *Salmonella* (mainly *S*. Enteritidis ST-11) was only isolated from a few seabirds at Livingston Island (Antarctic Peninsula) suggesting this bacterium is not indigenous to the region. The presence of *C. jejuni* and *S*. Enteritidis genotypes commonly found in humans and domestic animals, suggests reverse zoonosis (from humans to seabirds) probably through tourism and scientific activities. Nevertheless, this pathogens introduction to remote regions by other sources, such as the migration movements of seabirds, cannot be ruled out. We also show further spread of the bacteria among Antarctic wildlife is facilitated by substantial connectivity among populations of opportunistic seabirds, notably skuas.

On the other hand, in seagulls from Southwestern Europe we identified a high diversity of exclusive *Salmonella* pulsotypes (mainly *S*. Typhimurium) compared to the more predominant pulsotypes from poultry and humans, which likely indicates that seagulls are exposed to a higher variety of contamination sources. However, we detected 30 pulsotypes in common among isolates from two or three different host niches belonging to 12 different serovars: Bredeney, Derby, Enteritidis (ST-11), Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima, Rissen, Typhimurium (ST-19 and ST-34) and Virchow. This finding suggests the existence of generalist *Salmonella* strains circulating among different compartments. In addition, the presence of a wide repertoire of virulence-associated genes, regardless of the host of origin, may increase the capacity of these strains to infect different hosts and to adapt to new environments.

Our results demonstrate that seabirds can be carriers of *Campylobacter* and *Salmonella* strains of anthropogenic origin, some of them showing antimicrobial resistance and an important virulence potential. Our findings support that seabirds contribute to the amplification and maintenance of these pathogens in the environment. In addition, given the foraging and migratory movements of seabirds, they may play an important role in the spread of these zoonotic agents, but also of resistance and virulence genes by mobile genetic elements, to remote geographical areas and new animal hosts. It is necessary to increase the surveillance systems to wildlife, especially in seabirds, and to establish stricter environmental policies for the management of human wastes to limit the access of these birds to anthropogenic sources of contamination, which may help to control the dissemination of strains with potential hazard for the public and animal health.

Resum

Les espècies termòfiles de *Campylobacter* i serovars no tifoides de *Salmonella enterica* són els principals agents causals de gastroenteritis humana transmesa pels aliments a nivell mundial. Ambdós bacteris són capaços d'infectar un ampli ventall d'animals domèstics i salvatges. Una gran varietat d'aus silvestres, especialment les gavines, són portadores asimptomàtiques d'aquests agents zoonòtics a Europa, Amèrica i Austràlia. Tot i així, hi ha poca informació sobre aquests reservoris a Àfrica i a les regions remotes de l'Oceà Austral, i el paper de les aus silvestres en l'epidemiologia d'aquests patògens no es coneix del tot. Per tant, en el marc d'aquesta tesis doctoral hem investigat la prevalença, la susceptibilitat antimicrobiana, el potencial de virulència i l'estructura poblacional o la diversitat genètica de *Campylobacter* i *Salmonella* en espècies d'aus marines al llarg de la costa occidental de Sud-Àfrica i a les regions Antàrtica i Subantàrtica. També hem analitzat la relació genètica i el potencial de virulència d'aïllaments de diferents serovars de *Salmonella* procedents d'aus marines, aus de corral i humans, per tal d'avaluar la potencial circulació de les mateixes soques entre els diferents nínxols al sud-oest d'Europa.

A la província de *Western Cape* (Sud-Àfrica), vam detectar espècies termòfiles de *Campylobacter*, principalment *C. jejuni* i amb prevalences similars, en gavians de Lichtenstein i en xatracs crestats. La majoria de genotips (seqüències tipus o STs) de *C. jejuni* pertanyien al complex clonal (CC)-1275, que està relacionat principalment amb ambients aquàtics i aus salvatges. En canvi, vam observar una prevalença més alta de *Salmonella* en gavians que en xatracs, probablement degut als hàbits carronyaires dels gavians. Els serovars de *Salmonella* més freqüents van ser Anatum, Enteritidis i Hadar, però també vam trobar una gran diversitat d'altres serovars zoonòtics, especialment en colònies de gavines properes a zones urbanes. Mitjançant electroforesis en gel de camp polsat vam detectar genotips (pulsotips) iguals o molt similars en alguns aïllaments de *Salmonella* d'aus marines i d'altres d'origen clínic humà. La majoria dels aïllaments de

S. Enteritidis i *S*. Typhimurium pertanyien al ST-11 i ST-34, respectivament, genotips que es troben distribuïts globalment en una àmplia varietat d'hostes. A més del potencial de virulència, tant els aïllaments de *Campylobacter* com de *Salmonella* van mostrar resistència antimicrobiana a diversos agents, inclosos antimicrobians d'importància crítica (quinolones, tetraciclines i β- lactàmics) i multi-resistències en el cas de serovars de *Salmonella* aïllats de gavians.

També vam trobar espècies termòfiles de *Campylobacter* a totes les illes Antàrtiques i Subantàrtiques mostrejades, principalment *C. lari*, però també *C. jejuni*, especialment en paràsits subantàrtics, una de les principals espècies d'aus marines oportunistes a l'Oceà Sud. Cal destacar que vam aïllar genotips de *C. jejuni* pertanyents als CC-21, CC-45 i CC-206, que estan associats a animals domèstics i infeccions en humans. Tanmateix, només vam aïllar *Salmonella* (principalment *S*. Enteritidis ST-11) d'unes poques aus marines de l'illa de Livingston (Península Antàrtica), la qual cosa suggereix que aquest bacteri no és autòcton de la regió. La presència de genotips de *C. jejuni* i *S*. Enteritidis que habitualment es troben en humans i animals domèstics suggereix una zoonosi inversa (des d'humans cap a aus marines) probablement a través del turisme i les activitats científiques a la zona. Tot i així, no es pot descartar la introducció de patògens a regions remotes a través d'altres fonts, com ara els moviments migratoris de les aus marines. També vam observar una substancial connectivitat entre les poblacions d'aus marines oportunistes, especialment els paràsits subantàrtics, que poden facilitar la propagació dels bacteris entre la fauna silvestre de l'Antàrtida.

Per altra banda, vam identificar una gran diversitat de pulsotips únics de *Salmonella* (principalment de *S*. Typhimurium) en gavines del sud-oest d'Europa, en comparació amb els pulsotips predominants d'aus de corral i humans, la qual cosa probablement indica que les gavines estan exposades a una major varietat de fonts de contaminació. No obstant això, vam detectar 30 pulsotips en comú entre aïllaments de dos o tres nínxols d'hoste diferents pertanyents a 12 serovars diferents: Bredeney, Derby, Enteritidis (ST-11), Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima, Rissen,

Typhimurium (ST-19 i ST-34) i Virchow. Aquesta troballa suggereix l'existència de soques generalistes de *Salmonella* que circulen entre diferents compartiments. A més, la presència d'un ampli repertori de gens associats a la virulència, independentment de l'hoste d'origen, pot augmentar la capacitat d'aquestes soques per infectar diferents hostes i adaptar-se a nous entorns.

Els nostres resultats demostren que les aus marines poden ser portadores de soques de *Campylobacter* i *Salmonella* d'origen antropogènic, algunes d'elles amb resistència antimicrobiana i un important potencial de virulència. Les nostres troballes reforcen l'argument que les aus marines contribueixen a l'amplificació i el manteniment d'aquests patògens en el medi ambient. A més, degut als moviments migratoris i de recerca d'aliment les aus marines poden exercir un important paper en la disseminació d'aquests agents zoonòtics, però també de gens de resistència i virulència a través d'elements genètics mòbils, a àrees geogràfiques remotes i nous hostes. És necessari augmentar els sistemes de vigilància de la vida silvestre, especialment en aus marines, i establir polítiques ambientals més estrictes pel maneig dels residus humans per tal de limitar l'accés d'aquestes aus a font de contaminació antropogènica, la qual cosa pot ajudar a controlar la disseminació de soques amb potencial perill per la salut pública i animal.

Publications

The results presented in this thesis have been published or submitted for publication in international scientific peer-reviewed journals:

Moré, E., Ayats, T., Ryan, P.G., Naicker, P.R., Keddy, K.H., Gaglio, D., Witteveen, M., Cerdà-Cuéllar, M. (2017) Seabirds (Laridae) as a source of *Campylobacter* spp., *Salmonella* spp. and antimicrobial resistance in South Africa. *Environ. Microbiol.* 19(10):4164-4176.

Cerdà-Cuéllar, **M., Moré**, E., Ayats, T., Aguilera, M., Muñoz-González, S., Antilles, N., Ryan, P.G., González-Solís, J. Humans spread zoonotic enteric bacteria in Antarctica. *Submitted.*

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CHAPTER 1

General Introduction

1.1. ZOONOSES

Zoonoses are infectious diseases that can be transmitted directly or indirectly between animals and humans. Diseases transmitted from animals to humans are of concern for its impact in clinical medicine, while infections transmitted from humans to animals (i.e. reverse zoonoses) may put at risk species conservation. More than the 60% of pathogens affecting humans are shared with domestic or wild animals (Taylor *et al.*, 2001). The emergence of zoonoses is the result of the ecology and evolution of pathogens which exploit new niches and adapt to new hosts. The underlying causes that provide access to these novel niches seem to be mediated by human action in most cases, including changes in land use, extraction of natural resources, human population growth, animal production systems, antimicrobial drugs and vaccine use, international travel and trade, etc. (Karesh *et al.*, 2012).

According to the route of transmission, zoonotic diseases can be classified in vector borne zoonoses (e.g. malaria, West Nile fever, Lyme disease), direct zoonoses (e.g. influenza, Q fever, rabies) and indirect zoonoses (e.g. foodborne diseases). Nowadays, foodborne diseases, acquired through consumption of contaminated food and water, have an important health and economic impact worldwide. *Campylobacter* and *Salmonella* spp. are considered the main bacterial cause of foodborne diseases in humans (Figure 1.1) (EFSA and ECDC, 2017b). In the European Union (EU), these zoonotic pathogens were responsible for 340,837 gastroenteritis cases in 2016, and their economic cost is estimated in more than 5 billion euros per year. Therefore, *Campylobacter* and *Salmonella* spp. infections are of significant public health concern and there is a major global interest to reduce their incidence.

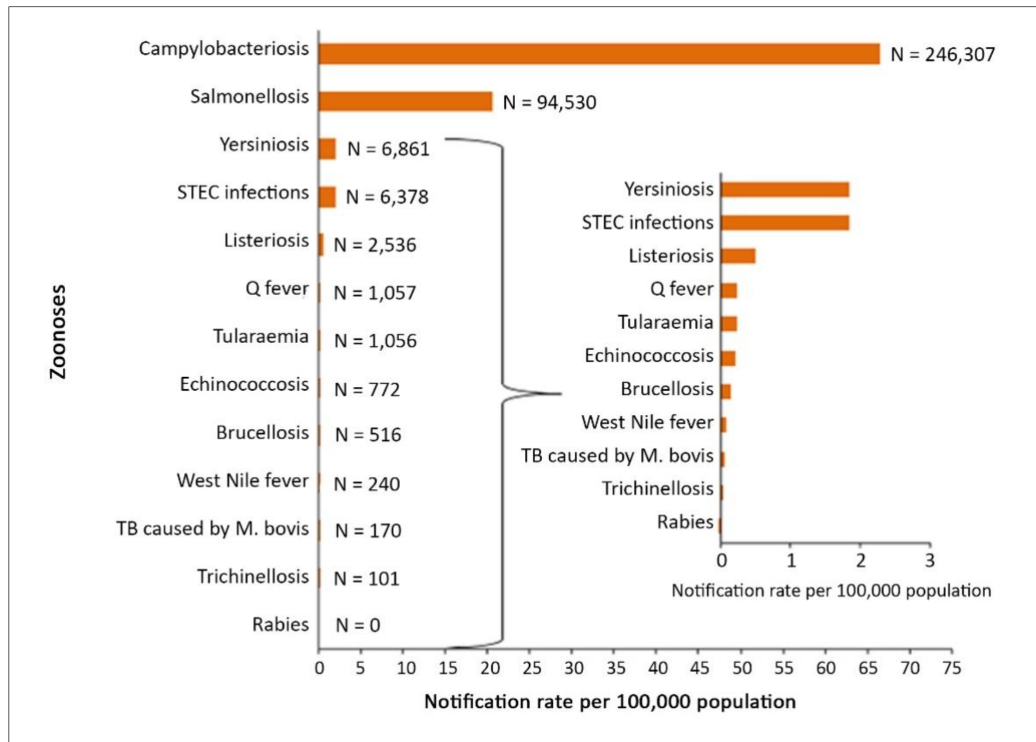


Figure 1.1. Reported numbers and notification rates of confirmed human zoonoses in the EU, 2016. Total number of confirmed cases is indicated at the end each bar. Source: EFSA and ECDC, 2017.

1.2. *CAMPYLOBACTER*

1.2.1. Discovery and taxonomy

It is believed that the first report regarding the bacterium that we now know as *Campylobacter* dates back to 1886, when Escherich observed non-culturable spiral-shaped bacteria in the colonic contents of children affected by what he called “cholera infantum” (Escherich, 1886). However, the first reliable identification is attributed to McFadyean and Stockman, who discovered a *Vibrio*-like bacterium in aborted ovine fetuses in 1913 (McFadyean and Stockman, 1913). Few years later, Smith and Taylor

found the same spiral bacteria associated to infectious abortions of bovines for which they proposed the name *Vibrio fetus* (Smith and Taylor, 1919). Afterward, closely related organisms were detected in faeces of cattle and pigs with enterocolitis and were classified as *V. jejuni* and *V. coli*, respectively (Jones *et al.*, 1931; Doyle, 1944).

Initially, this bacterium was studied mainly in the veterinary field due to the economic losses that it caused in livestock. A milk-borne outbreak of *V. jejuni* among prisoners in USA in 1938 is considered the first well documented instance of *Campylobacter* human infection (Levy, 1946). In 1957, King discriminated between *V. fetus* and the thermo-tolerant *V. jejuni* and *V. coli* associating the latter with human enteric diseases (King, 1957). The genus *Campylobacter* (meaning "curved rod" in Greek) was first proposed by Sebald and Véron in 1963, distinguishing them from *Vibrio* spp. by their DNA base composition, non-fermentative metabolism and microaerophilic growth requirements (Sebald and Véron, 1963). Later, Véron and Chatelain amended the taxonomy considering four distinct species in the genus: *C. fetus, C. jejuni, C. coli* and *C. sputorum* (Véron and Chatelain, 1973).

The difficulty of isolating and culturing *Campylobacter* from faeces, attributed to the fastidious nature of these bacteria and the overgrowth of competing coliforms, supposed a major hindrance to their research in human medicine. However, *Campylobacter* was successfully isolated from stools of humans employing a filtration method in 1972 (Dekeyser *et al.*, 1972). Few years later, the isolation procedure was refined using a selective medium supplemented with a mixture of antibiotics (Skirrow, 1977). The improvement in diagnostic methods represented an important breakthrough and allowed the retrieval of *Campylobacter* from a wide range of human, animal and environmental sources, and gradually novel taxa were proposed during the 1980s. Finally, *Campylobacter* became recognized as the main cause of bacterial gastroenteritis in humans, despite having been ignored in clinical microbiology for so many decades (Olson *et al.*, 2008).

In the wake of the description of new *Campylobacter* species, the taxonomic structure was rearranged and the novel bacterial family Campylobacteriaceae was proposed, which contains the genus *Campylobacter*, in addition to the closely related *Arcobacter*, *Dehalospirillum* and *Sulfurospirillum* genera (Vandamme and De Ley, 1991). Ever since, the taxonomy of *Campylobacter* genus has undergone many changes and, nowadays there are still some controversies that remain to be resolved (Debruyne *et al.*, 2008).

Currently, the genus consists of 28 species, nine subspecies and three biovars known according to the LPSN (http://www.bacterio.net/campylobacter.html) (Table 1.1). The species most commonly associated to human gastroenteritis are *C. jejuni* and *C. coli*, but other species related to livestock animals, such as *C. lari*, *C. upsaliensis*, *C. lanienae, C. fetus*, *C. sputorum* and *C. hyointestinalis,* can occasionally cause human infections as well. Some non-zoonotic species isolated from humans are implicated in periodontal diseases, for example, *C. curvus, C. rectus, C. showae* and *C. concisus*. However, other species, such as *C. canadensis, C. cuniculorum*, *C. iguaniorum* and *C. mucosalis*, have been isolated from animals but do not cause human illness.

The research of *Campylobacter* in hitherto little explored habitats, such as poles ecosystems or new hosts in which the presence of the bacterium was suspect, has revealed the existence of novel species. It is the case of *C. peloridis* found in molluscs, *C. volucris* in gulls and *C. subantarcticus* in albatrosses and penguins from Antarctic regions (Debruyne *et al.*, 2009, 2010a, 2010b). However, novel species have also been discovered in extensively researched hosts: *C. avium* in chickens and turkeys, and more recently, *C. hepaticus* in chickens with spotty liver disease (Rossi *et al.*, 2009; Van *et al.*, 2016).

Table 1.1. Described and validated *Campylobacter* species, their respective sources and human-associated diseases.

***Campylobacter* species**	**Source**	**Human disease**
C. avium	Poultry	NP
C. canadensis	Wild birds	NP
C. coli	Pigs, sheep, cattle, poultry, wild birds	G, S, M
C. concisus	Humans, domestic pets	G, P, A
C. corcagiensis	Primates	NP
C. cuniculorum	Rabbits	NP
C. curvus	Humans	P, G
C. fetus		
subsp. *fetus*	Cattle, sheep, reptiles	G, S
subsp. *testudium*	Reptiles	G
subsp. *veneralis*	Cattle, sheep	S
C. geochelonis	Reptiles	NP
C. gracilis	Humans	P, A
C. helveticus	Dogs, cats	G
C. hepaticus	Poultry	NP
C. hominis	Humans	G
C. hyointestinalis		
subsp. *hyointestinalis*	Cattle, pigs	G
subsp. *lawsonii*	Pigs	NP
C. iguaniorum	Reptiles	NP
C. insulaenigrae	Marine mammals	G
C. jejuni		
subsp. *doylei*	Humans	G, S
subsp. *jejuni*	Cattle, sheep, pigs, poultry, wild birds	G, S, M, GBS
C. lanienae	Cattle, pigs	G
C. lari		
subsp. *concheus*	Shellfish	G
subsp. *lari*	Dogs, cats, poultry, wild birds	G, S
C. mucosalis	Pigs	NP
C. peloridis	Shellfish	G
C. rectus	Humans	P, A
C. showae	Humans	P, A
C. sputorum		
biovar *faecalis*	Sheep, bulls	NP
biovar *paraureolyticus*	Cattle	G
biovar *sputorum*	Cattle, pigs	A, G
C. subantarticus	Wild birds	NP
C. upsaliensis	Dogs, cats	G, S
C. ureolyticus	Humans	G, S, A
C. volucris	Wild birds	NP

A: abscesses; G: gastroenteritis; GBS: Guillain-Barré syndrome; M: meningitis; NP: none present as yet; P: periodontal disease; S: septicemia.

Source: http://www.bacterio.net/campylobacter.html

1.2.2. General characteristics

Campylobacter is a Gram-negative, small bacterium (0.2-0.8 µm x 0.5-5 µm) with a slightly curved or spiral-shaped appearance, non-spore-forming, but in old cultures or under stress conditions can take on coccoid body or viable but non-culturable (VBNC) form (Rollins and Colwell, 1986). In general, cell has a single polar unsheathed flagellum at one or both ends that enable to generate a corkscrew-like motion, while some species are non-motile (*C. gracilis, C. hominis*) or have multiple flagella (*C. showae*) (Ferrero and Lee, 1988; Etoh *et al.*, 1993; Vandamme *et al.*, 1995; Lawson *et al.*, 2001).

This fastidious bacterium neither ferment nor oxidize carbohydrates, instead it obtains energy from amino acids or tricarboxylic acid cycle intermediates. Most species have catalase and oxidase but not urease activity (Debruyne *et al.*, 2008). *Campylobacter* is essentially microaerophilic, it grows at an atmosphere with reduced oxygen and elevated carbon dioxide levels (5% O_2, 10% CO_2, and 85% N_2) since it is susceptible to oxygen radicals and peroxide (Garénaux *et al.*, 2008). Moreover, several species of the human oral cavity can grow in anaerobic conditions.

The growth temperature for *Campylobacter* is 30°C to 37°C, although thermotolerant species grow better between 37°C and 42°C. Thermophilic species, including *C. jejuni, C. coli, C. lari* and *C. upsaliensis* are causal agents of campylobacteriosis and their high growth temperature may be a result of adaptation to warm-blooded animals. The thermal stress response of bacteria is mostly due to the induction of the expression of heat-shock proteins which promote the folding of cellular proteins and the proteolysis of potentially deleterious proteins. Although unable to multiply below 30°C due to the absence of cold-shock proteins, *Campylobacter* can survive to refrigeration and freezing temperatures (Sampers *et al.*, 2010). *Campylobacter* is sensitive to desiccation, heat, ultra-violet radiation and other environmental stresses, even so it can persist in some environments, such as manure, for prolonged periods (Inglis *et al.*, 2010). The survival

time depends on the bacterial strain and the environmental conditions (e.g. light, temperature, oxygen, nutrients and biotic interactions).

1.2.3. Detection, isolation and confirmation

Campylobacter is typically a fragile bacterium difficult to isolate and culture in laboratory due to the special requirements for growth. The isolation methods are based on complex selective media containing oxygen scavengers (horse or sheep blood, charcoal), growth promoting reagents (ferrous sulphate, sodium metabisulphite, sodium pyruvate) and antibiotics (cefoperazone, amphotericin B, polymyxin B, cycloheximide, rifampicin, trimethoprim lactate and vancomycin). An enrichment step in a liquid medium, prior to isolation on selective agar plates, usually provides better recovery when cells are either low in number, injured or stressed (e.g. in food samples) (Williams *et al.*, 2009). Some of the most frequently employed enrichment broth media are Bolton, Preston, Park-Sanders and Exeter. Numerous selective solid media also exist for *Campylobacter*, some of the most common ones are: mCCDA (modified charcoal cefoperazone deoxycholate), Preston, Skirrow, Butzler, Karmali and Campy-Cefex. Agar plates with different selective principles in parallel can be used to increase the yield. According to the standardized method for the detection and enumeration of *Campylobacter* (ISO 10272-1:2017), Bolton broth is recommended for enrichment and mCCDA is the selective agar of choice. Incubation is performed at 42°C in a microaerobic atmosphere.

For confirmation, presumptive colonies can be stained and examined microscopically regarding their morphology and motility, and biochemical (oxidase, catalase, nitrate reductase) or serological (latex agglutination) tests can be performed. Hippurate hydrolysis test can be used to discriminate between *C. jejuni* (positive) and *C. coli* (negative), but some false-negatives could be wrongly classified (Adzitey and Corry, 2011). Since conventional phenotypic methods may be often atypical and difficult to interpret, the use of molecular techniques is more reliable. The polymerase chain

reaction (PCR), based on 16S rRNA, 23S rRNA, *mapA, ceuE* or *lpxA* genes, among others, is more sensitive and specific and allows a rapid confirmation and identification of *Campylobacter* species, and the detection of the bacteria without culture (Linton *et al.*, 1996; Fermér and Engvall, 1999; Denis *et al.*, 2001; Klena *et al.*, 2004; Katzav *et al.*, 2008). However, direct PCR amplification of *Campylobacter* from environmental samples can be complicated due to the presence of low numbers of the bacteria or inhibitory substances, and thus, a prior enrichment or DNA purification step, respectively, may be necessary. Recently, more rapid and sensitive detection methods have been developed such as real-time quantitative PCR (qPCR) or fluorescence *in situ* hybridization (FISH) (Poppert *et al.*, 2008; Leblanc-Maridor *et al.*, 2011).

1.2.4. Clinical manifestations

Thermophilic *Campylobacter* spp. generally cause enteric infections in humans which ranges from a mild watery diarrhoea to a severe inflammatory bloody diarrhoea. Campylobacteriosis usually occurs within two to five days after exposure to the pathogen and can be accompanied by other general symptoms including headache, malaise, abdominal pain, cramping, nausea, vomiting and fever (van Vliet and Ketley, 2001). Some infected people do not have any symptoms. The illness is typically self-limiting and lasts less than one week, but the bacterial shedding often persists after clinical symptoms have ended. Campylobacteriosis may be more severe in infants, elderly or immunocompromised patients, in which the pathogen occasionally spreads to the bloodstream and causes a serious life-threatening infection (WHO, 2017a).

Complications such as bacteraemia, hepatitis, pancreatitis and miscarriage may occur, but are uncommon especially when compared to those associated with *Salmonella* (see section 1.3.4) (Moore *et al.*, 2005). *Campylobacter* infection may also result in long-term sequelae such as rheumatologic disorders (e.g. reactive arthritis) and peripheral neuropathies (e.g. Guillain-Barré syndrome (GBS), Miller Fischer syndrome) (Nachamkin, 2002). Probably, one of the most important immune-mediated disorders

reported in 0.1% of campylobacteriosis cases is the GBS, a flaccid paralysis that may cause respiratory and severe neurological dysfunctions. Molecular mimicry of *Campylobacter* lipopolysaccharides (LPS) with gangliosides in nervous tissue is considered to induce cross-reactive antibodies that lead to GBS (Godschalk *et al.*, 2004).

Most of campylobacteriosis cases do not require specific treatment other than rehydration and electrolyte replacement. Antimicrobial therapy is restricted for severe cases or patients with high risk of invasive disease. In these cases, macrolides (e.g. erythromycin, azithromycin), tetracycline and fluoroquinolones (e.g. ciprofloxacin) are commonly used as first-line treatment (Gilbert *et al.*, 2017). Antimicrobial susceptibility testing helps to choose appropriate therapy since some antimicrobial resistance might compromise the efficacy of the treatment.

1.2.5. Epidemiology

Campylobacter is the most frequent bacterial cause of foodborne diarrheal disease in humans worldwide. In 2010, more than 95.6 million human campylobacteriosis cases and 21 thousand deaths were estimated, which represents the 27.4% of global bacterial foodborne illnesses (Havelaar *et al.*, 2015). In the EU, a total of 246,307 confirmed cases of campylobacteriosis was reported in humans in 2016 (66.3 cases per 100,000 population) (EFSA and ECDC, 2017b). This represents an increase of 6.1% compared with the rate in 2015 and a statistically significant increasing trend over the period 2008–2016. *Campylobacter* has a clear seasonality with a sharp increase of cases in summer and early autumn. Thermophilic *Campylobacter* species, mainly *C. jejuni* (83.6%) and *C. coli* (8.5%), followed by *C. lari, C. fetus* and *C. upsaliensis,* are the most commonly reported. Despite the high number of cases, *Campylobacter* infections are sporadic and their severity in terms of case fatality is low (0.03% in 2016).

The most important route of *Campylobacter* transmission to humans is the consumption of contaminated food, mainly undercooked chicken meat; raw milk is a

common source of outbreaks (EFSA and ECDC, 2017b). International travels, environmental exposure and direct contact with domestic animals are also important risk factors for infection. The level of risk for travel-related campylobacteriosis appears to be associated with the travel destination (Mughini-Gras *et al.*, 2014). In developing countries, *Campylobacter* is often hyperendemic and seasonality is less marked or absent (Coker *et al.*, 2002). Besides, asymptomatic infections are common and diarrhoea is usually limited to children, suggesting that a high level of exposure in early life leads to the development of protective immunity. Due to the ubiquitous nature of the pathogen, risk factors in poor regions are more diffusely associated with exposure to the environment, including contaminated drinking water. Although it is not as common, person-to-person transmission via faecal-oral or fomites also occurs.

Campylobacter spp. normally inhabit the intestinal tract of warm-blooded animals, and thus are frequently detected in foods derived from these animals (Horrocks *et al.*, 2009; Kaakoush *et al.*, 2015). Poultry are the main reservoir of *C. jejuni*, *C. coli*, and to a lesser extent *C. lari, C. upsaliensis* and *C. concisus*. In cattle, *C. jejuni, C. coli, C. lari* and *C. lanienae* are frequently found, while pigs are more readily colonized by *C. coli.* Sheep and goats have also been reported as carriers of *Campylobacter* species but with lower prevalence. *Campylobacter* is also present in animal pets, such as dogs and cats (mainly *C. upsaliensis*), hamsters, ferrets, rabbits and reptiles. Wild animals are potential reservoirs of the pathogen, and among them, wild birds are most likely to carry *Campylobacter* species (see section 1.7). Campylobacters have also been found in shellfish.

Although *Campylobacter* is unable to grow outside of a suitable host, it can survive in different environmental sources, including soil, manure and surface waters, which in turn, are the most likely sources of infection to domestic and wild animals (Murphy *et al.*, 2006; Bronowski *et al.*, 2014). This bacterium is found in abundance on farms and their surrounding environment. Despite of biosecurity measures, the bacterium can enter the farm, and both rodents and insects have also been identified as possible

vectors (Hald *et al.*, 2008). Once established, the bacterium is difficult to eliminate since transmission within individuals occurs rapidly, especially in poultry farms (Sahin *et al.*, 2002; Urdaneta, 2016). Water is also an effective vehicle of transmission of *Campylobacter* to animals and humans. *Campylobacter* is omnipresent in rivers, ponds, lakes, streams and coastal waters, mostly in those which are exposed to direct contamination with animal faeces, agricultural run-off and sewage effluents (Whiley *et al.*, 2013).

1.2.6. Pathogenesis

Campylobacteriosis severity depends on the virulence of the strain and other host-specific factors such as age, gastric acidity level and the host immune-response to the infection. A low dose of *Campylobacter*, about 500 cells, is enough to induce infection in humans (Kothary and Babu, 2001). *Campylobacter* enter through the oral route, cross the stomach and attain the small intestine thanks to their resistance to gastric and bile acids. At first, the bacterium colonizes the small intestine and then moves to the colon that is the target organ. Motility is necessary to resist peristalsis and survive in the gastrointestinal environment, as well as to circumvent the intestinal mucus layer. Therefore, the flagella of *Campylobacter* play an essential role for intestinal colonization, along with the bacterial chemosensory system that drives flagellar movement based on the environmental signals.

To establish infection, *Campylobacter* must attach to the intestinal epithelial cells and subsequently invade them (Figure 1.2). *Campylobacter* adhesion is not mediated by appendages like fimbria or pilus as occurs in *Salmonella* and *Escherichia coli*, although the precise molecular mechanism of the attachment for *Campylobacter* is still unclear (Rubinchik *et al.*, 2012). It seems that outer membrane proteins of *C. jejuni* specifically bind to fibronectin, a glycoprotein of the extracellular matrix, located on the basolateral surface of epithelial cells. The mechanisms that controls the bacterial invasion are also confuse and controverted since different results have been observed *in vitro* depending

on the *C. jejuni* strain and the culture cell model used (Ó Cróinín and Backert, 2012). *C. jejuni* effectors induce rearrangements of eukaryotic cell cytoskeleton to facilitate the bacterium uptake. All strains require the polymerization of microtubules (tubulin subunits) for maximal invasion, while some strains also require the polymerization of microfilaments (actin subunits). Besides, it has been demonstrated that *C. jejuni* flagella are involved not only in motility, but also in the secretion of flagellar proteins and invasion effectors acting as a type III secretion system (T3SS) (Guerry, 2007).

Once internalized, a *Campylobacter*-containing vacuole is developed avoiding the delivery into lysosomes. *C. jejuni* may evade the host immune response within the endocytic vacuole although its role is not yet well established. Invasion by *C. jejuni* induce interleukin (IL)-8, one of the earliest pro-inflammatory cytokines that sign the recruitment of polymorphonuclear leukocytes (PMNL), mainly neutrophils, to the gut lumen (Young *et al.*, 2007; Janssen *et al.*, 2008). The interaction of phagocytes, including macrophages and dendritic cells, with the bacteria results in a massive pro-inflammatory response and increases the cytokine production.

While adherence of *Campylobacter* and enterotoxins production alter the fluid resorption of the intestine resulting in secretory diarrhoea, the intestinal inflammation and the mucosal damage, probably along with the effect of bacterial cytotoxins, results in the inflammatory diarrhoea frequently observed in humans (Wassenaar, 1997; Janssen *et al.*, 2008). The best characterized toxin of *Campylobacter* is the cytolethal distending toxin (CDT) which arrests eukaryotic cell cycle inducing cellular distension and apoptosis (Asakura *et al.*, 2008).

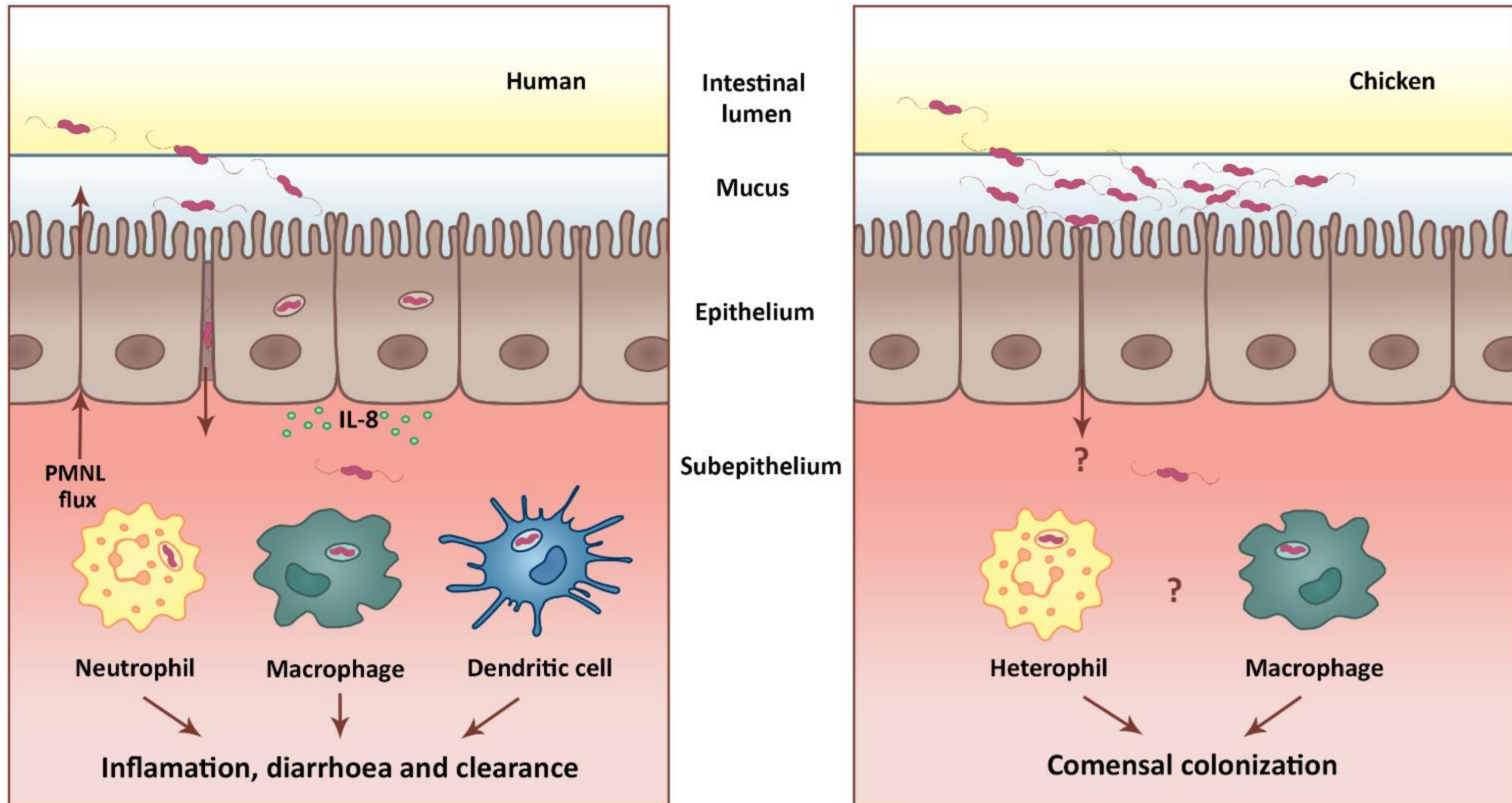


Figure 1.2. Pathogenesis of *C. jejuni* in human (left) and chicken (right). Question marks indicate processes that are not yet clear. PMNL: polymorphonuclear leukocytes.

By contrast, *Campylobacter* infection in chickens typically does not lead to the same symptoms and pathological inflammatory response that are seen in humans (Figure 1.2). In birds, the primary site of colonization are the deep crypts of the caecum, where *Campylobacter* replicates in the mucosal layer reaching high numbers, up to 10^{10} colony-forming units (CFU) per gram of infected intestine (Lee and Newell, 2006). Invasion of the intestinal epithelium is not typically reported in chickens, although some studies *in vitro* have demonstrated that *C. jejuni* is able to invade chicken enterocytes (Byrne et al., 2007; Van Deun et al., 2008). It has been suggested that chicken intestinal mucus may effectively attenuate *C. jejuni* invasiveness and contribute to the asymptomatic nature of infection in chickens (Byrne *et al.*, 2007). Heterophils and macrophages might also have a role in the establishment of *Campylobacter* colonization in chickens but unknown factors either dampen the immune response or redirect it towards tolerance (Young *et al.*, 2007). However, a recent study demonstrates that *C. jejuni* infection can lead to disease in some chicken breeds causing damage to gut mucosa and diarrhoea (Humphrey *et al.*, 2014). Thus, currently it is not clear if the bacterium is a harmless commensal in chickens or if it can cause damage under certain circumstances.

1.2.6.1. Virulence factors

Virulence factors refer to intrinsic attributes that enable a microorganism to establish itself within a host and enhance its potential to cause disease, including those involved in motility, chemiotaxis, drug resistance, host cell adherence and invasion, alteration of the host cell signalling pathways, induction of host cell death, evasion of the host immune system defences, intracellular survival and acquisition of iron and nutrients. These bacterial properties are determined by the coordinate expression of many virulence-associated genes in response to specific signals present in the surrounding. Unfortunately, the bacterial factors involved in *Campylobacter* pathogenesis are poorly understood compared to other enteric pathogens. Several virulence factors have been described but the role of many of them in pathogenesis is only hypothetic (Bolton, 2015). The most relevant virulence factors in *Campylobacter* are shown in Table 1.2.

Table 1.2. Most relevant *Campylobacter* virulence factors.

Gene	Virulence factor	Virulence role	References
flaA & flaB	Major and minor flagellin proteins	Motility	Nachamkim *et al.,* 1993
cj1321-cj1325/6	Proteins of *O*-linked flagellin glycosylation system	Motility	Champion et al., 2005
luxS	Autoinducer AI-2 biosynthesis enzyme	Chemotaxis	Quiñones et al., 2009
acfB	MCP-type signal transduction protein	Chemotaxis	Wodall et al., 2005
cadF	Outer membrane fibronectin-binding protein	Adhesion	Konkel *et al.,* 1997
capA	Autotransporter lipoprotein A	Adhesion	Ashgar et al., 2007
pglA-pglF	Proteins of N-linked glycosylation system	Adhesion and colonization	Karlyshev et al., 2004
kpsE	CPS export system inner membrane protein	Adhesion and invasion	Bachtiar et al., 2007
dnaJ	Chaperone protein	Colonization	Konkel *et al.,* 1998
racR	TCRS protein	Colonization	Brás *et al.,* 1999
hcp	Hemolysin co-regulated protein of T6SS	Invasion	Bleumink-Pluym *et al.,* 2013
pldA	Phospholipase A	Invasion	Grant *et al.,* 1997
virB11	T4SS secretion protein	Invasion	Bacon *et al.,* 2000
ciaB	Invasion antigen B	Invasion	Konkel *et al.,* 1999
ceuE	Enterochelin uptake substrate-binding protein	Iron uptake	Palyada *et al.,* 2004
cdtA, cdtB & cdtC	CDT subunits	Toxicity	Picket *et al.,* 1996
cgtB & wlaN	LPS 1,3-galactosyltransferases	Toxicity	Gilbert et al., 2000; Linton *et al.,* 2000

MCP: methyl-accepting chemotaxis protein; CPS: capsular polysaccharide; TCRS: two-component regulatory system; T6SS: type VI secretion system; T4SS: type IV secretion system; CDT: Cytolethal distending toxin; LPS: lipopolysaccharide.

1.3. *SALMONELLA*

1.3.1. Discovery and taxonomy

Throughout history, there have been a great number of dire outbreaks of typhoid fever. Many scientists associated the disease with the consumption of contaminated food and drinks, and unsuccessfully tried to found the causal agent during years. It was Eberth who observed the bacillus for the first time in 1879 in mesenteric lymph nodes and spleen from a patient that died due to typhoid fever. Few years later, Salmon and Smith isolated the bacterium from pigs affected by hog cholera and it was consequently named "*Bacillus choleraesuis*". The genus *Salmonella* was proposed later in 1900 by Lignières in honour to Salmon's research group (*Salmonella* Subcommittee of the Nomenclature Comm. Int. Soc. Microbiol., 1934).

In 1934, the first Kauffman-White classification scheme was established in the basis of the serological identification of *Salmonella* surface structures (Kauffman, 1966). Initially, each serotype or serovar was considered a separate species and was named according to the caused disease or the animal from which the bacterium was isolated. However, when the absence of host specificity was observed, the new serovars began to be named according to the location at where they were isolated. Later, Crosa *et al.* (1973) demonstrated by DNA-DNA hybridization experiments that all serovars belonged to a single *Salmonella* species. As a result, the taxonomy of *Salmonella* underwent a series of modifications and a new nomenclature was proposed. "*Salmonella choleraesuis*" was the name accepted for the *Salmonella* type species and the six subgenera were considered to subspecies (Le Minor *et al.*, 1982). The only exception was *S. bongori* which was separated from the other subspecies and recognized as a distinct species (Reeves *et al.*, 1989). As the term "*S. choleraesuis*", which referred to both a species and a serovar, caused confusion, it was suggested to be changed to *S. enterica* since no serovar shared this name (Le Minor and Popoff, 1987). The nomenclature of Le Minor and Popoff (1987) was widely accepted and used in certain

countries, even though it has not been recognized nor validated by the Judicial Commission of the International Committee of Systematic Bacteriology. During years, two different systems of nomenclature were in use despite the attempts to unify them. Finally, the nomenclature of *Salmonella* was approved in 2005 and the White-Kauffman-Le Minor classification scheme was established (Judicial Commission, 2005; Grimont and Weill, 2007).

Currently, within the Enterobacteriaceae family, only two species comprise the genus *Salmonella*: *S. bongori* and *S. enterica.* Furthermore, *S. enterica* is divided into six subspecies: *enterica* (I)*, salamae* (II)*, arizonae* (IIIa)*, diarizonae* (IIIb)*, houtenae* (IV) and *indica* (VI) (Figure 1.3). *Salmonella* subspecies are further subtyped into serovars according to the immunological characterization of somatic (O), flagellar (H) and, to a lesser extent, capsular (K; Vi) antigens. The antigenic formulae of *Salmonella* serovars are available in the While-Kauffmann-Le Minor scheme, in continuous update by the World Health Organization Collaborating Centre for Reference and Research on *Salmonella* (WHOCC-Salm) of the Institut Pasteur (Issenhuth-Jeanjean *et al.*, 2014). The full name of a serovar is given as, for example, *Salmonella enterica* subsp. *enterica* serovar Typhimurium, but can be abbreviated to *S.* Typhimurium. Serovars of other subspecies are designated by their antigenic formulae, following the subspecies name. Currently, the taxonomic group contains more than 2,700 serovars of *Salmonella*.

While most *Salmonella* subspecies are widely distributed in the environment and cold-blooded animals, serovars belonging to *S. enterica* subsp. *enterica* can colonise a broad range of animal hosts, including mammals and birds. These serovars can cause human disease: serovars Typhi and Paratyphi are responsible for enteric fever, whereas the other serovars, denominated nontyphoidal *Salmonella* (NTS), can be non-invasive or invasive causing mild to moderate gastroenteritis or systemic infections, respectively (Figure 1.3).

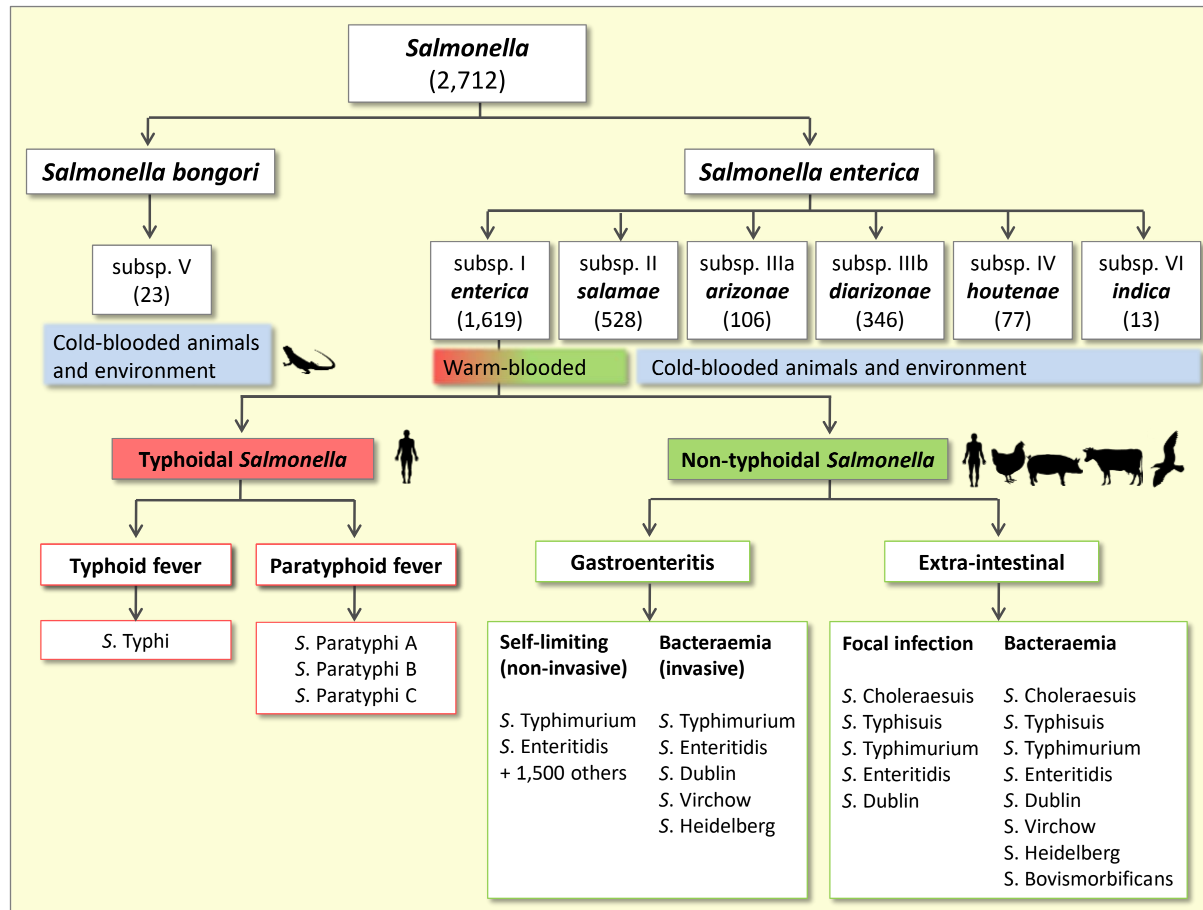


Figure 1.3. Taxonomic classification of *Salmonella* genus, sources and associated diseases. Blue, green and red colours represent environmental and cold-blooded animals, warm-blooded animals, and human origin, respectively. In parenthesis, number of serovars.

1.3.2. General characteristics

Salmonella is a Gram-negative, rod-shaped bacterium ranging 0.7-1.5 x 2.0-5.0 µm in size and non-spore-forming. The bacillus is predominantly motile by peritrichous flagella, except for the pathogenic avian-specific serovars Pullorum and Gallinarum, and other non-motile variants. *Salmonella* is facultative anaerobic, chemoorganotrophic bacterium, predominantly non-lactose fermenting and hydrogen sulphide producing. It also presents catalase but not oxidase nor urease activity (Bell and Kyriakides, 2002).

Salmonella is considered mesophilic with an optimum growth at 35-37°C, but some strains can survive at extremely low or high temperatures (2°C to 54°C). The induction of the multigenic cold shock response and the heat stress response controlled by the sigma factors allow a quick adaptation to temperature changes. These mechanisms can increase *Salmonella* survival rates when treated at low temperature prior freezing, or when exposed to heat treatment, especially in low water activity (a_w) foods (Mattick *et al.*, 2001; Dominguez and Schaffner, 2009). Moreover, *Salmonella* is resistant to desiccation, supports high salt concentrations (up to 4%) and can persist in extremely acid environments (pH 3.0-4.0) (Álvarez-Ordóñez *et al.*, 2012; Li *et al.*, 2012). *Salmonella* is incredibly adept and versatile in the strategies it employs to multiply or survive for prolonged periods under unfavourable environmental conditions outside the living hosts (e.g. faecal material, soil, water, pastures, foods) (Winfiel and Groisman, 2003; Spector and Kenyon, 2012).

1.3.3. Detection, isolation and confirmation

Salmonella is a non-fastidious bacterium that can grow in a simple glucose-salts medium or more rapidly in highly supplemented media. Standard *Salmonella* detection methods include a non-selective pre-enrichment, an enrichment in a selective medium and the subsequent plating onto two different selective media (ISO 6579-1:2017). Incubation in a pre-enrichment liquid medium, usually buffered peptone water or modified tryptone

soya broth, improves the recovery of bacteria when stressed, sub-lethally damaged or in low accounts (Valentín-Bon *et al.*, 2003). Subsequently, a selective enrichment broth, typically Müller-Kauffmann tetrathionate or Rappaport-Vassiliadis soya peptone, is used to favour the proliferation of *Salmonella* to the detriment of competing flora. The modified semi-solid Rappaport-Vassiliadis medium, which allows *Salmonella* to be distinguished from other non-motile bacteria, is the one demanded by ISO 6579-1:2017. The next step is the selection and differentiation of *Salmonella* by sub-cultivation onto different selective solid media, such as MacConkey, Xylose Lysine Desoxycholate (XLD), Xylose Lysine-Tergitol 4 (XLT4), Brilliant Green, Hektoen-Enteric or *Salmonella-Shigella*, among others. The production of hydrogen sulphide and the inability to ferment glucose are the main characteristics of *Salmonella* used for their detection in which these media are based.

Once *Salmonella* is isolated, its identity can be confirmed at subspecies level by biochemical tests (e.g. API-E20, VITEK®2, MUCAP test) and serovar can be determined by serological tests. Serotyping is performed by testing a bacterial suspension against commercial anti-sera by means of a series of slide agglutination tests. The type, order and repetition of sugar residues conforming the lipopolysaccharide (LPS) component of the outer membrane determine the O antigens. The H antigens are defined by the middle region of the flagellin protein constituting the bacterial flagellum. Monophasic serovars produce flagella always with the same antigenic specificity; instead, diphasic serovars can express in alternative phases two different flagellin types (H1 and H2). Most of the serovars of *S. enterica* subsp. *enterica* are diphasic, however, some diphasic serovars may become monophasic because of the loss or lack of expression of one of the flagellin genes (e.g. *S*. Typhimurium). Only a few serovars present the K antigens, which are polysaccharides located at the capsular surface, while the Vi antigens, a special subtype of K antigens, are only found in some pathogenic serovars (e.g. Typhi, Paratyphi C and Dublin). The combination of all these antigens, referred to as the

antigenic formula, is unique to each *Salmonella* serovar (Issenhuth-Jeanjean *et al.*, 2014).

Aside from the phenotyping methods that are laborious and time consuming, molecular techniques such as PCR or real-time PCR, mostly based on the target gene *inv*A, can also be used for a rapid detection and confirmation of *Salmonella* (Malorny *et al.*, 2003; González-Escalona *et al.*, 2012). Despite the high specificity and sensitivity of these techniques, live and dead cells cannot be distinguished and some samples need to be cultured to reach the detection limit threshold.

1.3.4. Clinical manifestations

In human infections, there are three clinical forms of salmonellosis: gastroenteritis, bacteraemia and enteric fever. Besides, there may be cases of chronic asymptomatic carriers of *Salmonella*. Gastroenteritis is the most common manifestation of food poisoning typically caused by nontyphoidal *Salmonella* (NTS) serovars. Enterocolitis symptoms usually begin six to 72 h after intoxication and include profuse water diarrhoea, nausea, vomiting and abdominal cramps. Myalgia, headache, chills and fever are also common in many patients. The symptoms can be mild to severe, but gastroenteritis is usually self-limiting and may last between two to seven days. Rehydration and electrolyte replacement is usually sufficient to overcome the bacterial infection and most patients recover without antibiotic treatment. In immunocompetent individuals, the disease typically remains localized in the terminal ileum, mesenteric lymph nodes and colon. However, infant, elderly and immunocompromised patients are more susceptible to NTS infections and have a higher risk of developing severe symptoms and complications (WHO, 2017b).

Bacteraemia occurs when invasive nontyphoidal *Salmonella* (iNTS) pass through the intestinal barrier, enter the bloodstream and disperse to other organs. Almost all serovars can be invasive, but some of them, such as Choleraesuis, Dublin and Heidelberg

are markedly more likely to cause hospitalization, systemic infections or death (Jones *et al.*, 2008). High fever is the characteristic symptom of bacteraemia which can lead to septic shock and other severe extra-intestinal complications (e.g. infections of liver, spleen, biliary or urinary tract, pneumonia, arthritis or endocarditis, meningitis) (Hohmann, 2001). Some comorbidities predispose to iNTS diseases, especially human immunodeficiency virus (HIV), malaria and malnutrition in developing countries (Feasey *et al.*, 2012).

Serovars Typhi and Paratyphi cause life threatening systemic infections, known as enteric fever or typhoid fever, regardless of the immunocompetence of the patient. Most infections concur five to nine days after contamination and are characterised by prodromal symptoms (e.g. headache, malaise, myalgia, abdominal pain and diarrhoea or constipation) followed by the onset of low fever that highly increases in the second week. Besides fever, infected patients may also display bradycardia, hepatosplenomegaly, rose spots on their chest and abdomen, meningism and neuropsychiatric manifestations, among others (Kuvandik *et al.*, 2009). Some complications like those mentioned for *Salmonella* bacteraemia can occur, including pancreatitis, hepatitis and cholecystitis. The most severe complications are encephalopathy and haemorrhage due to gastrointestinal bleeding and intestinal perforation (Huang and DuPont, 2005).

Currently, it is recommended to reserve the antimicrobial therapy for patients with severe disease or with a high risk for invasive disease. In life-threatening infections, fluoroquinolones and third-generation cephalosporins are administered for empiric therapy until the antimicrobial susceptibilities of the pathogen agent are known (Gilbert *et al.*, 2017). Nowadays, two typhoid vaccines of demonstrated safety and efficacy are available: the oral vaccine based on a live attenuated mutant strain of *S*. Typhi (Ty21a) and the injectable Vi capsular polysaccharide vaccine (ViCPS) (WHO, 2008).

1.3.5. Epidemiology

The global human health impact of NTS is high, with an estimation of 78.7 million illness cases and more than 59 thousand deaths in 2010 (Havelaar *et al.*, 2015). Despite improvements in sanitation and water supplies, the incidence of NTS infections continues to increase worldwide, being one of the major causes of bacterial foodborne diseases in both industrialized and developing countries (Figure 1.4) (WHO, 2015b). In the EU, a total of 94,530 confirmed salmonellosis cases were reported in 2016 (20.4 cases per 100,000 population), which represents an increase of 1.9% compared with the previous year (EFSA and ECDC, 2017b). However, there was an overall significant declining trend of salmonellosis between 2008 and 2015, probably because of the implementation of National Control Programmes, except in Czech Republic, France and Spain where the trend was upward. Most cases were reported during summer months following a seasonal pattern. Although the incidence of salmonellosis is lower than campylobacteriosis, the fatality rate is higher (0.25% in 2016).

Salmonella is widely distributed in the environment (e.g. water, soil, faecal material, foods) where it can survive for long time periods (Winfiel and Groisman, 2003). However, its natural environment is the intestinal tract of a broad range of domestic and wild animals which act as reservoirs and excrete the bacteria in their faeces for weeks or months (Hoelzer *et al.*, 2011; Hilbert *et al.*, 2012). The main source of *Salmonella* infection in humans is the consumption of contaminated food of animal origin, mainly poultry meat, eggs and milk, or vegetables and water contaminated with animal wastes (EFSA and ECDC, 2017b). *Salmonella* infection occurs when the bacteria are capable of multiplying on foodstuffs due to inadequate storage temperatures, insufficient cooking and cross-contamination of ready-to-eat food. To a lesser extent, transmission through direct contact with infected animals, environment or person-to-person can also occur.

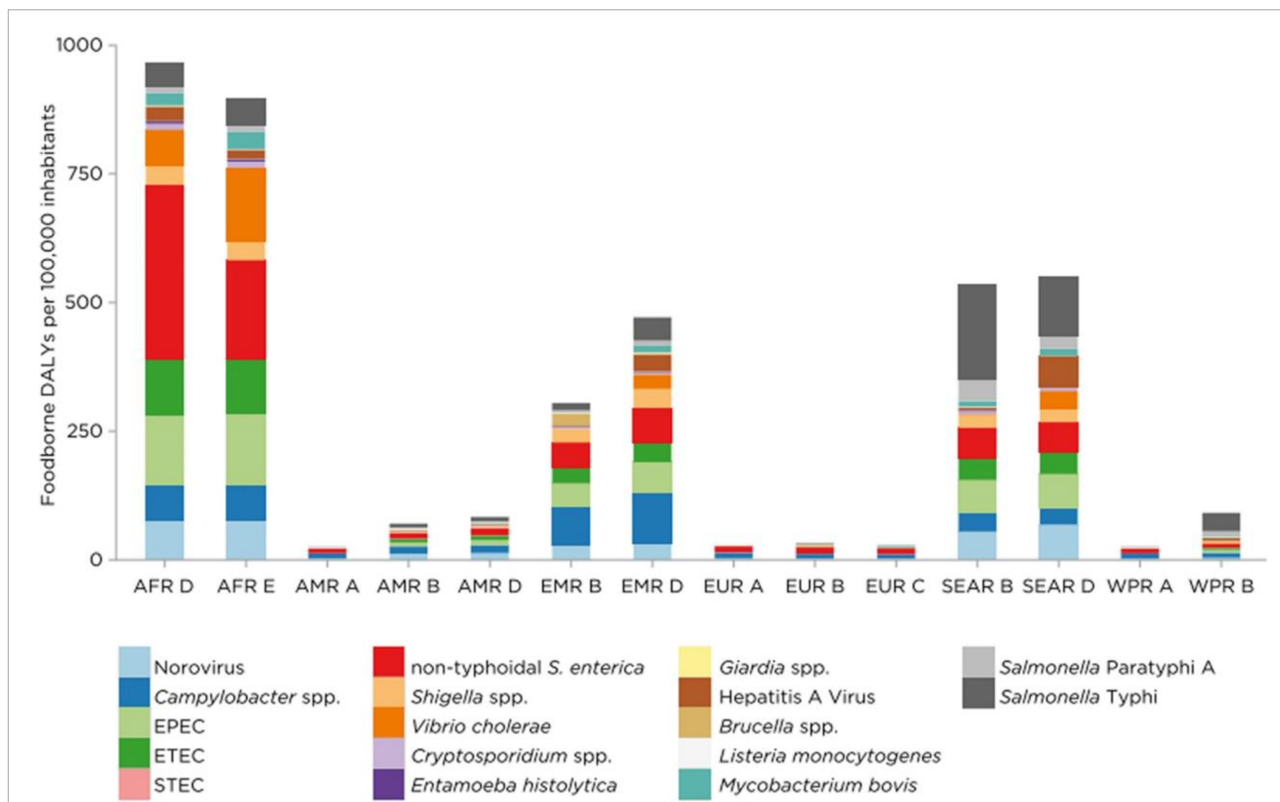


Figure 1.4. The global burden of foodborne disease by regions (DALYs per 100,000 population) caused by enteric hazards, 2010. DALYs: Disability Adjusted Life Years. AFR: African Region; AMR: American Region; EMR: Eastern Mediterranean Region; EUR: European Region; SEAR: South-East Asian Region; WPR: Western Pacific Region. Subregions are defined on the basis of child and adult mortality, as described by Ezzati et al. (2002). A: very low child and adult mortality; B: low child mortality and very low adult mortality; C: low child mortality and high adult mortality; D: high child and adult mortality; E: high child mortality and very high adult mortality. Source: WHO, 2015.

In the last years, *S*. Enteritidis followed by *S*. Typhimurium (including its monophasic variant 1,4,[5],12:i:-) have been the most common serovars detected in humans in the EU (EFSA and ECDC, 2017b). Although these serovars represent the 70% of human confirmed cases, other serovars are also of public health concern (Figure 1.5). *S*. Enteritidis is generally associated with poultry and products thereof; whereas *S*. Typhimurium is related with a wider host range, including cattle, pigs and poultry. Since the implementation of *Salmonella* control programmes in the EU in 2003 (Regulation (EC) No 2160/2003), the prevalence of *Salmonella* target serovars (Enteritidis, Typhimurium, Infantis, Virchow and Hadar) in poultry production have successfully reduced nowadays (EFSA and ECDC, 2017b). *S*. Infantis is the most frequently reported serovar in *Gallus gallus* followed by Enteritidis, but non-target serovars are also prevalent both in chickens and turkeys. Surveillance of *Salmonella* infection in food producing animals is vitally important since these are the first links in the food-chain. *Salmonella* epidemiology is complex due to the existence of multiple sources of infection and reservoirs of the pathogen, which results in significant challenges for public health authorities to control salmonellosis in humans.

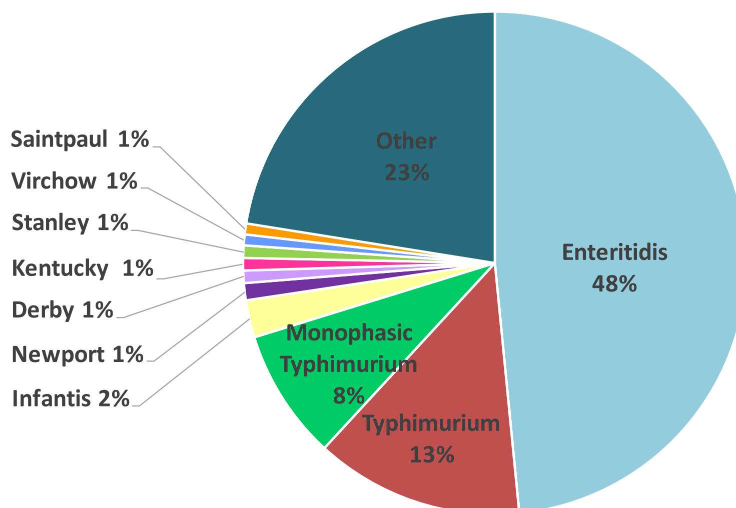


Figure 1.5. Distribution of the 10 most frequently reported *Salmonella* serovars in humans in the EU in 2016. Source: EFSA and ECDC, 2017.

In contrast to the worldwide distribution of NTS infections, enteric fever occurs predominantly in underdeveloped countries and it is associated with a high morbidity and mortality rates. The WHO estimated more than 7.5 and 1.7 million cases of typhoid and paratyphoid fever, respectively, which resulted in more than 52 and 12 thousand deaths in 2010 (Havelaar *et al.*, 2015). Typhoidal *Salmonella* serovars are endemic throughout Africa and Asia and persists in the Middle East, Central and South America and in some South-East European countries (Figure 1.4). Typhoidal *Salmonella* is carried only by humans and is transmitted through the faecal–oral route usually by contaminated water and food. The risk of infection is higher among impoverished and overcrowded populations with unsanitary conditions and exposed to unsafe water supplies due to ineffective sewage disposal. In Europe and USA, the incidence of enteric fever is low and most cases are imported by foreigners or travellers returning from endemic areas (Jensenius *et al.*, 2013)

1.3.6. Pathogenesis

The pathogenicity of *Salmonella* depends on the serovar, the virulence of the strain, the inoculum dose, the host-status and its immune response. The infective dose of *Salmonella* is 10^3 to 10^9 cells, usually in the order of 10^6 cells (Blaser and Newman, 1982). The inherent tolerance of *Salmonella* to the extreme stomach pH and the bile acids allows it to reach the small intestine. The bacterium must also resist digestive enzymes, secretory IgA, antimicrobial peptides and other innate immune defences and compete with indigenous microbiota (Álvarez-Ordóñez *et al.*, 2011). Flagella motility regulated by a complex chemotaxis system enables the bacterium to penetrate the intestinal mucus layer and colonize the ileum and to a lesser extent the colon.

In the lumen, *Salmonella* selectively attaches to the apical domain of enterocytes by fimbrial adhesins and invade the cell by bacterial-mediated endocytosis, crossing the intestinal barrier to reach the gut-associated lymphoid tissue (GALT) (Figure 1.6). *Salmonella* preferentially enters through microfold (M) cells of the follicle-associated

epithelium, which transport it to the lymphoid cells (T and B) in the underlying Peyer's patches (Tahoun *et al.*, 2012). Adhesion is a highly specific process and involves a complete repertoire of fimbrial biosynthesis genes. The different *Salmonella* serovars may possess a set of specific adhesins which facilitate the bacterial attachment to a multitude of eukaryotic cell types encountered in various hosts (Wagner and Hensel, 2011).

Salmonella invasion of intestinal cells is mainly conferred by the T3SS injectosome which allows the bacteria to mediate its own uptake. T3SS apparatus is composed of multi-channel proteins assembled in a needle-like structure projected from the bacterial surface that puncture the membrane of the host cell and inject effector proteins into the cellular cytoplasm. These translocated proteins modulate actin polymerization and trigger a local rearrangement of the host cell cytoskeleton leading to formation of membrane ruffles that engulf the bacterium. Other effector proteins secreted by a second T3SS modulate the *Salmonella*-containing vacuole (SCV) blocking the fusion of the lysosomes which supports bacterial survival and multiplication within the host cell (Haraga *et al.*, 2008; Moest and Méresse, 2013).

Invasion by NTS serovars induces the expression of several cytokines, such as Toll-like receptor (TLR)-dependent IL-8 and IL-12/interferon-gamma (IFN- γ), evoking an acute inflammatory response. As a result, a transmigration of PMNL into the gut lumen takes place, thereby preventing systemic spread of the bacteria (Patel and McCormick, 2014). The secretion of fluids and the influx of electrolytes to the lumen evoke to the diarrhoea onset. Some *Salmonella* strains can also produce enterotoxins that may stimulate intestinal secretion increasing the intestinal damage. NTS serovars have evolved to use inflammation-derived metabolites (e.g. nitrate, tetrathionate) enhancing their growth in the inflamed intestine (Winter *et al.*, 2010). In addition, *Salmonella* produces several toxins in different amounts depending on the serovar, including the CDT (Miller and Wiedmann, 2016).

While the NTS infection generally elicits a local inflammatory reaction, typhoidal serovars overcome the innate immune response in the intestinal mucosa and cause a systemic infection in healthy individuals (Raffatellu *et al.*, 2008; Gal-Mor *et al.*, 2014) (Figure 1.6). When *S.* Typhi crosses the epithelial barrier, it induces a recruitment of intestinal macrophages. Bacteria can survive and multiply into the microbicidal environment of these phagocytic cells and subsequently migrate to mesenteric lymph nodes and disseminate to liver, spleen and bone marrow. The mononuclear phagocyte system (MPS) provides the periodic recirculation of the pathogen to new foci of infection. Some patients can become asymptomatic carriers of *Salmonella* and excrete large amounts of bacteria in their faeces, having the potential to re-infect (Ruby *et al.*, 2012).

The carrier state has also been described in livestock animals and is associated with foodborne outbreaks. Birds can be infected by host-specific and non-host-specific *Salmonella* serovars showing differences in their pathogenesis. *S*. Gallinarum and *S*. Pullorum are adapted and restricted to birds, especially poultry, in which they cause fowl typhoid and pullorum disease, systemic infections with high mortality rates (Shivaprasad and Barrow, 2008). Conversely, non-host-specific *Salmonella* serovars readily colonize the cecal tonsils, the upper part of the small intestine, the gizzard and proventriculus of birds, but only produce severe disease in some special cases (e.g. during the laying period, after viral diseases or in two weeks-old chicks). Therefore, most birds become symptomless carriers of *Salmonella* for some weeks or months and shed it in the faeces sometimes intermittently (Revolledo and Ferreira, 2012).

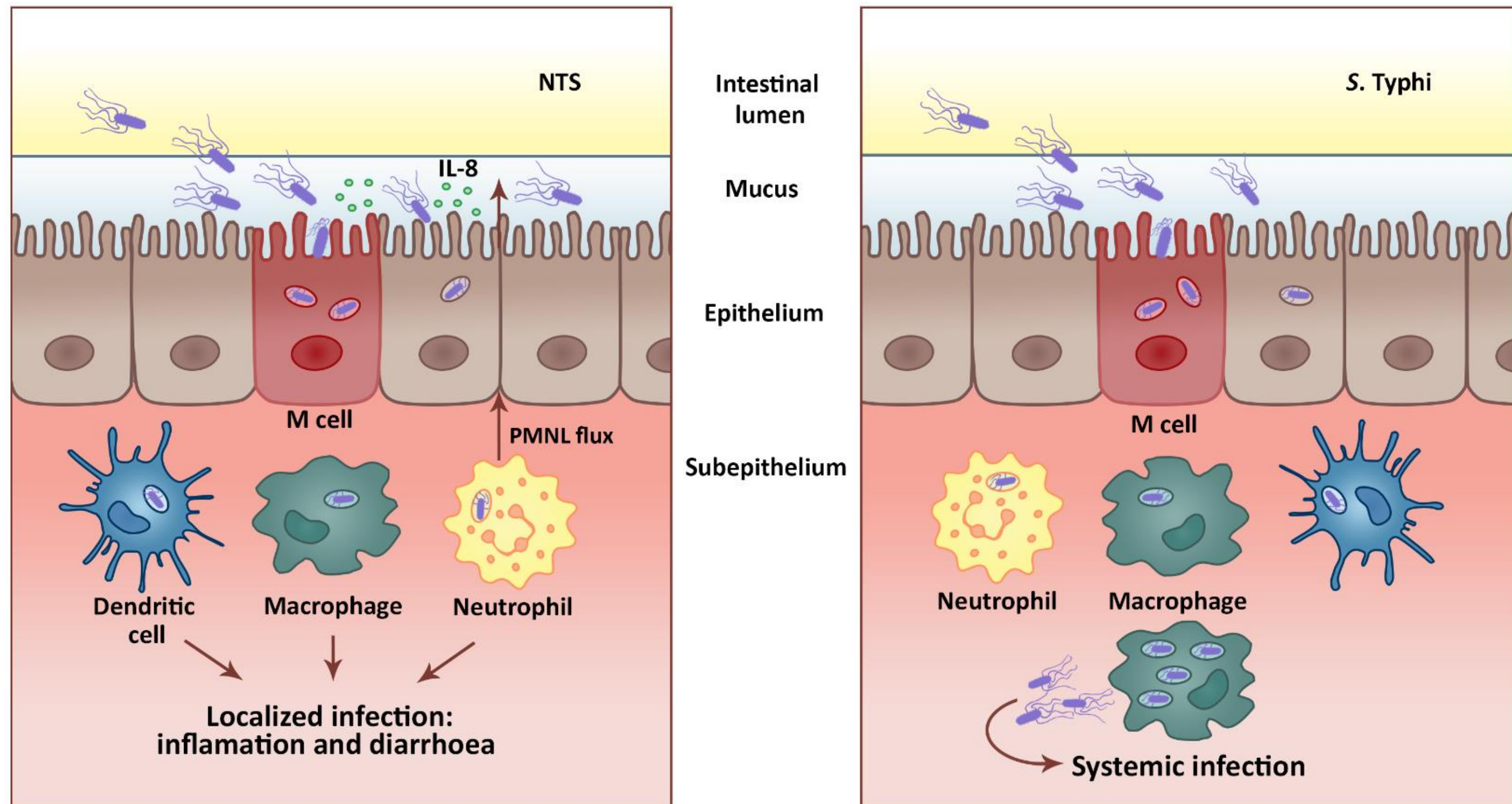


Figure 1.6. Pathogenesis of nontyphoidal *Salmonella* (NTS) serovars (left) and *S*. Typhi (right) in humans. PMNL: polymorphonuclear leukocytes

1.3.6.1. Virulence factors

Virulence factors of *Salmonella* are under a complex genetic control and involve multiple genes encoded both on the bacterial chromosome and on large plasmids. Most virulence-associated genes are clustered together in distinct large regions (10-200 kb) called *Salmonella* pathogenicity islands (SPIs) distributed along the chromosome. SPIs have been acquired by horizontal gene transfer from other species, have a G+C content different from that of the host core genome and often contain mobile genetic elements. The number and content of the SPIs differ among *Salmonella* serovars, partly explaining their heterogeneity in host specificities and disease outcome. Currently, 21 different SPIs have been identified, some of them are conserved throughout the genus *Salmonella* (e.g. SPI-1 and SPI-2), while others are specific for certain serovars (e.g. SPI-7 of *S*. Typhi) (Sabbagh *et al.*, 2010). The SPI-1 contains at least 35 genes encoding T3SS and several effector proteins involved in bacterial invasion of host cells. A larger region of SPI-2 encodes a second T3SS which secrete effector proteins implicated in *Salmonella* intracellular survival and replication within the SCV. Other SPIs (e.g. SPI-3, SPI-4 and SPI-5) also appear to facilitate the invasion and survival of the bacterium within host cells. However, many SPIs have been recently identified and much less is known about their distribution across serovars and the role they play in disease. In addition to SPIs, virulence determinants can be encoded in pathogenicity islets (smaller clusters), fimbrial operons, prophages or randomly dispersed in the genome, as well as in virulence plasmids (Table 1.3).

Table 1.3. Most relevant *Salmonella* virulence factors.

Gene	Location	Virulence factor	Virulence role	Reference
agfA	Fimbrial operon	Major subunit of SEF17 fimbriae	Adhesion and invasion	Collinson *et al.,* 1996
agfC	Fimbrial operon	Accessory protein for SEF17 assembly	Adhesion and invasion	Gibson *et al.,* 2007
lpfA	Fimbrial operon	Major subunit of long polar fimbriae	Adhesion and invasion	Bäumler and Heffron, 1995
lpfC	Fimbrial operon	Accessory protein for long polar fimbrial assembly	Adhesion and invasion	Bäumler and Heffron, 1995
sefC	Fimbrial operon	Accessory protein for SEF14 fimbriae assembly	Adhesion and intracel. survival	Clouthier *et al.,* 1993
sitC	SPI-1	Iron/manganese ABC transporter permease	Iron uptake	Janakiraman&Slauch, 2000
invA	SPI-1	Inner-membrane protein of T3SS	Invasion	Galán et al., 1992
orgA	SPI-1	Oxygen-regulated protein of T3SS	Invasion	Klein *et al.,* 2000
prgH	SPI-1	Inner-membrane ring protein of T3SS	Invasion	Klein *et al.,* 2000
avrA	SPI-1	Acetyltransferase, effector protein of T3SS	Intracel. survival	Wu *et al.,* 2013
spiC	SPI-2	Accessory protein for translocation of T3SS effectors	Intracel. survival	Yu *et al.,* 2004
mgtC	SPI-3	P-type ATPase, effector protein of T3SS	Intracel. survival	Alix *et al.,* 2007
misL	SPI-3	Autotransporter outer-membrane protein	Colonization and intracel. survival	Dorsey *et al.,* 2005
orfL	SPI-4	Autotransporter protein of T1SS	Colonization and intracel. survival	Niedergang *et al.,* 2000
sopB	SPI-5	Inositol phosphatase, effector protein of T3SS	Invasion and intracel. survival	Zhou *et al.,* 2001
pipD	SPI-5	Dipeptidase, effector protein of T3SS	Invasion	Wood *et al.,* 1998
iroN	Chromosome	Siderophore receptor protein	Iron uptake	Bäumler *et al.,* 1998
tolC	Chromosome	Major outer-membrane efflux protein	Colonization and invasion	Horiyama *et al.,* 2010
sifA	Chromosome	Effector protein of T3SS	Intracel. survival	Beuzón *et al.,* 2000
cdtB	Chromosome	Cytolethal distending toxin B	Toxicity and intracel. survival	Rodriguez-Rivera *et al.,* 2015

Continued

Table 1.3. Continued.

Gene	Location	Virulence factor	Virulence role	Reference
pefA	Plasmid	Major subunit of plasmid-encoded fimbriae	Adhesion and colonization	Friedrich *et al.,* 1993
spvB	Plasmid	Actin ADP-ribosyltransferase, effector protein of T3SS	Intracel. survival	Lesnick *et al.,* 2001
spvC	Plasmid	Phosphothreonine lyase, effector protein of T3SS	Intracel. survival	Haneda *et al.,* 2012
sopE	SopEΦ prophage	Effector protein of T3SS	Invasion	Mirold *et al.,* 1999
gipA	GIFSY-1 prophage	Putative transposase	Intracel. survival	Stanley *et al.,* 2000
gogB	GIFSY-1 prophage	Effector protein of T3SS	Intracel. survival	Pilar *et al.,* 2012
gtgB	GIFSY-2 prophage	Effector protein of T3SS	Intracel. survival	Figueroa-Bossi *et al.,* 2001

T3SS: type III secretion system; ADP: adenosine diphosphate; ATP: adenosine triphosphate; ABC: ATP-binding cassete

1.4. ANTIMICROBIAL RESISTANCE

Emergence of resistance to antimicrobials is a natural process that has been observed since the first antibiotics were discovered. However, antimicrobial resistance (AMR) has become a growing concern in recent times since the overuse of antimicrobials in both humans and food-producing animals has accelerated the selection and spread of resistant bacteria. Consequently, antimicrobial drugs have become less effective or even ineffective, limiting available treatment options. AMR affects every country regardless of its level of income and development and it is estimated to account for more than 700,000 deaths per year worldwide (O'Neill, 2016).

The quantity of antimicrobials used in poultry and livestock is dramatic and often includes drugs important for the treatment of common human infections (Van Boeckel *et al.*, 2015). Worryingly, much of their use in food-producing animals is not to treat and control disease, but instead either to prevent infections (sometimes to compensate for poor farming practices) or for production purposes. In these cases, antimicrobials are usually administered at lower and imprecise doses, via medicated feed or drinking water, for long periods, potentially enhancing selection for AMR in bacterial populations. The use of antimicrobials as prophylactic and growth promoters is not allowed in EU since 2006, but are still used for this purpose in many countries including USA, where they can be obtained without veterinary prescription (Maron *et al.*, 2013). Antimicrobial resistant bacteria from animal origin has significant public health implications since they can be transmitted to humans through food products, the environment or, less often, direct contact.

Bacterial resistance to antimicrobials can appear as a result of mutations or by acquisition of resistance conferring genes via horizontal gene transfer (e.g. plasmids, conjugative transposons) (Von Wintersdorff *et al.*, 2016). The latter occurs frequently in the host intestinal tract due to high amounts of different bacteria and the close contact among them, but also in the environment, especially the aquatic one (e.g. sewage). The

transfer of antimicrobial resistance genes among bacteria increase the dispersion of AMR and contributes to the emergence of multi-resistant bacteria.

To combat this increasing concern, it is necessary to coordinate efforts of the different institutions and apply international measures and national strategies. In 2015, the WHO adopted a global action plan to: raise awareness of AMR, strengthen the surveillance and research, reduce the incidence of infections through effective sanitation, hygiene and infection prevention measures; optimize the use of antimicrobial agents in human and animal health, and increase development of new diagnostic tools, vaccines and other alternative products (e.g. phage therapy, lysins, probiotics) (WHO, 2015a).

1.4.1. *Campylobacter* antimicrobial resistance

Intrinsic resistance in *C. jejun*i and *C. coli* has been described against penicillins and most of the cephalosporins, as well as trimethoprim, sulfamethoxazole, rifampicin and vancomycin (Fitzgerald *et al.*, 2008). On the other hand, *C. lari* belongs to the nalidixic acid resistant thermophilic *Campylobacter* (NARTC) group (Benjamin *et al.*, 1983).

In the early 1990s, when enrofloxacin was taken into use in animal production, especially in poultry and pigs, *Campylobacter* fluoroquinolone resistance started to increase worldwide among animals and human isolates at the same time (Engberg *et al.*, 2001). There are still growing trends in resistance to fluoroquinolones in EU, where *C. coli* and *C. jejuni* strains present high resistance levels to ciprofloxacin (EFSA and ECDC, 2017a). Given the high level of acquired resistance to fluoroquinolones in EU this antimicrobial can no longer be considered appropriate for routine empirical treatment of human *Campylobacter* infections.

By contrast, the macrolide resistance of *Campylobacter* has remained at a low and stable level for a long time. However, there is also evidence that resistance rates to erythromycin and other macrolides (e.g. azithromycin, clindamycin) are slowly

increasing in several countries, mostly among *C. coli* isolates from pigs (Gibreel and Taylor, 2006; EFSA and ECDC, 2017a). It has been observed that the use of tylosin as feed additive in veterinary medicine selects for a high level resistance to erythromycin in the *Campylobacter* population (Ladely *et al.*, 2007). Nevertheless, the acquisition of this resistance is a stepwise process and requires prolonged exposure, in contrast to the rapidly appearance of fluoroquinolone resistance, and may decrease in the absence of antibiotic selection pressure (Luangtongkum *et al.*, 2012). Since fluoroquinolone resistance is common, macrolides have become critical antimicrobials for the treatment of human campylobacteriosis. Even so, the recent identification of a transferable macrolide resistance mechanism in *Campylobacter* may pose a rapid means of spread of this resistance (Qin *et al.*, 2014).

High levels of resistance to tetracyclines, which is often plasmid mediated, are frequently observed in *Campylobacter* isolates from humans and animals in many EU countries (EFSA and ECDC, 2017a). Although tetracyclines are considered as an alternative to treat *Campylobacter* infections, in practice are not often used and are contraindicated in young children. As mentioned above, *Campylobacter* is resistant to many β-lactam antimicrobial agents, mainly penicillins and cephaloporins, but carbapenems are an exception and may be effective in the treatment of campylobacteriosis.

Multidrug resistance (MDR), defined as resistance to three or more families of antimicrobial agents, has so far been infrequent in *Campylobacter*. However, it has increased in recent years, probably due to the overuse of different antimicrobial agents in animal production along with horizontal transfer of AMR, posing a serious risk of treatment failures (Lin *et al.*, 2002; Qin *et al.*, 2014).

1.4.2. *Salmonella* antimicrobial resistance

Several *Salmonella* strains with MDR emerged during the late 1990s, and since then they have expanded worldwide. For instance, *S*. Typhimurium phage type DT104 is typically resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracycline (ACSSuT), and is able to acquire additional resistance to other clinically important antimicrobials (Helms *et al.*, 2005). The appearance of this strain raised an important public health concern because of its involvement in animals and human diseases. Besides, the genomic elements that confer the penta-resistance profile or other AMR (e.g. β-lactams) may spread horizontally among *Salmonella* serovars and other enteric pathogens (Douard *et al.*, 2010). Currently, the level of MDR in *Salmonella* clinical isolates is high in EU, and some of them are resistant to up to eight different antimicrobial classes (EFSA and ECDC, 2017a). MDR is especially notorious among monophasic *S.* Typhimurium isolates from humans, with a prevalence of 81.1% in 2015.

In EU, the highest proportions of resistance in human *Salmonella* isolates in 2015 were reported for sulphonamides/sulfamethoxazole (32.4%), tetracyclines (28.1%) and ampicillin (27.8%) (EFSA and ECDC, 2017a). Resistance to these antimicrobials was also observed among isolates from production animals and their derived products, probably as a consequence of their broad use in veterinary medicine. Resistance to fluoroquinolones (e.g. ciprofloxacin) and third-generation cephalosporins (e.g. cefotaxime, ceftazidime), which represent the clinically most important antimicrobial classes for treatment of human salmonellosis, was detected in 13.3% and 0.9% of human isolates, respectively. AMR levels for *Salmonella* are greatly influenced by the serovar, with some of them exhibiting high resistance to certain antimicrobials. For instance, resistance to colistin was present in 11.4% of the isolates (belonging to different serovars), mainly in *S*. Enteritidis which has been reported to have inherent resistance to this antimicrobial agent (Agersø *et al.*, 2012). However, *S*. Enteritidis is often more susceptible to antimicrobials than other serovars. Emergence of AMR and

MDR in *S*. Typhi has also been described in Africa and Asia, causing increased treatment failure cases (Zaki and Karande, 2011).

1.5. TYPING METHODS

Bacterial typing methods allow characterizing the intraspecies variability and are essential tools for conducting epidemiological studies: for example, for comparing different isolates to establish common origins and sources of infection for pathogenic bacteria traceability or studying bacteria population dynamics and diversity of strains in different geographical areas and host species. A proper typing technique must accomplish the following characteristics: typeability, discriminatory power, reproducibility and repeatability, epidemiological concordance and, if possible, speed and automation (van Belkum *et al.*, 2007). A wide variety of typing techniques are currently in use, thus, the more suitable one or a combination of two or more must be chosen according to the aims of the investigation and study design. Typing methods fall into two broad categories: phenotypic and genotypic methods.

Phenotypic methods are based on the gene expression of bacteria and include techniques such as serotyping, biotyping, phage typing, antimicrobial resistance typing, multilocus enzyme electrophoresis (MLEE), etc. The serovar determination is essential to unravel the epidemiology of *Salmonella* spp. (see section 1.3.3.), but it is ineffective for *Campylobacter* spp. (Allos *et al.*, 2004). The main drawback of phenotypic methods is that genes expression can vary according to growth conditions and may not reflect the genetic changes occurred in bacterial strains, therefore, they not provide a reliable and stable epidemiological marker (van Belkum *et al.*, 2007).

Genotypic methods are based on the analysis of bacterial genetic structure and provide more sensitivity and discrimination power, as well as higher levels of standardization

and reproducibility. Genotyping techniques can be classified in three categories according to their bases: (I) restriction sites in the bacterial DNA, (II) PCR amplification of genomic targets and, (III) polymorphisms in DNA sequences (Foley *et al.*, 2009). In this section, only the techniques used in this thesis for genotyping *Campylobacter* and *Salmonella* isolates are described in detail: enterobacterial repetitive intergenic consensus (ERIC)-PCR, PCR-restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). All of them are extensively used in current research of molecular epidemiology of foodborne bacteria.

1.5.1. Enterobacterial repetitive intergenic consensus PCR

The ERIC sequences are short imperfect palindromes (126 bp) with internal deletions or insertions, repeatedly interspersed throughout the genome of different bacterial species. These sequences were originally described in *E. coli* and *Salmonella,* and then were found to be conserved in other members of the Enterobacteriaceae, and even in unrelated bacteria from different phyla (Hulton *et al.*, 1991). As the position and number of copies of ERIC elements in bacterial genomes is variable, their PCR-amplification generates specific patterns that allow strains within Gram-negative enteric species to be distinguished (Versalovic *et al.*, 1991).

Given the simplicity, speed and low cost of this technique, ERIC sequences have been successfully used as a molecular marker in several *Salmonella* epidemiological studies for a long time (Chmielewski *et al.*, 2002; Ye *et al.*, 2011; Turki *et al.*, 2012; Wang *et al.*, 2014). However, other ERIC-PCR based-studies showed less discriminatory power compared with other molecular techniques such as PFGE (Fendri *et al.*, 2013; Almeida *et al.*, 2015). Moreover, it is not always possible to obtain repeatable profiles due to the appearance and disappearance of minor bands. This poor repeatability and reproducibility makes the comparison of ERIC-PCR results among different laboratories very difficult (Swanenburg *et al.*, 1998).

1.5.2. PCR-Restriction fragment length polymorphism

The *fla*A-RFLP technique consists in the amplification of a *fla*A gene region (1725 bp) and their further restriction with the *Dde*I enzyme to generate a fingerprint profile (Nachamkin *et al.*, 1993; Harrington *et al.*, 2003). The *fla*A gene, which encodes for the flagellin protein of bacterial flagella, has a genetic variability region flanked by conserved regions and provides a useful target for discriminating among *Campylobacter* isolates.

In addition to the high-resolution power, the relatively simplicity and the low cost of the *fla*A-RFLP technique has led to its widespread use for epidemiological studies of *Campylobacter*. The *fla*A-RFLP has been employed to determine the genetic diversity of *Campylobacter* in farms, to compare isolates from human and animal origin and to track outbreaks of campilobacteriosis, among others (Ring *et al.*, 2005; Corcoran *et al.*, 2006; Oporto *et al.*, 2007; Heuvelink *et al.*, 2009). However, the *fla*A gene is genetically instable and not species-specific. The recombination between the *fla*A and *fla*B genes within a strain can occur, as well as recombination intra and interspecies in co-infections, finding some alleles in both *C. jejuni* and *C. coli* (Harrington *et al.*, 1997; Dingle *et al.*, 2005). These limitations make this molecular typing unsuited for dynamic population or longer-term investigations. Nevertheless, *fla*A-RFLP may be appropriate as a first technique for distinguishing closely related strains of *Campylobacter*, especially when used in combination with other additional methods based on more conserved target genes, such as MLST (Behringer *et al.*, 2011).

1.5.3. Pulsed-field gel electrophoresis

PFGE is based on the enzymatic digestion of entire chromosomal DNA and the subsequent separation of restriction fragments of large size differences (30 - 1100 kb) applying an electrical field of alternating polarity. PFGE uses endonucleases with infrequent recognition sites to generate macrorestriction profiles: *Sma*I and *Kpn*I are

typically used for *Campylobacter; Xba*I and *Bln*I for *Salmonella* (Ribot *et al.*, 2001, 2006). Since decades, PFGE is the "gold standard" typing method used in national and international surveillance programs for tracking *Campylobacter, Salmonella* and other foodborne pathogens (PulseNet, http://www.pulsenetinternational.org). PFGE is a highly discriminatory genotyping tool, widely applied in epidemiological investigations to study the genetic diversity and to establish possible sources of infection (Fakhr *et al.*, 2005; Oloya *et al.*, 2009; Soyer *et al.*, 2010; Melero *et al.*, 2012).

However, this technique is time-consuming and demands intensive-labour and expensive equipment, which can suppose important limitations for some laboratories. Moreover, despite the use of standardized protocols, reproducibility can be difficult to achieve and variations in the interpretation of results can occur (Barrett *et al.*, 2006). The influence of genetic events on PFGE band patterns depends largely on whether they affect or not the restriction sites (Goering, 2010). Isolates sharing a recent common ancestor, after passing through the intestine of a host or even the same subcultured bacteria, may present differences in their PFGE profiles, especially in the case of bacteria with genomic instability such as *C. jejuni* (Hänninen *et al.*, 1999; Barton *et al.*, 2007). Therefore, although PFGE is a powerful technique for short-term epidemiological analysis or for a large number of isolates, results may poorly correlate with the true genetic relatedness among distant isolates in long-term or global epidemiological studies. Considering these limitations, alternative sequencing-based molecular methods for bacteria typing have been developed, such as MLST, which in combination with PFGE may facilitate appropriate interpretation of results (Barco *et al.*, 2013; Taboada *et al.*, 2013).

1.5.4. Multilocus sequence typing

MLST consists on the comparison of DNA sequences of gene fragments (450-500 bp) from several different housekeeping loci. Depending on the bacterial species, different target genes are analysed: *asp*A, *gln*A, *glt*A, *gly*A, *pgm*, *tkt* and *unc*A (=*atp*A) for *C. jejuni*

and *C. coli; adk* and *pgi* genes instead of *asp*A and *glt*A genes for *C. lari*; *aro*C, *dna*N, *hem*D, *his*D, *pur*E, *thr*A and *suc*A for *Salmonella* (Dingle *et al.*, 2001; Miller *et al.*, 2005; Achtman *et al.*, 2012). An allele number is assigned for each locus according to the allelic sequences available in the corresponding bacterial database (PubMLST, http://pubmlst.org; Enterobase, http://enterobase.warwick.ac.uk). Isolates that possess identical alleles for the seven loci correspond to a common sequence type (ST). In turn, STs sharing four or more identical alleles are grouped into clonal complexes (CC)s on the basis of eBurst (Feil *et al.*, 2004).

The main advantage of MLST compared to the abovementioned methods is that DNA sequence data are unambiguous and is possible to objectively compare the allelic profiles among different laboratories through an online global database. Since MLST measures nucleotide polymorphisms in relatively-stable housekeeping genes, which accumulate slowly and without selective pressure, it is a useful tool for long-term investigations. MLST is extensively used to identify major infection sources and study population structures and phylogeny of many bacteria including *Campylobacter* and *Salmonella* (Hughes *et al.*, 2010; Müllner *et al.*, 2010; Nielsen *et al.*, 2010; Antunes *et al.*, 2011; Keller and Shriver, 2014; Toboldt *et al.*, 2014; Papadopoulos *et al.*, 2016). MLST has also been proposed as a method to replace *Salmonella* serotyping (Achtman *et al.*, 2012).

Nevertheless, MLST is an expensive and time-consuming technique that requires high quality sequences. Besides, although MLST is very powerful in detecting groups of related organisms belonging to the same lineage, it may lack capacity to distinguish closely related isolates or highly clonal bacterial populations (Barco *et al.*, 2013; Taboada *et al.*, 2013). To enhance the discriminatory power among epidemic strains with common STs, the use of an additional subtyping method may be required, such as the sequencing of the flagellin short variable region (*flaA*-SVR) for *Campylobacter* (Sails *et al.*, 2003; Clark *et al.*, 2005).

1.5.5. Future prospects

Nowadays, next-generation sequencing (NGS) technologies have made possible to examine the complete or nearly entire genomes of bacterial isolates (Forde and O'Toole, 2013). Whole genome sequencing (WGS) methods can resolve isolates that differ at only a single nucleotide, thereby providing the highest level of resolution and phylogenetic accuracy for epidemiologic subtyping. Until recently, WGS-based analyses were limited to the characterization of a small number of strains of interest. However, rapid advances and relatively decreasing costs in NGS have made WGS increasingly accessible and applicable for tracking disease outbreaks with successful results. The WGS approach provides the possibility to obtain from one single assay many traditional typing results and other more complete analysis that yield critical information for bacterial surveillance. Some of them are the ribosomal MLST (rMLST) scheme, based on 53 ribosomal protein loci present in most bacteria; the core-genome MLST (cgMLST), which provides high-resolution data in groups of related but not identical isolates; and the whole genome MLST (wgMLST) that is applicable to single-clone bacteria or very closely related strains (Jolley *et al.*, 2012; Sheppard *et al.*, 2012).

1.6. ONE HEALTH

The improvement in detection and genotyping techniques has contributed to a greater understanding of the epidemiology of *Campylobacter* and *Salmonella*. However, the existence of multiple sources of infection and reservoir hosts reveals the complexity of the transmission cycle of these enteropathogens (Figure 1.7). It is well known that the main source of infection is the consumption of contaminated food. However, identification of the sources of environmental contamination may not always be possible due to the large number of animal species involved in the transmission of these zoonotic agents (Hoelzer *et al.*, 2011; Whiley *et al.*, 2013). Moreover, the bacterial

survival and persistence in soil, water and on a variety of surfaces provides an increased probability of infecting new hosts (Winfiel and Groisman, 2003; Bronowski *et al.*, 2014). Thus, transmission of these pathogens is a compelling example of the One Health paradigm, with reservoirs of the bacteria in humans, animals (both domestic and wildlife) and the environment.

The One Health concept recognized that human health and animal health are interdependent and bound to the health of ecosystems in which they exist (Zinsstag *et al.*, 2011). This concept is not new, was introduced by Rudolf Virchow and others in the late 19th century, but received relatively little attention at that time. Recently, the One Health approach involving human, animal, and environmental compartments is acquiring a great interest since it is critical to address current public health issues including these emerging infectious and zoonotic diseases.

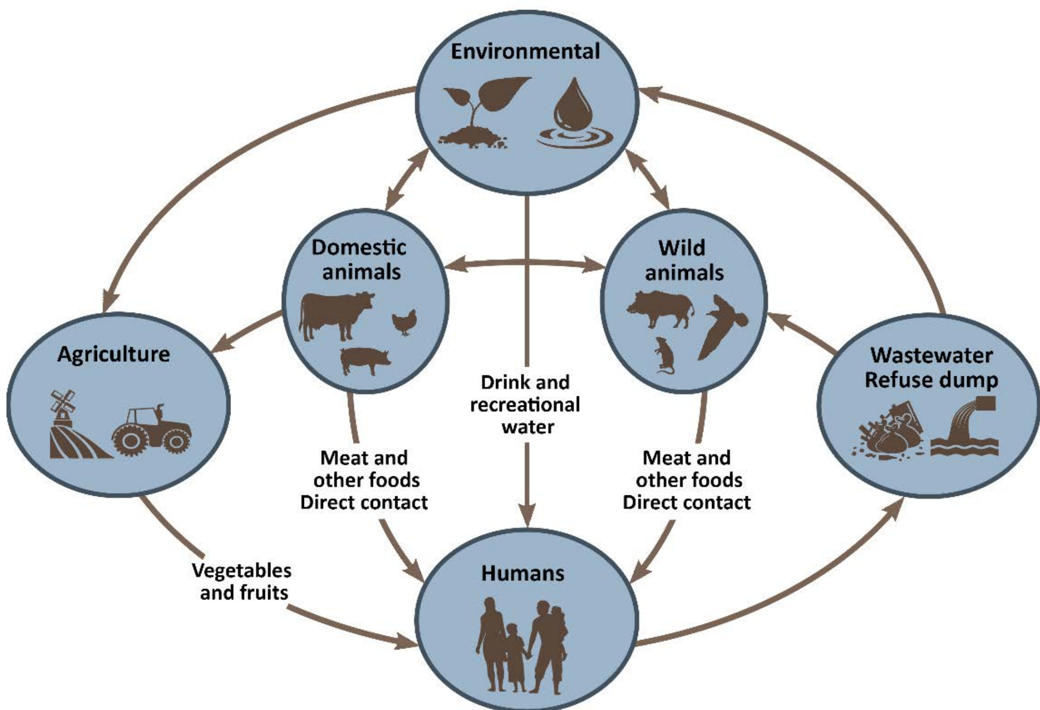


Figure 1.7. Sources and transmission of *Campylobacter* and *Salmonella*.

Currently, human campylobacteriosis and salmonellosis are still a major public health concern despite of routine surveillance strategies and the multiple efforts made to prevent and manage such infections. To reduce the incidence of these diseases in humans it is important to act at primary production level, the first link in the food chain. Since the implementation of national control programmes of *Salmonella* in poultry farms, human salmonellosis cases in Europe have significantly reduced (EFSA and ECDC, 2017b). However, the frequency of less common serovars also important in human medicine is increasing. In the case of *Campylobacter*, the biosecurity measures to restrict the environmental exposure in poultry farms are often insufficient to efficiently control the colonization of animals (Sahin *et al.*, 2002). This demonstrates that basic ecological principles govern the environmental niches occupied by pathogens, making it impossible to prevent these zoonotic infections without a clear understanding of One Health. Nevertheless, most studies of molecular epidemiology of *Campylobacter* and *Salmonella* focus on human clinical isolates or isolates from domestic animals and food origin. Comparatively, studies based on environmental samples or isolates from wild animals are scarce.

The role of wildlife in these zoonoses is of increasing interest (Greig *et al.*, 2015). It is likely that bacterial transmission between wild animals (e.g. insects, rodents, mammals and birds) and domestic animals takes place, although the impact of unidirectional or bidirectional transmission, and the role of wild animals as a reservoir are controversial issues (Meerburg and Kijlstra, 2007; Wales *et al.*, 2010; Antilles, 2014). Clearly, zoonotic bacteria have overcome species barriers and have adapted to new hosts, acquiring new combinations of virulence determinants via multiple horizontal transfers. Direct contact between humans and wildlife (e.g. birds, reptiles and wild boars), as well as the consumption of their meat, may also contribute to human campylobacteriosis and salmonellosis (Tsiodras *et al.*, 2008). In addition, contamination by wildlife vectors (e.g. insects, rodents and birds) have been postulated as a potential source for

contamination of non-animal based food (e.g. fresh fruits, nuts and vegetables) (Jay-Russell, 2013).

1.7. WILD BIRDS AS RESERVOIRS OF ZOONOTIC BACTERIA

Wild birds are important in the spread and maintenance of zoonotic pathogens in the environment, such as influenza A virus, West Nile virus, *Borrelia* and enteric bacteria (Bengis *et al.*, 2004). *Campylobacter* and *Salmonella* have been detected with variable prevalence in a wide range of wild birds including waterfowl, crows, sparrows, pigeons, raptors and seagulls (Chuma *et al.*, 2000; Molina-Lopez *et al.*, 2011; Gargiulo *et al.*, 2014; Antilles *et al.*, 2015; Jurado-Tarifa *et al.*, 2016; Konicek *et al.*, 2016).

Birds feeding habits appear to be the main factor influencing the exposure to these zoonotic bacteria and the proximity to farms and grazing fields imply an added risk of infection (Ramos *et al.*, 2010; Hald *et al.*, 2016). In some ecological guilds, such as insectivores, granivores and birds feeding exclusively on vegetable matter, the presence of enteropathogens is low or non-existent. On the contrary, high carriage rates have been found in other guilds including ground-foraging, raptors and opportunistic feeders. Ground-foraging bird species can become infected when ingesting food contaminated with droppings or eat filter-feeding molluscs in sewage contaminated habitats. Raptors can acquire these enteric bacteria from the intestines of their preys or from carcasses when they scavenge on carrion. Opportunistic birds are drawn to sewage sludge and refuse dumps that may be inherently rich sources of pathogenic agents (Raven and Coulson, 2001). In particular, seagulls are one of the most documented carriers of *Campylobacter* and *Salmonella* spp. probably due to their scavenging feeding habits. Over the past decades, populations of several gull species have increased drastically throughout Europe, North America and Australia, and have been largely attributed to greater availability of food because of anthropogenic activity (Hatch, 1996). Due to the

urbanization of coastal areas, interaction of seagulls with humans is increasingly close since flocks gather at areas where food scraps are abundant, especially around landfills, farms, processing factories and fishing boats.

Given the global loss of natural wetlands, seagulls and other seabirds have become more dependent on alternative habitats, including wastewater treatment plants (Murray and Hamilton, 2010). These behaviours may favour the transmission of antimicrobial resistant enteric bacteria of anthropogenic origin to wild birds. At the same time, seabirds are clearly of importance in maintaining these zoonotic bacteria in the environment during routine movements centralized on the place of residence from roosting or nesting sites to feeding sites. They often roost on nearby fields and pastures, and wash in local water bodies, in a way that bacteria ingested at feeding sites may enter again to the food chain, once excreted by the birds.

Furthermore, seabirds like other wild birds may act as effective dispersers of pathogens via faecal contamination due to their ability to cover long distances during annual movements. Twice a year, billions of wild birds, belonging to the 19% of extant species, migrate across national and intercontinental borders contributing to the potential establishment of new endemic foci of diseases (Reed *et al.*, 2003). Migration is a regular seasonal movement, often north and south along a flyway between breeding and wintering grounds, driven primarily by availability of food. The migration patterns are complex and variable between species or even different populations within the same species (Hockey *et al.*, 2005). For instance, Arctic tern (*Sterna paradisaea*) holds the long-distance migration record for birds, travelling up to 80,000 km between northern Scandinavia and Antarctica each year (Egevang *et al.*, 2010). Other trans-equatorial migrant birds are the long-tailed skua (*Stercorarius longicaudus*) and the Sabine's gull (*Larus sabini*), which breed in Arctic and spend the winter in the Southern Hemisphere, in close association with the cold waters of the Benguela Upwelling in the south-west coast of Africa (Stenhouse *et al.*, 2012; Gilg *et al.*, 2013). Bird migration involves long journeys and long distances which results in physiologic stress, and thus, a greater

susceptibility to infectious diseases that facilitates birds becoming a reservoir of pathogens. Moreover, the different ecosystems where birds stop over during the travel can also increase the risk of exposure to reservoir hosts and sources of zoonotic bacteria. For these reasons, migration may be a mechanism that facilitates the geographic distribution of pathogens and AMR that can pose a risk for public and animal health.

CHAPTER 2

Objectives

Despite the importance of seabirds and other wild birds as reservoirs of zoonotic bacteria and antimicrobial resistance traits, there are still gaps of knowledge of their role in the epidemiology of *Campylobacter* and *Salmonella* spp. Thus, the global aim of this thesis is to deepen the knowledge of the epidemiology of these zoonotic agents in seabirds from remote regions in the Southern Hemisphere and in the neaest Southwestern Europe.

In order to achieve this goal, the specific objectives are:

1. To determine the prevalence, genetic relatedness and antimicrobial resistance of thermophilic *Campylobacter* spp. and non-typhoidal *Salmonella* serovars in seabirds from the Western Cape coast of South Africa. **Study I**

2. To gain insight into the epidemiology, population structure and potential of virulence of thermophilic *Campylobacter* spp. and non-typhoidal *Salmonella* serovars from a range of seabird species from the Southern Ocean. **Study II and III**.

3. To assess the genetic relatedness and virulence potential of *Salmonella* isolates from human, poultry and seagulls from Southwestern Europe. **Study IV**.

CHAPTER 3

Study I: Seabirds (Laridae) as a source of *Campylobacter* spp., *Salmonella* spp. and antimicrobial resistance in South Africa

3.1. SUMMARY

Zoonotic thermophilic *Campylobacter* and nontyphoidal *Salmonella enterica* are a major cause of foodborne human gastroenteritis worldwide. There is little information about reservoirs of these zoonotic agents in Africa. Thus, chicks of kelp gulls (*Larus dominicanus*, n=129) and greater crested terns (*Thalasseus bergii*, n=100) were studied at five colonies on the Western Cape coast (South Africa) during summer 2013/2014. *Campylobacter* spp. occurrence was 14.0% ($CI_{95\%}$: 9.9-19.3), with *C. jejuni* the most frequently isolated species, whilst that of *Salmonella* was 27.5% ($CI_{95\%}$: 21.9-33.9) overall, with a higher prevalence in gulls (43.0%, $CI_{95\%}$: 34.8-52.4) than terns (7.0%, $CI_{95\%}$: 3.1-14.4). Among the 16 different *S. enterica* serovars found, Anatum, Enteritidis and Hadar were the most frequent. The same or highly similar pulsed-field gel electrophoresis genotype was found in some *Salmonella* isolates from seabirds and humans presenting with salmonellosis in Cape Town hospitals. Both *Campylobacter* and *Salmonella* isolates exhibited antimicrobial resistance to several agents, including critically important antimicrobials (quinolones, tetracyclines and β-lactams) and multidrug resistance in *Salmonella* serovars from kelp gulls. Our results highlight the importance of seabirds as reservoirs of *Campylobacter* and *Salmonella* resistant strains and their role in the maintenance and transmission of these bacteria in the environment, with implications for public health.

3.2. INTRODUCTION

Thermophilic *Campylobacter* and nontyphoidal *Salmonella enterica* are considered the most common causes of foodborne zoonotic infections worldwide, and are thus of economic and public health concern (Thorns, 2000; Havelaar *et al.*, 2015). These infections are usually self-limiting but may become more severe, causing complications such as dehydration, bacteraemia or occasionally long-term chronic sequels such as reactive arthritis, inflammatory bowel disease or Guillain-Barré syndrome (Batz *et al.*, 2013).

The occurrence of *Campylobacter* and *Salmonella* in aquatic environments (Levantesi *et al.*, 2012; Pitkänen, 2013) and in wildlife reservoirs is well documented (Hilbert *et al.*, 2012; Greig *et al.*, 2015). A wide variety of birds, including raptors, waterfowl, crows, pigeons and gulls, have been reported as asymptomatic carriers of these zoonotic agents (Molina-Lopez *et al.*, 2011; Gargiulo *et al.*, 2014; Antilles *et al.*, 2015; Jurado-Tarifa *et al.*, 2016; Konicek *et al.*, 2016), with their risk of infection linked to their opportunistic feeding habits and their proximity to farms or livestock pastures (Ramos *et al.*, 2010; Hald *et al.*, 2016). Although some *Campylobacter* and *Salmonella* strains display an important host-specificity (Heithoff *et al.*, 2008; Griekspoor *et al.*, 2013), many strains or serovars infectious to humans are adapted to a generalist lifestyle (e.g., certain *C. jejuni* and *C. coli* strains or *Salmonella* serovars Typhimurium and Enteritidis) and have a broad-host range (Hoelzer *et al.*, 2011; Dearlove *et al.*, 2016).

At the same time, the widespread use of antimicrobials during the last decade has selected for resistant microorganisms, with increasing reports of bacteria resistant to critically important antimicrobials (NARMS-FDA, 2016; EFSA and ECDC, 2017b). Antimicrobial resistant strains of zoonotic *Campylobacter* and *Salmonella* from domestic animals and humans can spread to wild animals, creating new host reservoirs of resistant bacteria in the environment. In addition, antimicrobial resistance genes can be transmitted to other pathogens or commensal bacteria by horizontal transfer (Davies

and Davies, 2010), contributing to the rapid amplification and mobilization of drug resistance (Carroll *et al.*, 2015). An example of this is the emergence of the plasmid-encoded mcr-1 gene conferring resistance to colistin that was initially reported in food animals and humans (Liu *et al.*, 2016). This new mechanism of resistance recently has been reported in wild fauna, including gulls, revealing the emergence and dissemination of this gene through wildlife (Liakopoulos *et al.*, 2016; Ruzauskas and Vaskeviciute, 2016). Due to the ability of many bird species to travel long distances, they can be effective spreaders of these zoonotic agents via faecal contamination of agricultural lands and surface waters used for drinking, recreation or irrigation (Reed *et al.*, 2003), or they may come in contact with food production animals. Hence, some wild bird species can play an important role in the epidemiology of humans and livestock campylobacteriosis and salmonellosis and potentially contribute to the maintenance and dissemination of antimicrobial resistance in the environment (Sippy *et al.*, 2012; Palomo *et al.*, 2013; Cody *et al.*, 2015).

Several studies have described the occurrence of *Campylobacter* and *Salmonella*, including resistant strains, in certain wild birds (e.g. gulls, raptors) from Europe, America and Australia (Hudson *et al.*, 2000; Cízek *et al.*, 2007; Dolejska *et al.*, 2016; Jurado-Tarifa *et al.*, 2016; Migura-Garcia *et al.*, 2017). However, there is little information about these zoonotic bacteria in wild birds in Africa. To assess whether seabirds in South Africa may play a role in the epidemiology of these zoonotic bacteria, we conducted a study in the Western Cape Province where some seabirds breed close to human settlements within the Benguela Upwelling Region. Here we report the presence, antimicrobial resistance and genetic diversity of *Campylobacter* and *Salmonella* spp. in kelp gulls (*Larus dominicanus*) and greater crested terns (*Thalasseus bergii*) along the southern coast of Africa, in colonies from semi-rural and urban areas. Both species are resident within the region, but some individuals undertake movements of hundreds to thousands of kilometres (Hockey *et al.*, 2005). We also investigated the genetic relatedness among

Salmonella isolates found in these birds with those of human clinical isolates from Cape Town, the major urban centre in the region.

3.3. MATERIALS AND METHODS

3.3.1. Sampling

A total of 229 kelp gull (N = 129) and greater crested tern (N = 100) chicks of 3-4 weeks old were sampled in the Western Cape, South Africa, between December 2013 and April 2014. Kelp gulls were sampled at four colonies at coastal wetlands: two colonies in semi-rural areas along the west coast (Velddrif and Yzerfontein) and two colonies close to urban centres: Strandfontein in Cape Town and Keurbooms River Mouth near Plettenberg Bay (Figure 3.1). The Velddrif colony of ca. 700 pairs is at a series of commercial salt pans adjacent to St Helena Bay (32°43'S, 18°12'E), 5 km north of Velddrif (population ca. 11,000 people in 2011; www.citypopulation.de/php/southafrica-westerncape.php). The Yzerfontein colony (ca 120 pairs) breeds at a natural salt pan (33°20'S, 18°10'E) ca. 1 km north of the small coastal resort town of Yzerfontein (1,100 people in 2011). Both these colonies are close to the coast in Strandveld vegetation, with agricultural lands inland supporting a mix of cereal crops and small stock farming (mainly sheep). The Strandfontein colony of ca. 1,250 pairs is on coastal dunes adjacent to a sewage treatment plant and a large refuse dump on the False Bay coast (34°05'S, 18°34'E), within greater Cape Town (3.4 million people in 2011); most adjacent areas are either green belts or developed for low income housing. Finally, ca. 1,400 pairs breed among coastal dunes at the Keurbooms River Mouth (34°02'S, 23°22'E), 3 km east of Plettenberg Bay (32,000 people in 2011), where non-breeding gulls also feed extensively at the municipal refuse dump (Whittington *et al.*, 2016; Witteveen *et al.*, 2017). Two colonies of greater crested terns were sampled on Robben Island, in Table Bay 10 km off Cape Town (Figure 3.1), where 4,000-8,000 pairs breed in most years: 50 chicks were sampled from a large colony (ca. 8,000 pairs) breeding next to an area with

large numbers of kelp gulls, and 50 from a smaller colony (ca. 800 pairs) breeding in a mixed-species colony with Hartlaub's gulls. Many Hartlaub's gulls commute to the city each day to feed. Faecal samples from gull and tern chicks were collected in duplicate using sterile swabs that were gently inserted into the cloaca, then placed in Amies transport medium with charcoal (Deltalab, Barcelona, Spain) and refrigerated until they were processed within two weeks of the date collected. Chicks were caught during a single visit to each colony. Tern chicks were sampled during an annual ringing operation that rounds up creching chicks into large holding pens, where they are held for up to 1-2 h until they are processed and released back into the colony. During this time the chicks continue to be fed by their parents. Kelp gull nests are more widely spaced, so chicks were caught individually by hand as the team of two researchers moved sequentially through each colony, sampling no more than one chick per brood. Chicks were sampled because they are much easier to catch than adult birds. Field protocols were approved by the University of Cape Town's Science Faculty Animal Ethics Committee (SFAEC 2013/V3/TC).

3.3.2. *Campylobacter* isolation and identification

Campylobacter isolation and identification from the swabs was performed as described by Urdaneta *et al.* (2015) Blood-free selective medium (mCCDA, modified charcoal cefoperazone desoxycholate agar, CM739 with selective supplement, SR0155E; Oxoid, Basingstoke, UK) was used. We subcultured up to four *Campylobacter*-presumptive colonies per positive bird onto blood agar plates (BioMérieux, Marcy l'Etoile, France) and *Campylobacter* species were identified by multiplex PCR using primers targeting the lipid A gene *lpxA* (Klena *et al.*, 2004). All isolates were preserved in brain hearth infusion broth with 20% of glycerol at -80°C for later analysis.

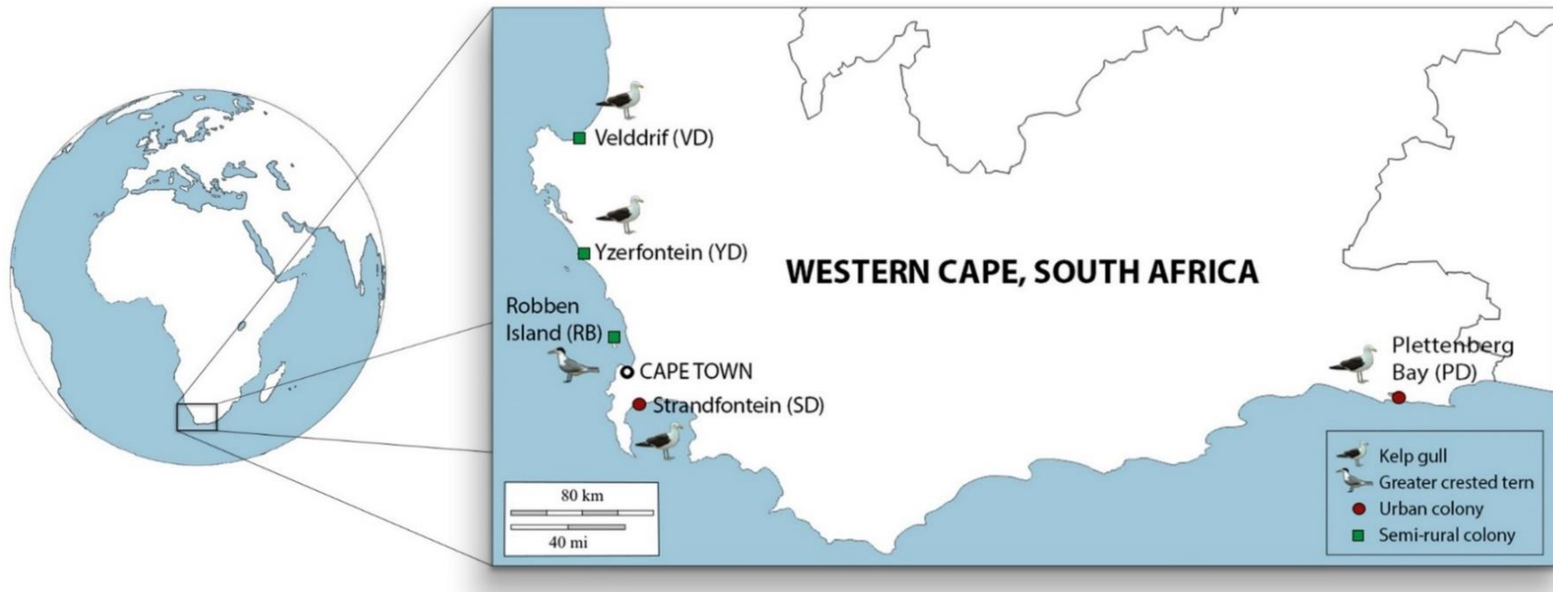


Figure 3.1. Map locations of the sampled seabird colonies in Western Cape (South Africa).

3.3.3. *Salmonella* isolation and identification

Salmonella isolation procedure was as described by Antilles *et al.* (2015). Briefly, isolation was performed by using buffered peptone water (Oxoid, Basingstoke, UK) pre-enrichment, followed by selective enrichment in Rappaport-Vassiliadis (Oxoid, Basingstoke, UK) and subculturing onto xylose lysine Tergitol 4 agar (Merck, Darmstadt, Germany); we subcultured up to four presumptive colonies onto MacConkey agar, and confirmed the lactose-negative colonies as *Salmonella* spp. with the Mucap (Biolife, Milano, Italy) and indole tests. All isolates were preserved in brain hearth infusion broth with 20% of glycerol at -80°C for later analysis.

Salmonella serotyping was carried out according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007) at the Laboratori Agroalimentari (Cabrils, Spain) of the Departament d'Agricultura, Ramaderia, Pesca i Alimentació.

3.3.4. Molecular typing of the isolates

Two different subtyping methods were carried out for genotyping both *Campylobacter* and *Salmonella* isolates. For *Campylobacter*, we used *fla*A-RFLP to determine the genotypic diversity among *Campylobacter* isolates within an individual host and within a gull colony. Isolates from the same bird showing identical *fla*A-RFLP profile were considered as the same strain and only one of them was selected for PFGE typing. With the same purpose, we conducted ERIC-PCR of all *Salmonella* isolates and representative isolates from the different *Salmonella* ERIC-PCR patterns identified per bird were analysed by PFGE.

3.3.4.1. *fla*A-RFLP

We carried out RFLP of the *fla*A gene following the CAMPYNET protocol as described previously (Harrington *et al.*, 2003). The *flaA* gene was amplified using the forward A1 (5'-GGA TTT CGT ATT AAC ACA AAT GGT GC-3') and reverse A2 (5'-CTG TAG TAA TCT TAA

AAC ATT TTG-3') primers (Nachamkin *et al.*, 1993). The amplified product of 1.7 kb PCR fragment was digested using the restriction enzyme *Dde*I (*Hyp*F3I; FastDigest®, Thermo Fisher Scientific, Waltham, MA, USA). Digest products were separated by electrophoresis on 2.5% agarose gel in 1x TAE buffer at 90V for 3 h.

3.3.4.2. ERIC-PCR

We performed ERIC-PCR as previously described (Antilles *et al.*, 2015), except that we used a 50ºC annealing temperature that is more adequate for Enterobacteriaceae. Primer pairs used were ERIC-F (5'-AAG TAA GTG ACT GGG GTG AGC G-3') and ERIC-R (5'-ATG TAA GCT CCT GGG GAT TCA C-3') (Versalovic *et al.*, 1991).

3.3.4.3. PFGE

Campylobacter and *Salmonella* isolates were typed by PFGE according to the standard operating procedure of PulseNet (www.pulsenetinternational.org/protocols/pfge/). Genomic DNA of *Campylobacter* isolates was digested with SmaI and KpnI restriction enzymes (Roche Applied Science, Indianapolis, IN), whilst for *Salmonella* digestion XbaI and BlnI enzymes (Roche Applied Science, Indianapolis, IN, USA) were used; the secondary enzyme (BlnI) was used only in a selection of isolates which showed identical XbaI-PFGE profiles. Electrophoresis was performed in a CHEF-DR III System (Bio-Rad, Hercules, CA, USA).

Isolates from the same bird that showed the same PFGE pattern with the primary restriction enzyme were considered the same clone, and only one was included in the analyses with the secondary enzyme.

We compared *Salmonella* isolates from seabirds with 47 human clinical isolates of serovars Enteritidis (n=24), Typhimurium (n=14), Hadar (n=8) and Anatum (n=1) collected during 2014 from patients from eight different hospitals in the Cape Town area. These isolates were randomly selected among those cases overlapping with the

bird sampling period. Human isolates were collected at the National Health Laboratory Service Microbiology laboratory, Groote Schuur Hospital and University of Cape Town; this study was approved by the University of Cape Town Human Research Ethics Committee (HREC REF:653/2016).

3.3.5. Analysis and comparison of band patterns

The RFLP, ERIC and PFGE band patterns were analysed using Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Similarity matrices were calculated using the Dice coefficient with tolerance and optimization values of 1.0%. A dendrogram was constructed based on an unweighted-pair group method with arithmetic mean (UPGMA) cluster analysis. A cut-off of 90% was used for the determination of the different profiles. PFGE patterns generated with the primary and secondary restriction enzymes were combined and named as pulsotype SK1, SK2 and so on for *Campylobacter*; and XB1, XB2 and so on for *Salmonella*. Some *Salmonella* isolates were genotyped only with the primary enzyme; in this case XbaI-PFGE profiles were named as pulsotype X1, X2, etc.

3.3.6. Antimicrobial susceptibility testing

Antimicrobial susceptibility was performed according to the Clinical Laboratory and Standard Institute (CLSI) disk diffusion method (M100-S26; CLSI, 2016) using Neo-Sensitabs™ (Rosco Diagnostica, Taastrup, Denmark) with CLSI potencies and interpretation zones according to the 2016 manufacturer's instructions. One *Campylobacter* and one *Salmonella* isolate per positive bird was tested, except in those cases were more than one PFGE profile was found in a single bird, where one isolate per profile was analysed.

Campylobacter was streaked to form a bacterial lawn onto Mueller-Hinton II agar supplemented with 5% defibrinated sheep blood (BioMérieux, Marcy l'Etoile, France)

and then incubated with antimicrobial disks at 37°C for 48 h under microaerobic conditions. The diameter of the bacterial growth inhibition was measured and designated as resistant or susceptible. *Campylobacter* isolates were tested for susceptibility to 7 antimicrobial agents which included two quinolones: nalidixic acid (30 µg, R < 18 mm), ciprofloxacin (5 µg, R < 26 mm); one aminoglycoside: gentamicin (10 µg, R ≤ 14 mm); one macrolide: erythromycin (15 µg, R < 20 mm); two tetracyclines: tetracycline (30 µg, R < 30 mm) and doxycycline (30 µg, R ≤ 20 mm); and one phenicol: chloramphenicol (30 µg, R ≤ 20 mm). Similarly, *Salmonella* isolates were streaked onto Mueller-Hinton agar (Difco, Madrid, Spain) to form a bacterial lawn and plates were incubated a 37°C for 24 h. A panel of 18 antimicrobial agents were evaluated, including three β-lactams: ampicillin (10 µg, R ≤ 13 mm), amoxycillin (30 µg, R ≤ 16 mm) and amoxicillin-clavulanate (20 + 10 µg, R ≤ 13 mm); one cephalosporin: ceftiofur (30 µg, R ≤ 17 mm); four aminoglycosides: apramycin (40 µg; R ≤ 19 mm), gentamicin (10 µg; R ≤ 12 mm), neomycin (120 µg, R ≤ 19 mm) and streptomycin (10 µg, R ≤ 11 mm); four quinolones: nalidixic acid (30 µg, R ≤ 1 3mm); ciprofloxacin (5 µg, R ≤ 15 mm), enrofloxacin (10 µg, R < 28 mm) and norfloxacin (10 µg, R ≤ 12 mm); two tetracyclines: tetracycline (30 µg, R ≤ 11 mm) and doxycycline (30 µg, R ≤ 10 mm); one polymyxin: colistin (150 µg, R < 15 mm); one phenicol: chloramphenicol (30 µg, R ≤ 12 mm); and three other antimicrobials: nitrofurantoin (300 µg, R ≤ 14 mm), lincomycin-spectinomycin (15 + 200 µg, R ≤ 16 mm), and trimethoprim-sulfamethoxazole (1.25 + 23.75 µg, R ≤ 10 mm).

3.3.7. Statistical analysis

The confidence intervals of *Campylobacter* and *Salmonella* prevalence were calculated with the Wilson score with continuity correction. Pearson's chi-squared test with Yates's correction for continuity was used to compare *Campylobacter* and *Salmonella* frequencies between different bird species and among gull colonies. Fisher's exact tests were performed when one or more of the observed values was lower than 5. $P < 0.05$

was considered statistically significant. The Deducer GUI of R software (www.R-project.org) was used for the statistical analysis.

3.4. RESULTS

3.4.1. *Campylobacter* and *Salmonella* occurrence

Thermophilic *Campylobacter* were isolated from 32 (14.0%, $CI_{95\%}$: 9.9-19.3) of the 229 chicks sampled: 12.4% ($CI_{95\%}$: 7.5-19.7) of kelp gulls and 16.0% ($CI_{95\%}$: 9.7-25.0) of greater crested terns. As no significant differences between the two sampled colonies of terns at Robben Island were found, both colonies were treated as a single entity. *Campylobacter* was found in gulls from all colonies except Yzerfontein (semi-rural; only a modest sample size) (Table 3.1), with a statistically significant higher frequency at Velddrif (semi-rural) and Strandfontein (urban) than at Plettenberg Bay (urban), where only one positive bird was detected. *C. jejuni* was the most frequently isolated species, but *C. lari* was identified in one tern from Robben Island and one gull from Strandfontein.

Salmonella was isolated in all seabird colonies studied with an overall occurrence of 27.5% ($CI_{95\%}$: 21.9-33.9), but was significantly more prevalent in kelp gulls (43.0%, $CI_{95\%}$: 34.8-52.4) than in greater crested terns (7.0%, $CI_{95\%}$: 3.1-14.4) (Table 3.1). *S. enterica* subsp. *salamae* was isolated in gulls from Strandfontein (urban), Yzerfontein and Velddrif (semi-rural). Sixteen different serovars of *S. enterica* subsp. *enterica* were detected (14 in kelp gulls and four in greater crested terns) (Table 3.1). Serovars Anatum and Enteritidis were the most frequent and were found at three of the four kelp gull colonies. Hadar was the third most frequent serovar and was found in greater crested terns as well as at two kelp gull colonies. Overall, most *Salmonella* positive birds carried a single serovar, but a few gulls from urban colonies carried two serovars: at Plettenberg Bay one gull was infected with both *S.* Manhattan and *S.* Anatum, and another gull

carried serovars Manhattan and Typhimurium; at Strandfontein one gull carried the serovars Enteritidis and Typhimurium.

Overall there was no significant difference in the occurrence of either *Campylobacter* or S*almonella* between gulls breeding at urban (Plettenberg Bay and Strandfontein) and semi-rural colonies (Velddrif and Yzerfontein). However, kelp gulls had a significantly higher occurrence of *Salmonella* than *Campylobacter*, whereas the opposite pattern was observed in greater crested terns. In most cases the birds carried either *Campylobacter* or *Salmonella*, but four gulls and one tern carried both pathogens.

3.4.2. Genetic diversity

Ninety *C. jejuni* isolates from 16 kelp gulls and 16 greater crested terns were genotyped by restriction fragment length polymorphism of the *fla*A gene (*fla*A-RFLP), revealing 22 different profiles (14 from gulls and eight from terns). In most cases, isolates from the same individual showed an identical RFLP profile, so only one isolate per bird was analysed by pulsed-field gel electrophoresis (PFGE). Of the 36 *C. jejuni* isolates genotyped by PFGE, ten different SmaI-PFGE pulsotypes were obtained. To increase discrimination power, the 36 *C. jejuni* isolates were genotyped using the KpnI enzyme, and the combination of both SmaI and KpnI-PFGE banding patterns resulted in 20 different pulsotypes (13 from kelp gulls and seven from greater crested terns; Figure 3.2). All but five *C. jejuni* isolates from kelp gulls and greater crested terns were grouped in three main clusters with a similarity level of over 60%. Most birds carried a single clone, except one greater crested tern in which two different pulsotypes were detected (SK5 and SK11). The same clone was found in kelp gulls from two colonies (SK9). In addition, one cluster (SK11 and SK12) grouped isolates from both bird species at a high similarity level (86%). By comparison, the two *C. lari* isolates found in a greater crested tern and a kelp gull showed a low similarity (67%) (data not shown).

A total of 235 *Salmonella* isolates from seabirds were analysed by enterobacterial repetitive intergenic consensus (ERIC)-PCR and 43 different profiles were detected (38 from kelp gulls and five from greater crested terns). Ninety isolates representing the different banding patterns identified were genotyped by PFGE using the XbaI enzyme. Isolates that showed an identical XbaI-PFGE profile were further analysed with the secondary enzyme BlnI. The combined analysis with XbaI and BlnI macrorestriction profiles resulted in 29 PFGE pulsotypes (26 from kelp gulls and four from greater crested terns) among the different serovars (Table 3.2). *S. enterica* subsp. *salamae* isolates from kelp gulls presented three different pulsotypes with a similarity of 36%; one of them (XB32) included isolates from three different gull colonies. The three *S.* Typhimurium isolates showed three different PFGE profiles. Five distinct pulsotypes were detected among the eight *S.* Enteritidis isolates, with one of them (XB7) found in both urban gull colonies. Genetic diversity was low among *S.* Anatum isolates; 28 isolates were grouped in three pulsotypes, with isolates from the two urban gull colonies clustering in one of them (XB1). All seven *S.* Hadar isolates from both bird species and three different colonies clustered together in the same pulsotype (XB12).

Genotyping of 47 human clinical isolates of *Salmonella* using the two restriction enzymes revealed 14 different PFGE profiles (Table 3.2), with 70% of the human isolates showing the same (23/47) or highly similar (10/47) pulsotype with bird isolates. The single *S*. Anatum clinical isolate was non-typeable with BlnI. The XbaI-PFGE profile of this isolate corresponded to the X1 pulsotype that included nine gull isolates, which also were non-typeable with the secondary enzyme. All *S*. Enteritidis of human origin were grouped in three pulsotypes which also included kelp gull isolates from urban colonies with a similarity of ≥ 87%. Despite the genetic diversity detected among *S*. Typhimurium isolates, a high similarity (84%) was observed between a kelp gull and a human isolate (pulsotypes XB22 and XB23). It should be noted that half of the human *S*. Enteritidis isolates and the human *S*. Typhimurium isolate that clustered together with isolates from kelp gulls were invasive. Regarding *S*. Hadar, almost all clinical isolates belonged

to the XB13 pulsotype which was closely related (88%) with XB12 that included isolates from different colonies and seabird species.

3.4.3. Antimicrobial susceptibility

One isolate (both for *Campylobacter* and for *Salmonella*) per pulsotype from each positive bird were tested for antimicrobial susceptibility to a panel of antimicrobials. Most of the 33 *Campylobacter* isolates tested were pansusceptible (72.7%). However, 37.5% of kelp gull isolates and 17.6% of greater crested tern isolates were resistant to at least one antimicrobial agent tested. All *Campylobacter* isolates were susceptible to gentamicin, erythromycin, doxycycline and chloramphenicol. In kelp gulls, the main antimicrobial resistances detected were to tetracycline (31.3%) and quinolones (12.5%). Tetracycline resistance was mainly found in gulls from Velddrif (66.7%). At Strandfontein, two isolates were resistant to both nalidixic acid and ciprofloxacin (22.2%), and one was also resistant to tetracycline (11.1%). In greater crested terns, three isolates showed resistance to quinolones (17.6%): one was resistant to nalidixic acid and two were resistant to both nalidixic acid and ciprofloxacin. Among those isolates resistant to nalidixic acid and ciprofloxacin, there were two *C. lari* from one kelp gull and one greater crested tern.

Of the 66 *Salmonella* isolates analysed, 74.2% were pansusceptible. All *Salmonella* isolates were susceptible to amoxicillin-clavulanate, ceftiofur, apramycin, gentamicin, neomycin, ciprofloxacin, enrofloxacin, norfloxacin and colistin. The only resistance detected in greater crested terns was to ampicillin and amoxicillin (14.3% each) (Table 3.3). The main resistance detected in kelp gulls were to tetracycline (13.6%) and streptomycin (10.2%). Overall, resistance to antimicrobials was greater in urban gull colonies (seven drugs) than gulls from semi-rural colonies (three drugs). Multidrug resistance, defined as resistance to three or more classes of antimicrobial agents, was present in three *Salmonella* isolates from kelp gulls, all from urban colonies. One *S.* Idikan isolate from Strandfontein was resistant to six groups of antimicrobials

(streptomycin, tetracycline, doxycycline, chloramphenicol, lincomycin-spectinomycin and trimethoprim-sulfamethoxazole), and two *S.* Paratyphi B var Java isolates from Plettenberg Bay showed resistance to four antimicrobials (streptomycin, lincomycin-spectinomycin, nitrofurantoin and trimethoprim-sulfamethoxazole).

Table 3.1. Frequency of *Campylobacter* species and *Salmonella* subspecies and serovars in kelp gull and greater crested tern chicks in the five colonies sampled in the Western Cape, South Africa.

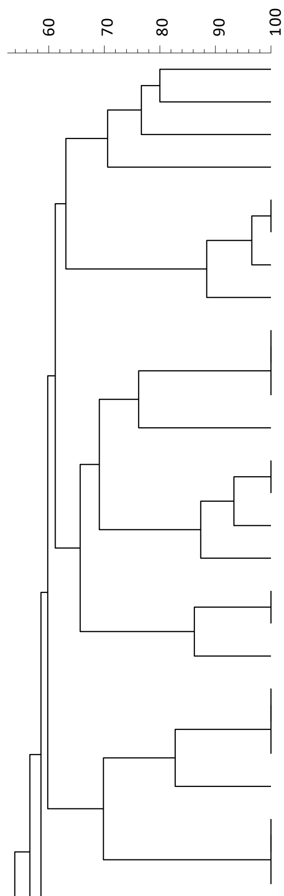
Species and serovars	Kelp gulls					Greater crested terns
	Urban		Semi-rural			
	Plettenberg Bay (n = 52)	**Strandfontein (n = 50)**	**Yzerfontein (n = 10)**	**Velddrif (n = 17)**	**Total (n=129)**	**Robben Island (n = 100)**
Campylobacter spp.						
C. jejuni	1.9 (0.1-11.6) [a]	16.0 (7.6-29.7)	0	35.3 (15.3-61.4)	11.6 (6.9-18.8)	15.0 (8.9-23.9)
C. lari	0	2.0 (0.1-12.0)	0	0	0.8 (0-4.9)	1 (0.1-6.2)
Total	1.9 (0.1-11.6)	18.0 (9.0-31.9)	0	35.3 (15.3-61.4)	12.4 (7.5-19.7)	16.0 (9.7-25.0)
Salmonella spp.						
S. enterica subsp. *salamae*	0	4.0 (0.7-14.86)	30.0 (8.1-64.6)	5.9 (0.3-30.8)	4.7 (1.9-10.3)	0
S. enterica subsp. *enterica*						
Anatum	26.9 (16.0-41.3)	4.0 (0.7-14.86)	0	11.8 (2.1-37.8)	14.0 (8.7-21.4)	0
Bovismorbificans	0	0	0	0	0	2.0 (0.4-7.7)
Corvallis	0	0	0	0	0	1.0 (0.1-6.2)
Derby	1.9 (0.1-11.6)	0	0	0	0.8 (0-4.9)	1.0 (0.1-6.2)
Enteritidis	1.9 (0.1-11.6)	12.0 (5.0-25.0)	0	5.9 (0.3-30.8)	6.2 (2.9-12.3)	0
Hadar	1.9 (0.1-11.6)	6.0 (1.6-17.5)	0	0	3.1 (1.0-8.2)	3.0 (0.8-9.2)
Heidelberg	0	2.0 (0.1-12.0)	0	0	0.8 (0-4.9)	0
Idikan	0	2.0 (0.1-12.0)	0	0	0.8 (0-4.9)	0
Manhattan	9.6 (3.6-21.8)	0	0	0	3.9 (1.4-9.3)	0
Muenchen	5.8 (1.5-16.9)	4.0 (0.7-14.86)	0	0	3.9 (1.4-9.3)	0

Continued

Table 3.1. Continued.

Species and serovars	Kelp gulls					Greater crested terns
	Urban		Semi-rural			
	Plettenberg Bay (n = 52)	Strandfontein (n = 50)	Yzerfontein (n = 10)	Velddrif (n = 17)	Total (n=129)	Robben Island (n = 100)
S. enterica subsp. *enterica*						
Ohio	0	2.0 (0.1-12.0)	0	0	0.8 (0-4.9)	0
Paratyphi B, var Java	3.8 (0.7-14.3)	0	0	0	1.6 (0.3-6.1)	0
Reading	0	0	10.0 (0.5-45.9)	0	0.8 (0-4.9)	0
Sandiego	1.9 (0.1-11.6)	0	0	0	0.8 (0-4.9)	0
Saintpaul	3.8 (0.7-14.3)	0	0	0	1.6 (0.3-6.1)	0
Typhimurium	1.9 (0.1-11.6)	4.0 (0.7-14.86)	0	0	2.3 (0.6-7.2)	0
Total	55.8 (41.4-69.3)	38.0 (25.0-52.8)	40.0 (13.7-72.6)	23.5 (7.8-50.2)	43.4 (34.8-52.4)	7.0 (3.1-14.4)

a) Percentage of infected chicks (95% confidence intervals).



SmaI-PFGE	KpnI-PFGE	Isolate	Pulsotype	Host	Location
		SD19-C1	SK1	Kelp gull	Urb-Strandfontein
		SD38-C1	SK2	Kelp gull	Urb-Strandfontein
		SD34-C2	SK3	Kelp gull	Urb-Strandfontein
		VD11-C1	SK4	Kelp gull	Srur-Velddrif
		RB25-C1	SK5	Greater crested tern	Robben Island
		RB66-C1	SK5	Greater crested tern	Robben Island
		RB94-C4	SK5	Greater crested tern	Robben Island
		RB49-C2	SK6	Greater crested tern	Robben Island
		RB87-C1	SK7	Greater crested tern	Robben Island
		RB89-C1	SK7	Greater crested tern	Robben Island
		RB93-C1	SK7	Greater crested tern	Robben Island
		SD41-C1	SK8	Kelp gull	Urb-Strandfontein
		SD16-C1	SK9	Kelp gull	Urb-Strandfontein
		VD8-C1	SK9	Kelp gull	Srur-Velddrif
		VD3-C1	SK9	Kelp gull	Srur-Velddrif
		VD16-C2	SK10	Kelp gull	Srur-Velddrif
		RB94-C1	SK11	Greater crested tern	Robben Island
		RB99-C1	SK11	Greater crested tern	Robben Island
		SD6-C1	SK12	Kelp gull	Urb-Strandfontein
		RB33-C1	SK13	Greater crested tern	Robben Island
		RB44-C2	SK13	Greater crested tern	Robben Island
		RB88-C1	SK13	Greater crested tern	Robben Island
		SD26-C1	SK14	Kelp gull	Urb-Strandfontein
		RB18-C1	SK15	Greater crested tern	Robben Island
		RB21-C1	SK15	Greater crested tern	Robben Island
		RB38-C1	SK15	Greater crested tern	Robben Island

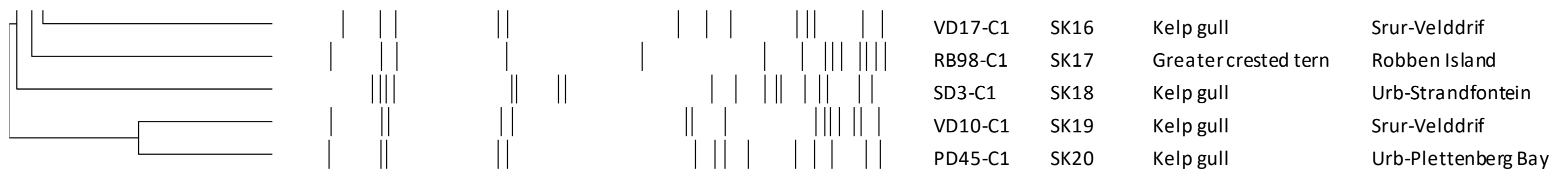


Figure 3.2. PFGE combined dendrogram of SmaI and KpnI patterns of *C. jejuni* isolates. The similarity matrices were calculated using the Dice coefficient and UPGMA clustering method. Profiles with a similarity ≥ 90% were considered same pulsotype. Urb: urban colony; Srur: semi-rural colony.

Table 3.2. *Salmonella* pulsotypes and serovars from seabirds and their relationship with clinical isolates.

Host	Pulsotype[a]	Serovar	Source[b]
Seabirds			
Kelp gulls	XB1	Anatum	Urb-PD (5), Urb-SD (2)
	XB2	Anatum	Srur-VD (2)
	XB5	Derby	Urb-PD (1)
	XB7[c]	Enteritidis	Urb-PD (1), Urb-SD (3)
	XB8	Enteritidis	Urb-SD (1)
	XB11	Enteritidis	Srur-VD (1)
	X3	Heidelberg	Urb-SD (1)
	X4	Idikan	Urb-SD (1)
	XB15	Manhattan	Urb-PD (5)
	XB16	Muenchen	Urb-PD (3)
	XB17	Muenchen	Urb-SD (2)
	X5	Ohio	Urb-SD (1)
	XB18	Paratyphi B	Urb-PD (2)
	X6	Reading	Srur-YD (1)
	XB19	Saintpaul	Urb-PD (2)
	X7	Sandiego	Urb-PD (1)
	XB22	Typhimurium	Urb-PD (1)
	XB28	Typhimurium	Urb-SD (1)
	XB29	Typhimurium	Urb-SD (1)
	XB31	subsp. *salamae*	Urb-SD (1)
	XB32	subsp. *salamae*	Urb-SD (1), Srur-YD (2), Srur-VD (1)
	XB33	subsp. *salamae*	Srur-YD (1)
Greater crested terns	XB3	Bovismorbificans	RB (2)
	X2	Corvalis	RB (1)
	XB4	Derby	RB (1)
Gulls and terns	**XB12**	Hadar	Urb-PD (1), Urb-SD (3), RB (3)

Continued

Table 3.2. Continued.

Host	Pulsotype[a]	Serovar	Source[b]
Humans and kelp gulls	X1	Anatum	Urb-PD (9), HCT (1)
	XB9	Enteritidis	Urb-SD (1), HCT(16)
	XB10	Enteritidis	Urb-SD (1), HCT (6)
Humans	**XB6**	Enteritidis	HCT (2)
	XB13	Hadar	HCT (7)
	XB14	Hadar	HCT (1)
	XB20	Typhimurium	HCT (2)
	XB21	Typhimurium	HCT (1)
	XB23	Typhimurium	HCT (1)
	XB24	Typhimurium	HCT (5)
	XB25	Typhimurium	HCT (1)
	XB26	Typhimurium	HCT (1)
	XB27	Typhimurium	HCT (1)
	XB30	Typhimurium	HCT (2)

a) Profiles with a similarity ≥ 90% were considered the same pulsotype. Pulsotypes referred as X were assigned by XbaI macrorestriction profies; XB indicates the combination of XbaI and BlnI profiles.

b) Urb-PD: urban colony at Plettenberg Bay; Urb-SD: urban colony at Strandfontein; Srur-YD: semi-rural colony at Yzerfontein; Srur-VD: semi-rural colony at Velddrif; RB: Robben Island colony. Number of isolates is given in brackets.

c) Pulsotypes with similarities of 84-89% among isolates from seabirds and humans are highlighted in bold: XB7, XB6 S. Enteritidis pulsotypes with 86% similarity; XB22, XB23 S. Typhimurium pulsotypes with 84% similarity; XB12, XB13 S. Hadar with 88% similarity.

Table 3.3. Antimicrobial resistance of *Salmonella* isolates according to the seabird species and colony.

Sampled colony	N[a]	Antimicrobial agents[b]									
		Amp	Amx	Str	Nal	Tet	Dox	Chl	Ni	Lisp	Sxt
Kelp gulls											
Urban											
Plettenberg Bay	31	0[c]	0	2 (6.5)	0	5 (16.1)	0	0	2 (6.5)	3 (9.7)	2 (6.5)
Strandfontein	20	0	0	2 (10.0)	0	3 (15.0)	3 (15.0)	1 (5.0)	0	1 (5.0)	3 (15.0)
Semi-rural											
Yzerfontein	4	0	0	2 (50.0)	0	0	0	0	0	0	0
Velddrif	4	0	0	0	1 (25.0)	0	0	0	1 (25.0)	0	0
Total	59	0	0	6 (10.2)	1 (1.7)	8 (13.6)	3 (5.1)	1 (1.7)	3 (5.1)	4 (6.8)	5 (8.5)
Greater crested terns											
Robben Island	7	1 (14.3)	1 (14.3)	0	0	0	0	0	0	0	0

a) Number of isolates tested.

b) Amp: ampicillin; Amx: amoxicillin; Str: streptomycin; Nal: nalidixic acid; Tet: tetracycline; Dox: doxycycline; Chl: chloramphenicol; Ni: nitrofurantoin; LiSp: lincomycin + spectinomycin; Sxt: trimethoprim + sulfamethoxazole. All isolates were susceptible to: amoxicillin + clavulanate, ceftiofur, apramycin, gentamicin, neomycin, ciprofloxacin, enrofloxacin, norfloxacin and colistin.

c) Number of isolates resistant (%).

3.5. DISCUSSION

The presence of zoonotic bacteria in wild birds has been described in many studies, with differences in the reported prevalence depending on the animal species and the location (Benskin *et al.*, 2009). However, little is known of the occurrence of zoonotic agents in African free-living birds. We report the presence, genetic diversity and antimicrobial resistance of the two most relevant foodborne zoonotic agents, *Campylobacter* and *Salmonella*, in seabirds in the Western Cape, South Africa. Birds have been reported as asymptomatic carriers of these pathogens, and seabirds nearby human settlements may be more prone to carry these bacteria, particularly those with scavenging feeding habits, such as gulls. *Campylobacter* occurrence in kelp gulls (12%) and greater crested terns (16%) was similar to that reported in yellow-legged gulls *Larus michahellis* in southern Europe (10%, Ramos *et al.*, 2010), but lower than in other gulls in the eastern coast of United States (laughing gulls *Leucophaeus atricilla*, 33%) and in northern Europe (black-headed gulls *Chroicocephalus ridibundus*, 28-36%) (Broman *et al.*, 2002; Keller *et al.*, 2011). The correlation between *Campylobacter* prevalence in wild birds and their feeding habits (Sensale *et al.*, 2010; Hald *et al.*, 2016), points to foraging in human waste and sewage as important risk factors of infection (Ramos *et al.*, 2010). Contrary to what would be expected, the highest *Campylobacter* prevalence was found in a colony (Velddrif) from a semi-rural area. In this site, sampling took place later in the season, during slightly warmer weather, when chicks were older on average and were moving around in an area where other birds had been active for a longer period. Thus, the area was more likely to be contaminated by these bacteria and could have contributed to an increased *Campylobacter* occurrence in chicks from this colony. On the other hand, *Campylobacter* species have been reported in livestock grazing in pastures (Oporto *et al.*, 2007; Moriarty *et al.*, 2008; Sproston *et al.*, 2011). Although no information is available about *Campylobacter* occurrence in livestock from South Africa, sheep present in the area may also have been the source of *C. jejuni* in this colony.

We detected a higher occurrence of *Salmonella* spp*.* in kelp gulls (43%) than in greater crested terns (7%). The prevalence of *Salmonella* shedding among wild birds appears to be quite variable. For instance, a high *Salmonella* occurrence (up to 75%) in gulls has been found in certain colonies of the western Mediterranean (Cerdà-Cuéllar, unpublished data). In contrast, recent studies report a moderate (13%-17%) to low (3%) *Salmonella* prevalence in other gull species in Australia and Europe (Palmgren *et al.*, 2006; Ramos *et al.*, 2010; Dolejska *et al.*, 2016; Masarikova *et al.*, 2016). The difference in *Salmonella* occurrence between kelp gulls and greater crested terns may be due to the different location of these bird colonies and their feeding habits. The greater crested tern's diet consists predominantly of pelagic fish, other marine prey and occasionally insects, whilst the kelp gull is a generalist that often scavenges at refuse dumps and sewage treatment plants (Hockey *et al.*, 2005; Gaglio *et al.*, 2017), which are commonly contaminated with pathogenic bacteria. Our results suggest that seabird feeding habits affects the incidence of *Salmonella* more than *Campylobacter*. We found the highest frequency of *Salmonella* at Plettenberg Bay, which is located close to a municipal refuse dump (Witteveen *et al.*, 2017) and a higher overall prevalence of *Salmonella* spp. than *Campylobacter* spp., especially in kelp gulls from urban areas, probably due to the increased presence and survival of *Salmonella* in gull breeding and feeding areas (Literák *et al.*, 1996). Also, the high survival rate of *Salmonella* in aquatic environments (Winfiel and Groisman, 2003) and the presence of this pathogen in surface waters (Levantesi *et al.*, 2012), may facilitate seabird infections. Contrary to *Salmonella*, *Campylobacter* is more susceptible to environmental stress (Murphy *et al.*, 2006) and its survival in the environment may be lower.

The wide variety of *Salmonella* serovars found in our study, particularly in urban kelp gull colonies, suggests a lack of host-specificity and the presence of different sources of *Salmonella* contamination. All the *Salmonella* serovars detected, except Corvallis and Paratyphi B var Java, previously have been found in South African farm animals, where the most common serovars are Typhimurium, Dublin, Enteritidis, Muenchen,

Heildelberg and Hadar (Magwedere *et al.*, 2015). Typhimurium, Enteritidis and Heidelberg are also the most frequently reported serovars in humans in South Africa (GERMS-SA, 2014). Anatum, Enteritidis and Hadar were the most frequent isolated serovars in our seabirds, all of them involved in salmonellosis outbreaks (Havelaar *et al.*, 2015). At the Strandfontein gull colony, located next to a major urban refuse dump, there was a high diversity of *Salmonella*, including the highest frequency of zoonotic serovars. This was also the case at the Plettenberg Bay gull colony, where we detected *S.* Paratyphi B var Java. This serovar, which is thought to be non-zoonotic, is very rare in South Africa (Smith *et al.*, 2016) but has been reported in stool samples from humans in the Western Cape in 2012 and the Eastern Cape in 2013 (GERMS-SA, 2013, 2014).

The high genetic diversity observed among *Campylobacter* isolates is common in this bacterium because of its genetic instability (Wassenaar *et al.*, 1998; Boer *et al.*, 2002). This genomic variability seems to be an important mechanism in environmental stress situations, generating population heterogeneity to improve fitness and survival in hostile environments and colonize other hosts (Ridley *et al.*, 2008). In our study, a higher genetic diversity was detected in *C. jejuni* isolates from kelp gulls than greater crested terns: 13 and seven PFGE profiles from 15 gulls and 15 terns, respectively. This greater *Campylobacter* genetic diversity in gulls may reflect different sources of infection probably due to their trophic plasticity. Even so, we detected a pulsotype in common in gulls from colonies located in semi-rural and urban areas, and two genetically close isolates from a greater crested tern and a kelp gull. This suggests a degree of connectivity among colonies and bird species, either directly or indirectly through a common contamination source.

Ten *S*. Anatum isolates (nine from gulls and one human clinical isolate) had identical XbaI-PFGE profiles, but none of them could be genotyped with the secondary restriction enzyme BlnI. This may be due to the lack of restriction sites or the presence of methylation patterns affecting the restriction enzyme recognition sites (Oyarzabal *et al.*, 2008). In *Salmonella*, genetic diversity depends largely on the serovar. Here, diversity

was greater among isolates of *S.* Typhimurium and *S.* Enteritidis, whereas clonality was frequent among *S.* Anatum and *S.* Hadar isolates. As for *Campylobacter*, the presence of pulsotypes of *Salmonella* in common to different colonies and seabird species indicates the likely transfer of strains among colonies or a common source of infection. On the other hand, the finding that 70% of human *Salmonella* isolates had pulsotypes the same or highly similar to those from bird isolates indicates an epidemiological link between them. Our results suggest that gulls breeding near urban areas can acquire human pathogenic *Salmonella* when they feed on anthropogenic food sources, including urban waste landfills or sewage, as has previously have been reported in other continents (Thorbjørn Refsum *et al.*, 2002; Palmgren *et al.*, 2006; Dolejska *et al.*, 2016; Hernandez *et al.*, 2016). Thus, gulls act as healthy carriers of strains involved in human disease and to some extent mirror *Salmonella* strains circulating in the humanised environment.

Despite around 70% of the isolates being pansusceptible, the notable frequency of antimicrobial resistant isolates detected is a cause of concern given that wild birds, unlike production animals, are not directly exposed to them. The main *Campylobacter* resistance detected was to quinolones and tetracycline (15%). Previous studies have reported high levels of tetracycline (22-100%) and quinolone (8-60%) resistant *Campylobacter* isolates in different production animals and farming systems in South Africa (Jonker and Picard, 2010; Bester and Essack, 2012). The broad use of tetracyclines in animal production systems in South Africa (Eagar *et al.*, 2012) may account for the high incidence of *Campylobacter* isolates resistant to this class of antimicrobial agents in livestock animals, which in turn may influence the resistance found in wildlife. To a lesser extent tetracyclines are also used in human medicine, although the drugs of choice as first line therapy for bacterial gastroenteritis are fluoroquinolones and macrolides. In recent years, there has been a dramatic increase in the proportion of *Campylobacter* clinical isolates in Cape Town resistant to nalidixic acid and ciprofloxacin (Lastovica, 2006). The simplicity of the resistance mechanism to quinolones and the

regular use of these agents may account for the rapid increase in *Campylobacter* resistant strains. The frequent use of enrofloxacin in animal production induces *Campylobacter* cross-resistance to other quinolones (Payot *et al.*, 2002; Takahashi *et al.*, 2005). *C. lari* strains isolated from kelp gulls and greater crested terns showed resistance to nalidixic acid, which is intrinsic in this *Campylobacter* species (Piddock *et al.*, 2003), and to ciprofloxacin; both resistance frequently are observed in the NARTC (nalidixic acid resistant thermophilic *Campylobacter*) biotype (Leatherbarrow *et al.*, 2007).

Even though *Campylobacter* and *Salmonella* share the same niche in birds, the use of quinolones in South Africa has not produced a similar resistance pattern among them. The main *Salmonella* resistance detected in our study were to tetracycline and streptomycin in kelp gulls and β-lactams in one greater crested tern. It is noteworthy that these drugs are used in human medicine (although tetracycline is not first choice). In gull colonies near urban areas, we found a greater number of *Salmonella* isolates resistant to a wide variety of antimicrobial drugs, including multidrug resistant strains, which may be indicative of anthropogenic pressure. Although greater crested terns feed on live prey captured mostly off shore, one isolate showed resistance to a β-lactam (ampicillin); this resistance is also found with some frequency in human clinical isolates (GERMS-SA, 2014). At Robben Island, greater crested terns often roost and breed in association to Hartlaub's gulls (*Chroicocephalus hartlaubi*) which are more commensal with humans than kelp gulls in the greater Cape Town area. Their interaction with this gull species may explain the ampicillin resistance found in terns.

In conclusion, our results suggest that kelp gulls and greater crested terns in the Western Cape of South Africa are a source of zoonotic thermophilic *Campylobacter* species and *Salmonella* serovars, with several *Salmonella* strains in common to both humans and gulls. Many isolates are resistant and multidrug-resistant (in the case of *Salmonella*) to antimicrobial agents, including critically important antimicrobials for human medicine (WHO-AGISAR, 2017). Our results highlight the importance of seabirds as reservoirs of *Campylobacter* and *Salmonella* resistant strains in the Western Cape

and their potential role in the maintenance and transmission of these bacteria in the environment, with significant implications for public health management. Further studies are needed to identify where these seabirds have acquired the zoonotic bacteria.

CHAPTER 4

Study II: Humans spread zoonotic enteric bacteria in Antarctica?

4.1. SUMMARY

Reports of enteric bacteria in Antarctic wildlife have suggested its spread from people to seabirds and seals, but evidence is scarce and fragmentary. We investigated the occurrence of zoonotic bacteria in poultry and seabirds across the Antarctic and Subantarctic region. Three findings suggest reverse zoonosis from humans to seabirds: the detection of a zoonotic *Salmonella* serovar Enteritidis and *Campylobacter* species (e.g. *C. jejuni*), typical of human infections; the resistance of *C. lari* isolates to ciprofloxacin and enrofloxacin, antibiotics commonly used in human and veterinary medicine; and most importantly, the presence of *C. jejuni* genotypes mostly found in humans and domestic animals. We also show further spread of zoonotic agents among Antarctic wildlife is facilitated by substantial connectivity among populations of opportunistic seabirds, notably skuas (*Stercorarius*). Our results highlight the need for even stricter biosecurity measures to limit human impacts in Antarctica.

4.2. INTRODUCTION

The global spread of pathogens is a growing conservation concern because their introduction into novel environments can have dramatic effects on wildlife (Van Riper *et al.*, 1986; Paxton *et al.*, 2016). Pathogens have been dispersed by migratory birds, fish, mammals and other taxa for millions of years, but in recent centuries humans have also contributed to their dispersal (Altizer *et al.*, 2011; Fuller *et al.*, 2012). Antarctica is the only continent where reverse zoonosis transmission has not been documented (Messenger *et al.*, 2014). Despite ongoing concern about human impacts in the region, diseases have not been identified as significant threats (Chown, Huiskes, *et al.*, 2012; Chown, Lee, *et al.*, 2012).

To date, the presence of pathogens in Antarctic wildlife has received limited attention (Barbosa and Palacios, 2009; Kerry and Riddle, 2009). It has been assumed that the region's isolation and relatively recent exploration by humans have protected Antarctic wildlife from novel pathogens, although there have been several outbreaks of infectious diseases at Southern Ocean islands (Weimerskirch, 2004; Cooper *et al.*, 2009; Kane *et al.*, 2012). The few surveys of pathogens in Antarctica have been opportunistic, and investigations of occasional mass mortality events to date have not established clear evidence of human-to-animal transmission (Gardner *et al.*, 1997; Frenot *et al.*, 2005; Iveson *et al.*, 2009; Kerry and Riddle, 2009; Vigo *et al.*, 2011; Hernandez *et al.*, 2012).

The mechanisms by which pathogens invaded the Southern Ocean wildlife remain uncertain. Human-mediated transport may be a legacy of exposure in the last few centuries to sealers and whalers or to their domestic animals (Gardner *et al.*, 1997; Griekspoor *et al.*, 2010), but several studies indicate that the main risk of pathogen invasion is the increase in tourism and research activities, which currently account for tens of thousands of visitors each year (Curry *et al.*, 2002; Hughes and Convey, 2010). In this regard, the Protocol on Environmental Protection to the Antarctic Treaty (1991), which came into force in 1996, included a number of measures to prevent the

introduction of novel pathogens (Committee for Environmental Protection, 2011). However, it may be of limited value if Antarctic wildlife disperses to areas outside the Antarctic region, where they can be exposed to a wide range of pathogens. Many Antarctic seabirds disperse across the Southern Ocean, coming into contact with domestic species in populated areas, and some species that visit the region during the Antarctic summer spend the winter in the Northern Hemisphere (e.g. Arctic Terns *Sterna paradisaea* and South Polar Skuas *Stercorarius maccormicki*). Such large-scale movements may introduce pathogens to Antarctica, and disperse them within the region. Climate change also may alter the migratory habits of animals, increasing the spread and contact between Antarctic, Subantarctic and temperate wildlife (Altizer *et al.*, 2013).

The zoonotic bacteria *Salmonella* spp. and thermotolerant *Campylobacter* spp. are amongst the most important foodborne diarrheal pathogens worldwide (Havelaar *et al.*, 2015). Both agents can spread rapidly in the environment through faecal contamination and can persist in soil or water for long enough to infect wild fauna. We explore the transfer of these zoonotic bacteria from humans and poultry to Subantarctic and Antarctic by sampling 24 seabird species over a broad geographical range, identifying bacterial species and comparing serovars and genotypes in seabirds with those commonly found in humans and domestic animals, and by testing their resistance to antibiotics commonly used in human and veterinary medicine. We also assess whether these pathogens are spreading across wildlife of the Southern Ocean.

4.3. MATERIAL AND METHODS

4.3.1. Sampling

From 2008 to 2011 we collected faecal samples from adult seabirds at four Southern Ocean localities: Livingston (Antarctica), Marion, Gough and the Falkland Islands (Figure

4.1.A; Table 4.1). Additionally, we also sampled backyard poultry at the Falklands, which support a permanent human settlement with a number of farms in close contact with Subantarctic and Antarctic wildlife. Birds were caught by hand and faecal samples were collected in duplicate using sterile swabs inserted into the cloaca. Samples were stored refrigerated in Amies transport medium with charcoal (Deltalab, Barcelona, Spain), transported to Spain within two to five weeks after the day of collection and cultured immediately upon arrival to the laboratory.

4.3.2. Bacterial isolation and identification

We performed *Salmonella* and *Campylobacter* isolation and identification by standard culture methods (Antilles *et al.*, 2015). *Salmonella* serotyping was performed according to the Kauffmann-White scheme (Grimont and Weill, 2007) and carried out at the Laboratori Agroalimentari (Cabrils, Spain) of the Departament d'Agricultura, Ramaderia, Pesca, Alimentació i Medi Natural. We identified *Campylobacter* isolates to species level by PCR using primers based on the *lpxA* gene (Klena *et al.*, 2004). A multiplex PCR for *C. jejuni* and *C. coli* identification was performed using forward primers lpxA-Cjejuni (5'-ACA ACT TGG TGA CGA TGT TGTA-3') and lpxA-Ccoli (5'-AGA CAA ATA AGA GAG AGA ATC AG-3') and a common reverse primer (lpxARKK2m: 5'-CAA TCA TGD GCD ATA TGA SAA TAH GCC AT-3'). *C. lari* identification was performed with a monoplex PCR using primers lpxA-Clari (5'-TRC CAA ATG TTA AAA TAG GCG A-3') and lpxARKK2m.

4.3.3. Antimicrobial susceptibility testing

We performed antimicrobial susceptibility testing for both *Salmonella* and *Campylobacter* isolates following the Clinical Laboratory and Standard Institute (CLSI) disc diffusion method (M100-S18) (CLSI, 2016) using Neo-Sensitabs™ (Rosco Diagnostica, Denmark) with CLSI potencies according to the manufacturer's instructions. For *Salmonella* isolates, we used Mueller-Hinton agar (Difco, Madrid, Spain) and plates were incubated at 37°C for 24 h. For *Campylobacter* isolates, we used

Mueller-Hinton II agar supplemented with 5% defibrinated sheep blood (BioMérieux, Marcy l'Etoile, France) and plates were incubated at 37°C for 48 h under microaerobic conditions.

Salmonella isolates were tested against 18 antimicrobials: ampicillin (33 µg), amoxicillin (30 µg), amoxicillin-clavulanate (30 + 15 µg), ceftiofur (30 µg), apramycin (40 µg), streptomycin (100 µg), gentamicin (40 µg), neomycin (120 µg), ciprofloxacin (10 µg), enrofloxacin (10 µg), nalidixic acid (130 µg), norfloxacin (10 µg), colistin (150 µg), chloramphenicol (60 µg), lincomycin-spectinomycin (15 + 200 µg), nitrofurantoin (260 µg), tetracycline (80 µg) and trimethoprim-sulfonamide (5.2 + 240 µg). *Campylobacter* isolates were tested against seven antimicrobials: nalidixic acid (30 µg), ciprofloxacin (5 µg), enrofloxacin (10 µg), tetracycline (80 µg), chloramphenicol (60 µg), erythromycin (15 µg) and gentamicin (10 µg).

4.3.4. *Salmonella* and *Campylobacter* genotyping

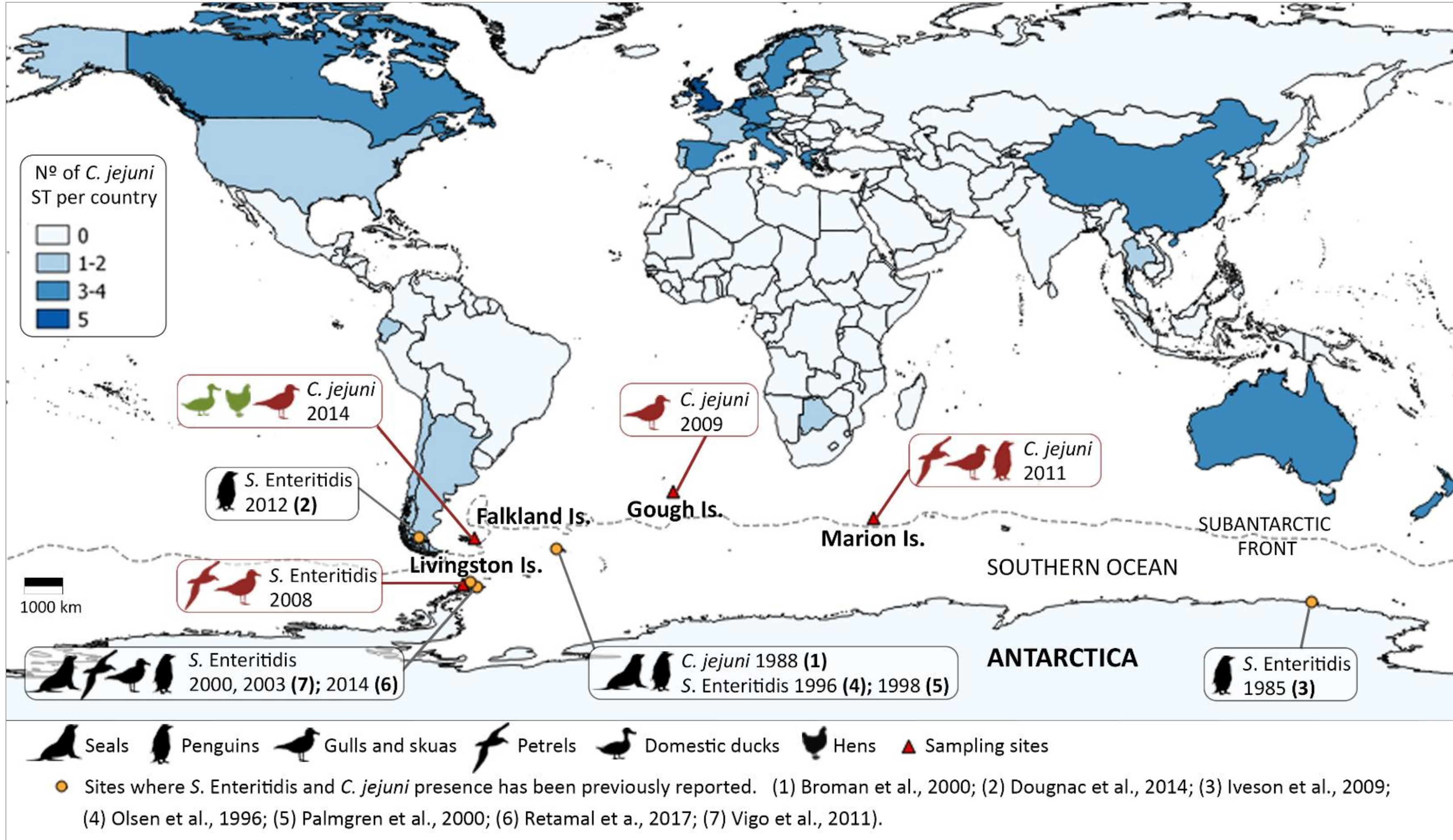
We typed representative bacterial isolates with pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). PFGE was carried out according to the standard operating procedure of PulseNet (www.pulsenetinternational.org). We performed restriction enzyme digests for PFGE with *Xba*I and *Bln*I enzymes for *Salmonella*, and with *Sma*I and *Kpn*I enzymes for *Campylobacter* (Roche Applied Science, Indianapolis, IN, USA). *Salmonella* Braenderup H9812 restricted with *Xba*I was used as molecular size standard for both *Campylobacter* and *Salmonella*. We analysed the resulting PFGE patterns using Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Banding patterns were compared with the UPGMA (Unweighted Pair Group Method with Arithmetic averages) clustering method using the Dice correlation coefficient with a band position tolerance of 1%.

We further characterized *S. enterica* and thermotolerant *Campylobacter* using MLST, which is based on sequencing of seven housekeeping genes (Dingle *et al.*, 2001; Miller

et al., 2005; Achtman *et al.*, 2012). Primers used for *Salmonella* were those described in the MLST public database (http://mlst.warwick.ac.uk/mlst) and those used for *Campylobacter* species are indicated in the corresponding MLST database (www.pubmlst.org/campylobacter) and in Miller *et al.* (2005). The sequence types were determined according to the scheme provided on these sites.

To explore potential spill-over from domestic to wild birds, we compared *C. jejuni* and *C. lari* isolates found in the present study with others from ducks and hens from Falkland Is., using PFGE and MLST.

Figure 4.1. *Salmonella* and *Campylobacter* findings in the Southern Ocean. A) Antarctic and Subantarctic sampling sites marked with red triangles. B) Distribution of *S.* Enteritidis and *C. jejuni* reported in the Southern Ocean. Boxes show animal host, year and zoonotic bacteria found in the Southern Ocean region (in red and green: information from wild and domestic birds, respectively, described in this study; in black: information from wild animals previously reported in other studies). C) Worldwide frequency distribution of *C. jejuni* ST reported in this study.



Nº of C. jejuni ST per country
0
1-2
3-4
5
C. jejuni 2014
C. jejuni 2009
C. jejuni 2011
S. Enteritidis 2012 (2)
Falkland Is.
Gough Is.
Marion Is.
SUBANTARCTIC FRONT
Livingston Is.
SOUTHERN OCEAN
S. Enteritidis 2008
1000 km
S. Enteritidis 2000, 2003 (7); 2014 (6)
C. jejuni 1988 (1)
S. Enteritidis 1996 (4); 1998 (5)
ANTARCTICA
S. Enteritidis 1985 (3)
Seals
Penguins
Gulls and skuas
Petrels
Domestic ducks
Hens
Sampling sites
Sites where S. Enteritidis and C. jejuni presence has been previously reported. (1) Broman et al., 2000; (2) Dougnac et al., 2014; (3) Iveson et al., 2009; (4) Olsen et al., 1996; (5) Palmgren et al., 2000; (6) Retamal et a., 2017; (7) Vigo et al., 2011).

Table 4.1. Sampled birds at the four studied localities and *Campylobacter* occurrence.

Seabirds	Livingston Is.	Gough Is.	Marion Is.	Falkland Is.
Order Sphenisciformes				
Adelie penguin (*Pygoscelis adeliae*)	2			
Chinstrap penguin (*Pygoscelis antarctica*)	21			
Gentoo penguin (*Pygoscelis papua*)	57 (1 Cl)[a]			113
King penguin (*Aptenodytes patagonicus*)			27 (1 Cj)	
Magellanic penguin (*Spheniscus magellanicus*)				35
Macaroni penguin (*Eudyptes chrysolophus*)			29 (1 Cj, 1 Cl)	
Northern rockhopper penguin (*Eudyptes moseleyi*)		24		
Southern rockhopper penguin (*Eudyptes chrysocome*)			23	54
Order Pelecaniformes				
Imperial shag (*Phalacrocorax atriceps*)				5
Order Charadriformes				
Kelp gull (*Larus dominicanus*)	17			
Brown skua (*Stercorarius antarcticus*)	13 (10 Cl)	15 (1 Cj, 10 Cl)	14 (6 Cj, 7 Cl)	25 (1 Cj, 3 Cl)

Continued

Table 4.1. Continued.

Seabirds	Livingston Is.	Gough Is.	Marion Is.	Falkland Is.
Order Procellariiformes				
Atlantic yellow-nosed albatross (*Thalassarche chlororhynchos*)		13		
Black-browed albatross (*Thalassarche melanophris*)				20
Sooty albatross (*Phoebetria fusca*)		5		
Atlantic petrel (*Pterodroma incerta*)		21		
Soft-plumaged petrel (*Pterodroma mollis*)		32		
Northern giant petrel (*Macronectes halli*)			16	
Southern giant petrel (*Macronectes giganteus*)	29 (1 Cl)	9	15 (2 Cl)	2
White-chinned petrel (*Procellaria aequinoctialis*)			1	
Broad-billed prion (*Pachyptila vittata*)		3		
Great shearwater (*Ardenna gravis)*		16		
Sooty shearwater (*Ardenna grisea*)				10
Total	139	138	125	264

a) Number of *Campylobacter* positive birds in brackets. Cj: *C. jejuni*, Cl: *C. lari*.

4.4. RESULTS

4.4.1. *Salmonella* and *Campylobacter* spp. in seabirds

We sampled 666 seabirds from 24 species at Livingston (n= 139), Gough (n= 138), Marion (n= 125) and the Falkland Islands (n= 264) (Figure 4.1.A; Table 4.1), and isolated three *Salmonella* serovar Enteritidis, 10 *C. jejuni* and 35 *C. lari*. The only other *Salmonella* serovar detected was one Oakey; no other thermotolerant *Campylobacter* species were found.

We isolated *Salmonella* Enteritidis from two kelp gulls and one southern giant petrel from Livingston Is.; *C. jejuni* from one macaroni penguin, one king penguin, and six brown skuas at Marion Is. and from single brown skuas at Gough and the Falkland Is. (Figure 4.1.B); and *C. lari* from one gentoo penguin, one southern giant petrel and 10 brown skuas at Livingston Is.; from one macaroni penguin, two southern giant petrels and seven brown skuas at Marion Is.; from 10 brown skuas at Gough Is.; and from three brown skuas at the Falkland Is. Marion Is. showed the highest diversity of positive seabird species to *Campylobacter* and was the only locality where co-infections occurred of both *C. jejuni* and *C. lari* (n=3 skuas).

4.4.2. Antimicrobial resistance

We did not detect any antimicrobial resistance in isolates of *Salmonella* or *C. jejuni*. Among *C. lari* isolates, besides nalidixic acid resistance which is characteristic of this species and was found in all tested isolates, we found ciprofloxacin resistance in isolates from one macaroni penguin and two Subantarctic skuas from Marion Is., and from three skuas from Gough Is. Ciprofloxacin and enrofloxacin resistance was detected in two *C. lari* from skuas at Livingston Is.

4.4.3. Genetic diversity

All three *Salmonella* Enteritidis isolates exhibited identical PFGE patterns and MLST sequence type (ST-11). Among *C. jejuni* isolates, PFGE analysis clustered together three isolates: two from brown skuas from the Falklands and Marion Is. and one from a domestic duck from the Falklands. MLST showed these isolates to belong to the widespread ST-45. Four other *C. jejuni* ST (ST-137, ST-227, ST-696 and ST-883) were isolated from skuas and penguins at Gough and Marion Is. (Figure 4.2). These ST have been mostly reported in several hosts in developed countries of the Northern Hemisphere, Australia and New Zealand (Figure 4.1.C).

Among *C. lari* isolates**,** PFGE genotyping showed highly similar isolates (> 80% similarity) from several skuas at Livingston, Marion and Gough Is. and from a giant petrel at Marion Is. One cluster was formed by three (GH128-C1, GH131-C1 and MAR5-C1) nearly identical isolates (≥ 95% similarity) found in skuas from Gough and Marion Is. belonging to the same novel ST (Figure 4.3). In addition, the same genotype was found in two different seabird species, a brown skua and a gentoo penguin from Livingston Is. (isolates AN138-C7 and AN32-C1), which were closely related (81% similarity) to an isolate from a duck (FK72-C1) from the Falklands. One cluster grouped isolates from distant localities, i.e. one isolate from a skua at the Falklands and one from a penguin at Marion Is. (FK54-C1 and MAR18-C1), with an 88% similarity.

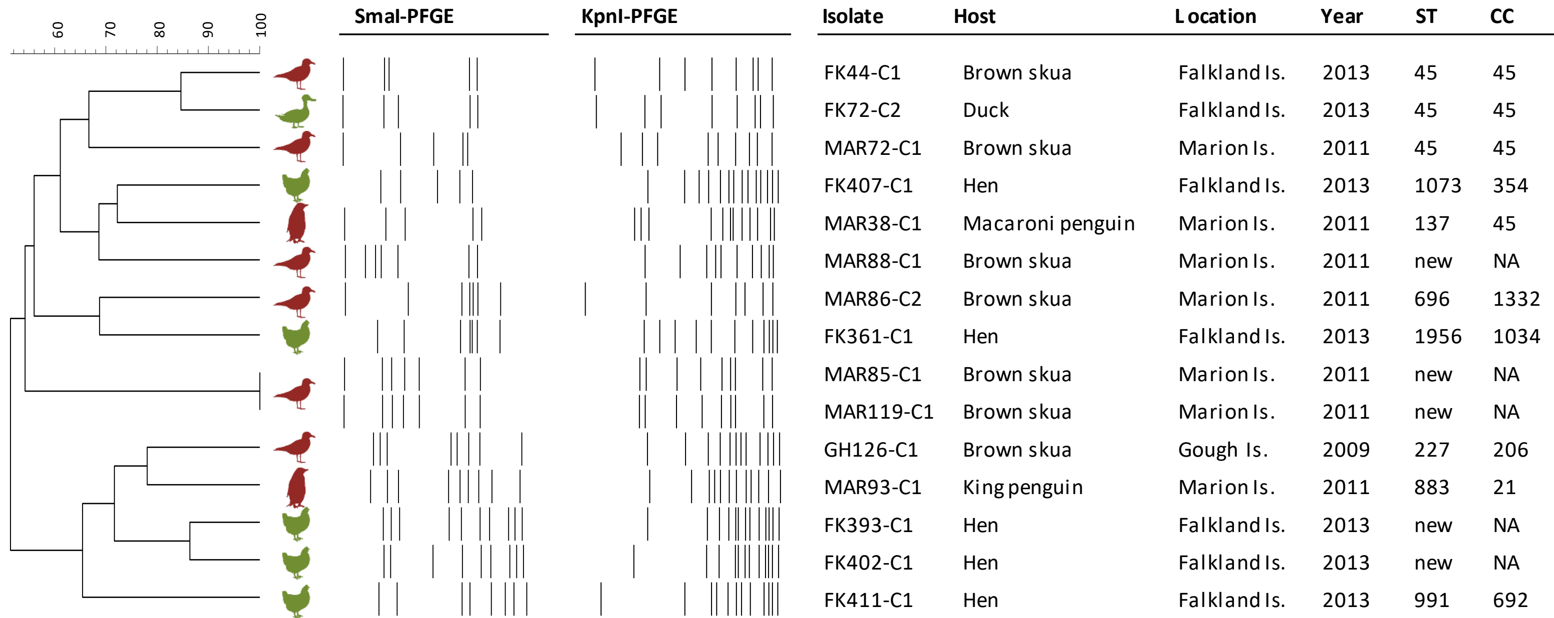


Figure 4.2. PFGE combined dendrogram of SmaI and KpnI profiles of *C. jejuni* isolates from wild (in red) and domestic (in green) birds.

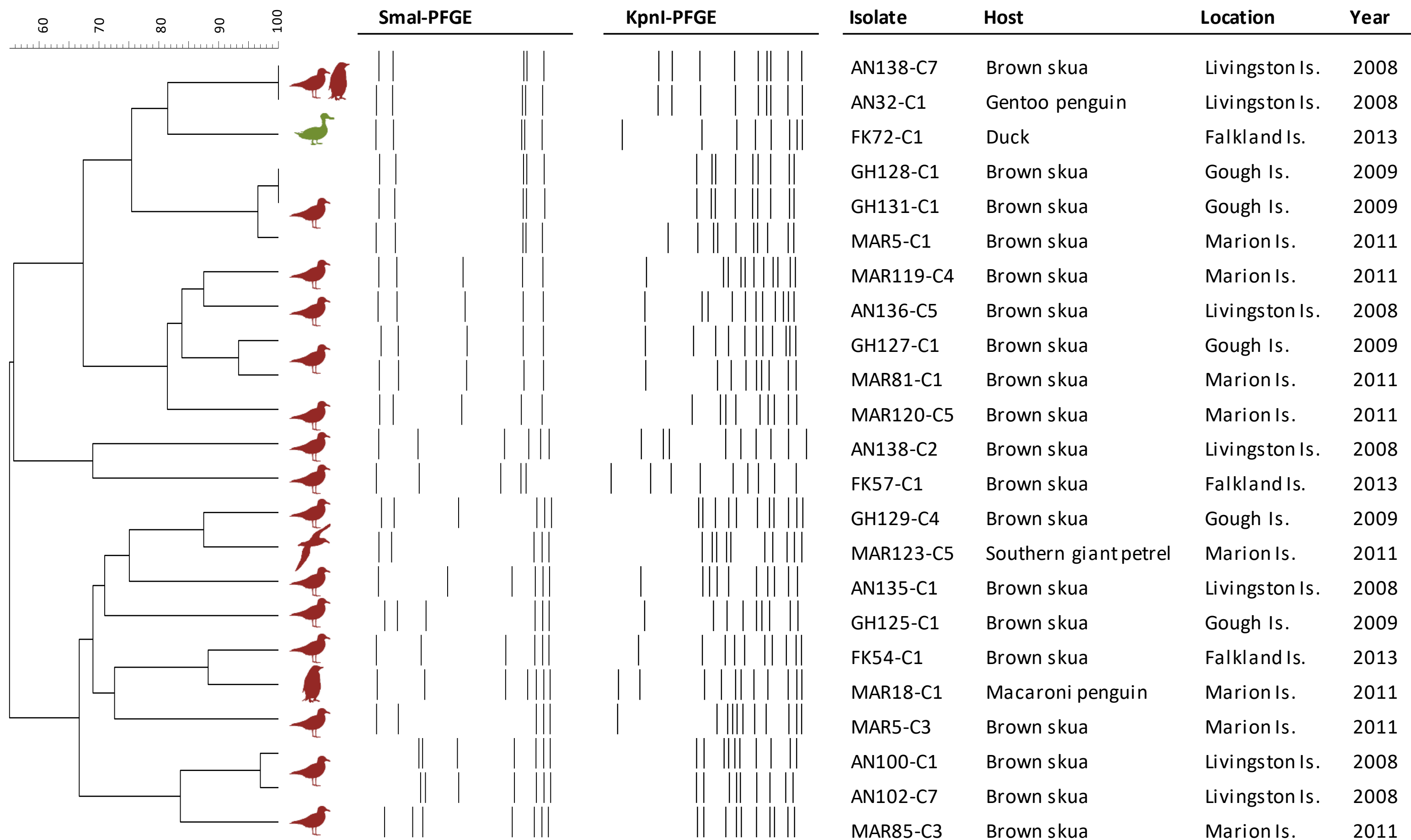


Figure 4.3. PFGE combined dendrogram of SmaI and KpnI profiles of *C. lari* isolates from wild (in red) and domestic (in green) birds.

4.5. DISCUSSION

Three lines of evidence strongly suggest a reverse zoonosis in Antarctica, whereby zoonotic enteric bacteria have been introduced by humans to Southern Ocean ecosystems: the detection in seabirds of *Salmonella* serovars (e.g. Enteritidis) or *Campylobacter* species (e.g. *C. jejuni*) typically associated with humans (Figure 4.1.B), the antibiotic resistance of some strains, and most importantly, the occurrence of several *Campylobacter* genotypes (ST-45, ST-137, ST-227, ST-696 and ST-883) previously reported almost exclusively in humans and domestic animals from developed countries. *Salmonella* was only isolated from a few seabirds at Livingston Is. (Antarctic Peninsula), suggesting *Salmonella* is not indigenous to seabirds in the region. *Salmonella* Enteritidis serovar is, together with Typhimurium, the most common serovar causing salmonellosis in humans worldwide (Hendriksen *et al.*, 2011). Our results agree with the scarcity of *Salmonella* isolates previously reported in seabirds and mammals of the Southern Ocean, which mainly belong to serovars commonly found in humans (Figure 4.1B) (Olsen *et al.*, 1996; Palmgren *et al.*, 2000; Iveson *et al.*, 2009; Vigo *et al.*, 2011; Dougnac *et al.*, 2015; Retamal *et al.*, 2017). The *Salmonella* serovar found typically occurs in scavenging birds associated with urban areas, such as gulls and raptors, and is relatively uncommon in wildlife from less transformed areas (Ĉîẑek *et al.*, 1994; Ramos *et al.*, 2010; Jurado-Tarifa *et al.*, 2016). All our *Salmonella* isolates had the same PFGE macrorestriction profile and the same MLST type (ST-11), which has also been reported from seabirds and seals in the Antarctic Peninsula (Vigo *et al.*, 2011), and it is the most abundant and widespread ST of *S*. Enteritidis worldwide, further suggesting the clonal spread of this serovar from other continents to Antarctica.

We found thermophilic *Campylobacter* species in all sampled localities, mainly *C. lari*, but also *C. jejuni*, which is a major cause of foodborne diarrhoeal illness in humans worldwide (Havelaar *et al.*, 2015). *C. jejuni* has been isolated only once in penguins from the same colony (3/100; 3/446 of all sampled birds) in the broader Antarctic region, at South Georgia (Broman *et al.*, 2000). In non-remote areas, prevalence of this

Campylobacter species from scavenging seabirds has been reported at much higher rates (Kapperud and Rosef, 1983; Keller *et al.*, 2011; authors, unpublished data). We found *C. jejuni* mainly in brown skuas, one of the main opportunistic seabird species of the Southern Ocean. When given the chance, skuas often scavenge on human waste, providing a plausible mechanism for the transfer of *C. jejuni* to this species.

Antimicrobial resistance was generally low, but the presence of at least certain resistance is worrying given that they were found in some of the most remote areas on Earth. A few *C. jejuni* and *C. lari* isolates from poultry at the Falklands (authors, unpublished data) and some *C. lari* isolates from a macaroni penguin and skuas from three islands were resistant to fluoroquinolones (ciprofloxacin, enrofloxacin). These agents belong to the so-called critically important antimicrobials and are therefore seldom used in human or veterinary medicine (WHO AGISAR, 2017). As a result, the development of resistance in backyard poultry or wild seabird populations is very unlikely, strongly suggesting contamination by a resistant strain of anthropogenic origin. Interestingly, the domestic duck which carried a *C. lari* resistant isolate was free ranging most of the day, a practice that may facilitate transmission between the domestic and the wildlife compartments. Resistance also may have developed through spontaneous mutation, acquired by horizontal gene transfer from other microorganisms that constitute natural sources of drug-resistant genes, or may have been imported into the Southern Ocean through bird migration. However, the detection in skuas of several *C. jejuni* genotypes almost exclusively found in humans and livestock supports the likelihood of reverse zoonosis. MLST analysis showed some strains from skuas from Marion Is. to belong to new STs. They could represent host specific strains or strains endemic of the Southern Ocean. However, several other genotypes belonged to ST almost exclusively associated with human disease and asymptomatic infection in livestock (ST-45, ST-137, ST-227, ST-696 and ST-883) from northern developed countries, strongly supporting their human origin. At Gough and Marion Is., introduction likely occurred through personnel based at the South African scientific stations, despite

strict biosecurity controls for more than two decades. The introduction of these human-associated strains to these remote islands by migrating birds infected during migrating movements cannot be ruled out, but seems less plausible.

The case of the Falkland Is. is particularly relevant, since ST-45 was isolated from a skua and a domestic duck. This ST is very common in humans and livestock but has only been reported once in a single bird in the Southern Ocean in a remote site of the Subantarctic region (Olsen *et al.*, 1996; Griekspoor *et al.*, 2010), suggesting movement from the domestic to the wildlife compartment. Inhabited areas close to the Antarctic region with free-ranging livestock, such as Patagonia, the Falklands and Tristan da Cunha, are of particular concern, since in these localities domestic animals come in close contact with Antarctic wildlife, potentially facilitating the spread of infectious diseases. Many Antarctic birds and mammals regularly visit these areas or mix with the local fauna in common wintering grounds (Shirihai, 2007).

It is also plausible that zoonotic enteric bacteria and other pathogens can spread and circulate through wildlife across the Southern Ocean. *C. lari*, the most abundant *Campylobacter* species recovered at all four sites, has been reported previously in Southern Ocean penguins, gulls, skuas and seals (Bonnedahl *et al.*, 2005; Leotta *et al.*, 2006; García-Peña *et al.*, 2010, 2017). The widespread distribution of *C. lari* among host species and localities and its high genetic diversity suggest that it has long been circulating in the region. The genetic similarities among isolates from skuas, penguins and gulls in our study also suggest substantial connectivity across Southern Ocean localities and therefore potential for spreading new pathogens.

Our results provide compelling evidence for reverse zoonosis of pathogens in Antarctica and suggest that zoonotic enteric bacteria can be spread by wildlife across the Southern Ocean. The increasing spread of pathogens, underpinned by globalization and climate change, now affects the most remote areas on Earth. Strict measures to limit human

impacts in Antarctica (Chown, Huiskes, *et al.*, 2012; Chown, Lee, *et al.*, 2012) should be expanded to zoonotic bacteria and to settled areas in the peri-Antarctic region.

CHAPTER 5

Study III: Genetic diversity, population structure and virulence potential of *Campylobacter* and *Salmonella* spp. from Southern Ocean seabirds

5.1. SUMMARY

Seabirds can be carriers of zoonotic agents such as *Campylobacter* and *Salmonella* spp., which are the most reported cause of bacterial foodborne illnesses to humans worldwide. Foraging and migrating movements of seabirds can contribute to the dispersal of these pathogens. There is little information on the genetic diversity, population structure and pathogenic potential of these agents of seabird origin, especially in remote regions. We studied 77 *Campylobacter* (50 *C. lari*, 26 *C. jejuni* and one *C. coli*) and 23 *Salmonella* spp. isolates from seabirds from several Southern Ocean localities and the South African coast. The isolates were typed by pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) and the presence of virulence-associated genes were investigated. A high genetic diversity and new sequence types (ST) were observed in the *C. lari* population, although some common genotypes were found among different bird species and islands. Most *C. jejuni* STs from South African seabirds belonged to clonal complex CC-1275, which is mainly found in aquatic environments and wild birds. Conversely, CC-45, CC-21 and CC-206, associated with domestic animals and human infections, were often detected in *C. jejuni* isolates from Southern Ocean seabirds. These seabirds could have acquired these strains during their northward migration, although it is more likely that infection has occurred locally due to the introduction by human activities in those remote localities. We also found *Salmonella* serovar Enteritidis ST-11 and *S*. Typhimurium ST-34, among others; these genotypes have a worldwide distribution and a broad-range of hosts. Many isolates of *C. jejuni* and different *Salmonella* serovars presented multiple genes involved in pathogenicity. Our results revealed that seabirds from remote regions of the Southern Ocean and from South Africa can be carriers of genotypes of livestock and human origin and show an important virulence potential.

5.2. INTRODUCTION

Wild birds can be carriers of human pathogens such as *Campylobacter* and *Salmonella* spp. which are major bacterial causes of gastroenteritis worldwide (Havelaar *et al.*, 2015). Laridae are amongst the wild birds most frequently reported to be infected with these zoonotic agents in high levels (Ramos *et al.*, 2010; Antilles, 2014; Dolejska *et al.*, 2016; Masarikova *et al.*, 2016; Migura-Garcia *et al.*, 2017). The habitats of these marine birds often overlap with human activities and their scavenging feeding habits have been associated with an increased risk of pathogen infection.

Campylobacter and *Salmonella* spp. have been found in seabirds from Western Cape province of South Africa, with some *Salmonella* strains related to those causing human salmonellosis in Cape Town hospitals (Moré *et al.*, 2017). These pathogens have also been found in seabirds from pristine environments in remote areas. Some surveys have reported the presence of *C. lari* and *S. enterica* subsp. *enterica* serovar Enteritidis in penguins, skuas, gulls and petrels from Antarctic regions and circumpolar islands (Palmgren *et al.*, 2000; Bonnedahl *et al.*, 2005; Leotta *et al.*, 2006; Iveson *et al.*, 2009; Vigo *et al.*, 2011; García-Peña *et al.*, 2017; Chapter 4). *C. jejuni* has seldom been detected in penguins and brown skuas (*Stercorarius antarcticus*), one of the main opportunistic seabird species of the Southern Ocean (Broman *et al.*, 2000; Chapter 4).

The remoteness of Antarctica and the Subantarctic, and the recent arrival of humans to the region, has not prevented the spread of novel pathogens to these areas. The increase in human presence, due to scientific research and tourism, may be the main reason why these pathogens invaded Southern Ocean wildlife (Curry *et al.*, 2002; Smith and Riddle, 2009). On the other hand, many seabirds come into contact with non-native wildlife, domestic animals and humans outside the region, specifically in stopover sites and wintering areas during their seasonal migrations. For instance, each year brown skuas leave the Southern Ocean to spend the winter months in mid– to low-latitudes, often reaching the coasts of Africa, South America and Australia; many south polar skuas

(*Stercorarius maccormicki*) cross the equator to winter in the Northern Hemisphere (Weimerskirch *et al.*, 2015; Delord *et al.*, 2018). Moreover, climate change may have contributed to an increase in pathogen dispersal by altering the migratory routes of wild birds among Antarctic, Subantarctic and temperate regions and by allowing pathogens to adapt to new environments (Altizer *et al.*, 2013).

Many pelagic seabirds of the Southern Ocean migrate to the waters off South Africa in the austral winter to feed in the nutrient-rich waters of the Benguela upwelling system, where they come into contact with local seabirds (Crawford *et al.*, 1991). Therefore, the same or closely related *Campylobacter* and *Salmonella* strains found in seabirds from the Southern Ocean could be found in seabirds from the Western Cape of South Africa. Using a robust method such as multi-locus sequence typing (MLST) it is possible to establish the genetic relatedness among these bacterial strains and to compare them with those circulating at a global scale (Urwin and Maiden, 2003). This molecular analysis can contribute to a better understanding of the global epidemiology of these zoonotic agents.

Some *Campylobacter* and *Salmonella* spp. are generalists, widely distributed and commonly related to human gastroenteritis (e.g. *C. jejuni*, *C. coli*; *Salmonella* serovars Enteritidis and Typhimurium), while others are more adapted to certain hosts and only occasionally cause human infections (e.g. *C. lari* in seabirds and marine mammals, *S. enterica* subsp. *salamae* in reptiles) (Abbott *et al.*, 2012; Miller *et al.*, 2014; Havelaar *et al.*, 2015). The ability to cause infection in a particular host is mainly determined by the virulence potential of the bacterial strain, in addition to host susceptibility. Virulence factors involved in motility, adhesion, colonization, invasion and survival within host cells are encoded in chromosomal and plasmid genes that can be conserved or specific at species, serovar or genotype level, and can be transferred between them by mobile genetic elements (Foley *et al.*, 2013; Bolton, 2015).

In this study, we investigated the genetic diversity, population structure and potential pathogenicity of *Campylobacter* and *Salmonella* spp. isolates carried by seabirds from remote Antarctic and Subantarctic islands, and from seabirds from the Western Cape coast of South Africa where they breed close to human settlements within the Benguela Upwelling Region.

5.3. MATERIALS AND METHODS

5.3.1. Bacterial isolates

Campylobacter and *Salmonella* spp. isolates were recovered from stock cultures stored at −80°C in cryovials containing Brain Heart Infusion broth (BHI; Merck KGaA, Darmstadt, Germany) supplemented with 20% glycerol. These isolates had been recovered from cloacal swabs of seabird species sampled at various locations during 2008-2014 (Moré *et al.*, 2017; Chapter 3) (Figure 5.1.). Overall, 110 *Campylobacter* (*C. lari*, *C. jejuni* and *C. coli*) and 64 *Salmonella* isolates were analysed: 33 *Campylobacter* isolates from kelp gulls (*Larus dominicanus*) and greater crested terns (*Thalasseus bergii*) from the Western Cape (South Africa) and 77 *Campylobacter* isolates from kelp gulls, brown skuas, southern giant petrels (*Macronectes giganteus*), gentoo penguins (*Pygoscelis papua*), king penguins (*Aptenodytes patagonicus*) and macaroni penguins (*Eudyptes chrysolophus*) from Southern Ocean islands (Falkland Is., Livingston Is., Gough Is. and Marion Is.); *Salmonella* isolates included 64 *Salmonella enterica* (two subsp. *salamae* and 62 subsp. *enterica* of different serovars) from kelp gulls and greater crested terns from the Western Cape (South Africa) and kelp gulls and a southern giant petrel from Livingston Is. For comparison purposes, isolates from domestic ducks (*Anas platyrhynchos*) and hens (*Gallus gallus*) from the Falkland Is. were also included in the analyses (Chapter 4). Fresh cultures of *Campylobacter* isolates were obtained by streaking a loop of the frozen stock cultures onto blood agar plates (BioMérieux, Marcy

l'Etoile, France); plates were incubated at 37°C for 48 h under a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2; Anaerocult©, Merck, Darmstadt, Germany). *Salmonella* isolates were cultured onto Trypticase Soy Agar (TSA; Difco, Madrid, Spain) at 37°C for 24 h in aerobic conditions.

5.3.2. Genotyping

Campylobacter and *Campylobacter* and *Salmonella* isolates had previously been genotyped by pulsed-field gel electrophoresis (PFGE) using the restriction enzymes SmaI and KpnI, and XbaI and BlnI, respectively, according to the PulseNet protocols (Moré *et al.*, 2017; Chapter 3). We performed a comparison analysis of all PFGE profiles using Fingerprinting II v3.0 software. Similarity matrices were calculated by the Dice coefficient (tolerance and optimization values of 1.0%) and dendrograms were constructed using the UPGMA method. Isolates with a similarity ≥ 90% were considered as the same pulsotype (referred to as SK for *Campylobacter* and XB for *Salmonella*). A set of isolates with representative pulsotypes from the different bird species and regions were selected for further analysis.

We characterized by MLST a selection of 77 *Campylobacter* isolates (41 *C. lari*, 35 *C. jejuni* and one *C. coli*) and 23 *Salmonella enterica* isolates (two subsp. *salamae* and 21 subsp. *enterica* serovars Anatum, Bovismorbificans, Enteritidis, Hadar, Manhattan, Muenchen, Saintpaul, Typhimurium and Paratatyphi B var Java). For *Campylobacter*, PCR amplification and sequencing of fragments of seven housekeeping genes (*aspA, glnA, gltA, glyA, pgm tkt* and *uncA/atpA*) were carried out using the primer set described by Miller et al. (2005). We required alternative primers to amplify and sequence *tkt* and *pgm* genes of some isolates (Korczak *et al.*, 2009). In the case of the *C. lari* MLST scheme, *aspA* and *gltA* genes were substituted by *adk* and *pgi* (Miller *et al.*, 2005). Primers employed to obtain sequences of *Salmonella* loci (*aroC, dnaN, hemD, hisD, purE, sucA* and *thrA*) were those recommended in the *Salmonella enterica* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). We used InstaGene® Matrix (Bio-Rad,

Hercules, CA, USA) for DNA template extraction and the ultrafiltration kit NucleoFast® 96 PCR (Macherey-Nagel, Düren, Germany) for cleaning PCR products, according to the manufacturer's instructions. Sanger sequence data were analysed by Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Alleles and sequence types (STs) were assigned based on the MLST scheme provided on the *Campylobacter* PubMLST databases (http://pubmlst.org/campylobacter), as well as on the *Salmonella enterica* MLST database. Novel alleles and STs of *Campylobacter* were submitted to the database mentioned above. To represent the relationship among *Campylobacter* isolates, we generated a complete minimum spanning tree (MST) using the geoBURST algorithm from the PHYLOViZ 2.0 software package (Nascimento *et al.*, 2017). For *Salmonella* isolates, the phylogenetic analysis was conducted with MEGA 7.0 software (Kumar *et al.*, 2016) using the maximum likelihood method based on the concatenated MLST loci.

The 16S rDNA sequencing of two *C. lari* isolates showing an allelic profile of seven exclusive loci was performed to confirm species identity. Primers and PCR conditions followed those described by Lane (1991). Sequences were analysed using BioEdit v7.0.5 software and compared with those from the National Center for Biotechnology Information (NCBI) Database.

5.3.3. Virulence-associated genes

The presence of virulence-associated genes was studied in those isolates characterized by MLST. For *Campylobacter* isolates, we analysed 14 virulence genes involved in cell adhesion and colonization (*flaA, flaB, cadF, dnaJ* and *racR*), invasion (*hcp, pdlA, virB, ciaB* and *ceuE*) and toxin production (*cdtA, cdtB, cdtC* and *wlaN*). For *Salmonella*, we investigated 27 virulence genes, involved in adhesion, colonization, invasion, iron uptake and intracellular survival and replication, located in the *Salmonella* pathogenicity island (SPI)-1 (*orgA, sitC, invA, prgH* and *avrA*), SPI-2 (*spiC*), SPI-3 (*misL, mgtC*), SPI-4 (*orfL*), SPI-5 (*pipD* and *sopB*), pathogenicity islets (*tolC, iroN, sifA* and *cdtB*),

fimbrial operons (*agfA, agfC, lpfA, lpfC* and *sefC*), plasmids (*spvB, spvC* and *pefA*) and prophages (*sopE, gipA*, *gogB* and *gtgB*).

For both bacteria, PCRs were performed in a 25 µL reaction mixture that included 12.5 µL of a ready-to-use PCR Master Mix solution (Promega, Wisconsin, USA), 0.4 µM of each primer and 2.5 µL of DNA. Amplification cycles were the following: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing temperature corresponding to each gene for 30 s, and extension at 72°C for 2 min; final extension at 72°C for 10 min. Details of the primers employed alone or in multiplex PCRs and annealing temperatures are listed in Table 5.1. Amplified products were separated out by electrophoresis in a 1.8% agarose gel in 1x TAE buffer with 0.2 µg/ml of ethidium bromide and viewed under UV light to determine the presence of a PCR product. Isolates with the same combination of virulence-associated genes were considered to have the same virulotype (VT).

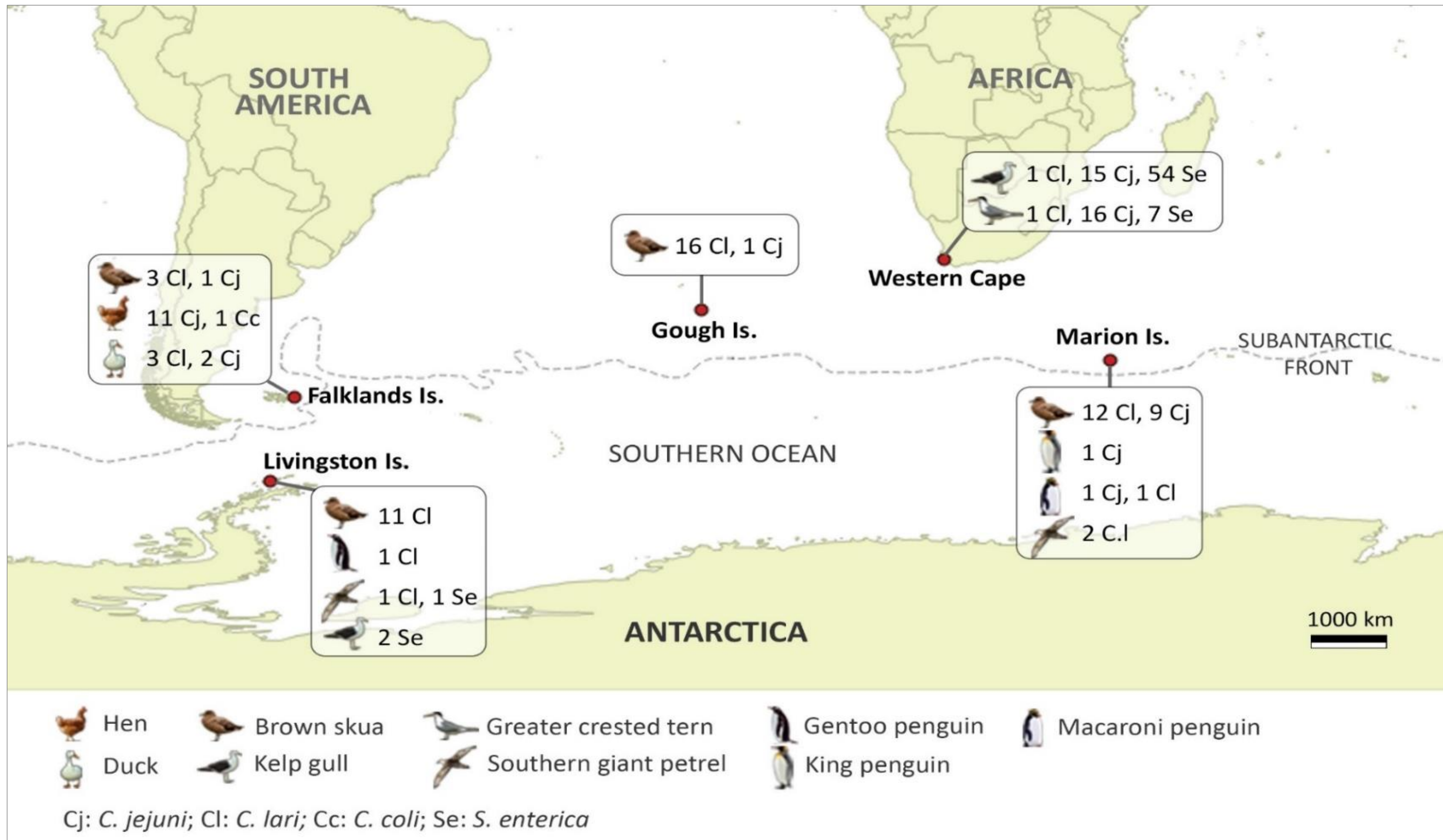


Figure 5.1. Locations and host origin of *Campylobacter* and *Salmonella* isolates from birds sampled in the Southern Ocean.

Table 5.1. PCR primers used for *Campylobacter* and *Salmonella* virulence-associated gene detection.

Gene	Sequence forward / reverse primers (5' to 3')	PCR	Annealing T (°C)	Amplicon size (bp)	Reference
Campylobacter					
cadF	TTGAAGGTAATTTAGATATG / CTAATACCTAAAGTTGAAAC	monoplex	55	400	Konkel et al., 1999
cdtA	CCTTGTGATGCAAGCAATC / ACACTCCATTTGCTTTCTG	monoplex	55	370	Hickey et al., 2000
cdtB	CAGAAAGCAAATGGAGTGTT / AGCTAAAAGCGGTGGAGTAT	monoplex	55	620	Datta et al., 2003
cdtC	CGATGAGTTAAAACAAAAAGATA / TTGGCATTATAGAAAATACAGTT	monoplex	55	182	Datta et al., 2003
ceuE (Cc) [a]	CCTGCTCGGTGAAAGTTTTG / GATCTTTTTGTTTTGTGCTGC	monoplex	50	794	Bang et al., 2003
ceuE (Cj)	ATGAAAAAAATATTTAGTTTTTGCA / ATTTTATTATTTGTAGCAGCG	monoplex	55	894	Bang et al., 2003
ciaB	TTTTTATCAGTCCTTA / TTTCGGTATCATTAGC	monoplex	45	986	Datta et al., 2003
dnaJ	AAGGCTTTGGCTCATC / CTTTTTGTTCATCGTT	monoplex	40	720	Datta et al., 2003
flaA	AATAAAAATGCTGATAAAACAGGTG / TACCGAACCAATGTCTGCTCTGATT	monoplex	55	855	Datta et al., 2003
flaB	AAGGATTTAAAATGGGTTTTAGAATAAACACC / GCTCATCCATAGCTTTATCTGC	monoplex	55	260	Goon et al., 2003
hcp	CAAGCGGTGCATCTACTGAA / TAAGCTTGCCCTCTCTCCA	monoplex	56	463	Harrison et al., 2014
pldA	AAGCTTATGCGTTTTT / TATAAGGCTTTCTCCA	monoplex	53	913	Datta et al., 2003
racR	GATGATCCTGACTTTG / TCTCCTATTTTTACCC	monoplex	40	584	Datta et al., 2003
virB11	TCTTGTGAGTTGCCTTACCCCTTTT / CCTGCGTGTCCTGTGTTATTTACCC	monoplex	45	494	Datta et al., 2003
wlaN	TTAAGAGCAAGATATGAAGGTG / CCATTTGAATTGATATTTTTG	monoplex	53	672	Linton et al., 2000
Salmonella					
agfA	TGCAAAGCGATGCCCGTAAATC / TTAGCGTTCCACTGGTCGATGGTG	multiplex V	56	151	Bäumler et al., 1997
agfC	CTTTATTGCTCCTTGCCGC / GAAGGCGGCCATTGTTGTGA	multiplex IV	56	310	Gibson et al., 2007
avrA	CCTGTATTGTTGAGCGTCTGG / AGAAGAGCTTCGTTGAATGTCC	multiplex V	56	425	Huehn et al., 2010
cdtB	ACAACTGTCGCATCTCGCCCCGTCATT / CAATTTGCGTGGGTTCTGTAGGTGCGAGT	monoplex	56	268	Skyberg et al., 2006
gipA	GCAAGCTGTACATGGCAAAG / GGTATCGGTGACGAACAAAT	multiplex VII	56	212	Mikasova et al., 2005

Continued

Table 5.1. Continued.

Gene	Sequence forward / reverse primers (5' to 3')	PCR	Annealing T (°C)	Amplicon size (bp)	Reference
Salmonella					
gogB	GCTCATCATGTTACCTCTAT / AGGTTGGTATTTCCCATGCA	multiplex VII	56	598	Drahovska et al., 2007
gtgB	TGCACGGGGAAAACTACTTC / TGATGGGCTGAAACATCAAA	multiplex VII	56	436	Mikasova et al., 2005
invA	CTGGCGGTGGGTTTTGTTGTCTTCTCTATT / AGTTTCTCCCCCTCTTCATGCGTTACCC	monoplex	56	1070	Skyberg et al., 2006
iroN	ACTGGCACGGCTCGCTGTCGCTCTAT / CGCTTTACCGCCGTTCTGCCACTGC	multiplex IV	56	1205	Skyberg et al., 2006
lpfA	TTGCTCTGTCTGCTCTCGCTGTAG / CATGATTCTCTTCCTGAGCCTCC	multiplex II	56	250	Bäumler et al., 1995
lpfC	GCCCCGCCTGAAGCCTGTGTTGC / AGGTCGCCGCTGTTTGAGGTTGGATA	multiplex V	56	641	Skyberg et al., 2006
mgtC	TGACTATCAATGCTCCAGTGAAT / ATTTACTGGCCGCTATGCTGTTG	multiplex IV	56	655	Soto et al., 2006
misL	GTCGGCGAATGCCGCGAATA / GCGCTGTTAACGCTAATAGT	monoplex	56	561	Hughes et al., 2008
orfL	GGAGTATCGATAAAGATGTT / GCGCGTAACGTCAGAATCAA	monoplex	56	332	Hughes et al., 2008
orgA	TTTTTGGCAATGCATCAGGGAACA / GGCGAAAGCGGGGACGGTATT	multiplex VI	56	255	Skyberg et al., 2006
pefA	GCGCCGCTCAGCCGAACCAG / GCAGCAGAAGCCCAGGAAACAGTG	multiplex III	56	157	Skyberg et al., 2006
pipD	CGGCGATTCATGACTTTGAT / CGTTATCATTCGGATCGTAA	multiplex II	56	399	Hughes et al., 2008
prgH	GCCCGAGCAGCCTGAGAAGTTAGAAA / TGAAATGAGCGCCCCTTGAGCCAGTC	multiplex VI	56	657	Skyberg et al., 2006
sefC	GCGAAAACCAATGCGACTGTAG / CCCACCAGAAACATTCATCCC	monoplex	56	1103	Bäumler et al., 1997
sifA	TTTGCCGAACGCGCCCCCACACG / GTTGCCTTTTCTTGCGCTTTCCACCCATCT	multiplex III	56	449	Skyberg et al., 2006
sitC	CAGTATATGCTCAACGCGATGTGGGTCTCC / CGGGGCGAAAATAAAGGCTGTGATGAAC	multiplex II	56	768	Skyberg et al., 2006
sopB	CGGACCGGCCAGCAACAAAACAAGAAGAAG / TAGTGATGCCCGTTATGCGTGAGTGTATT	multiplex III	56	220	Skyberg et al., 2006
sopE	TCAGTTGGAATTGCTGTGGA / TCCAAAAACAGGAAACCACAC	monoplex	56	642	Hughes et al. 2008
spiC	CCTGGATAATGACTATTGAT / AGTTTATGGTGATTGCGTAT	monoplex	56	301	Hughes et al., 2008
spvB	CTATCAGCCCCGCACGGAGAGCAGTTTTTA / GGAGGAGGCGGTGGCGGTGGCATCATA	multiplex I	56	717	Skyberg et al., 2006
spvC	CTTGCACAACCAAATGCGGAAGAT / CTCTGCATTTCACCACCATCACG	multiplex I	56	571	Agron et al., 2001
tolC	TACCCAGGCGCAAAAAGAGGCTATC / CCGCGTTATCCAGGTTGTTGC	multiplex VI	56	161	Skyberg et al., 2006

a) Cc: *C. coli*; Cj: *C. jejuni.*

5.4. RESULTS

5.4.1. Genetic diversity and population structure

PFGE profiles of 110 *Campylobacter* and 64 *Salmonella* isolates from Southern Ocean and South African seabirds were analysed and compared. Based on the results obtained, we selected isolates representative of different bird species and locations to assess their genetic relationship by MLST. In those cases where isolates from the same bird or the same bird species and colony showed a pulsotype in common, only one isolate was selected for further analysis. The selection included 77 *Campylobacter* and 23 *Salmonella enterica* isolates. Overall, 68 *Campylobacter* pulsotypes (SK) (35 *C. lari*, 32 *C. jejuni* and one *C. coli*) and 20 *Salmonella* pulsotypes (XB) were identified (Figures 5.2, 5.3 and 5.4). One *C. jejuni* isolate (MAR119-C5) non-typeable by PFGE with either of the two restriction enzymes was included in the selection. One isolate of *S*. Anatum non-typeable with the secondary enzyme BlnI was also included.

MLST of *Campylobacter* isolates revealed 62 STs (35 of *C. lari*, 26 of *C. jejuni* and one of *C. coli*), of which 65% corresponded to novel STs (33 of *C. lari* and seven of *C. jejuni*) (Figures 5.5 and 5.6). We described 53 new *C. lari* alleles (five of *adk*, eight of *atpA*, seven of *glnA*, three of *glyA*, nine of *pgi*, 11 of *pgm* and 10 of *tkt* genes) and four new *C. jejuni* alleles (two of *glnA*, one of *glyA* and one of *pgm* genes). We also identified four *C. lari* and four *C. jejuni* new allelic combinations. However, one *C. lari* isolate (FK57-C1) was not typeable by MLST since it was not possible to obtain the sequence of the *tkt* gene and thus the allelic profile was incomplete (*adk*63-*atpA*72-*gln*A61-*glyA*57-*pgi*72-*pgm*67-*tkt*X).

A high genetic diversity was observed among *C. lari* isolates, and most STs had three or more loci variants (Figure 5.5). No *C. lari* ST belonged to a previously defined clonal complex (CC). We found two STs, one in a southern giant petrel from Marion Is. (ST-54) and one in a skua from the Falkland Is. (ST-62), with exclusive allelic profiles (i.e. all

seven loci were different from those found in all other STs detected). The former was identified by 16S rDNA sequence analysis as *C. lari* subsp. *lari*, whilst the latter was a *C. lari* subsp. *concheus*. Despite the high genetic diversity, the same STs were found in different seabird species and islands: ST-37 was detected in one skua and one gentoo penguin from Livingston Is., ST-33 was found in three skuas from Gough Is. and Marion Is., ST-43 was present in two skuas from Marion Is. and Livingston Is., and one southern giant petrel carried ST-41, a single-locus variant (SLV) of ST-43. We also found other SLVs, such as ST-27 and ST-35, in one skua and one macaroni penguin, respectively, from Marion Is. Although most brown skuas carried unrelated STs, some clusters of SLVs and double loci variants (DLVs) were observed in this bird species from Livingston Is. (ST-38, ST-39 and ST-40) and Gough Is. (ST-47, ST-48 and ST-51). Interestingly, *C. lari* isolates from domestic ducks at the Falkland Is. showed a low similarity between them and among those from wild birds. We did not observe relevant genetic similarity among *C. lari* isolates from Western Cape and Southern Ocean islands.

Regarding *C. jejuni*, the CCs most frequently observed were CC-1275 and CC-45, followed by CC-21 and CC-206 (Figure 5.6). Five CCs (CC-283, CC-354, CC-692, CC-1034 and CC-1332) were represented by just one ST which appeared only once (ST-267, ST-1073, ST-991, ST-1956 and ST-696, respectively), while six STs (ST-2353, ST-2654, ST-8572, ST-8577, ST-8588 and ST-8590) belonged to unassigned CCs. The predominant CC-1275 encompassed seven STs, all of which were detected in Western Cape seabirds. ST-1275 is the central type of the CC, but ST-1223 and ST-1268 were the most prevalent. The latter and ST-2654 were found in both Western Cape kelp gulls and greater crested terns. ST-45 was detected in skuas from Marion and the Falkland Is., as well as one domestic duck from the Falkland Is. ST-45 is the ancestral type of the CC-45 which contains other SLVs, such as those found in one skua (ST-8589) and one macaroni penguin (ST-137) from Marion Is., and one Western Cape greater crested tern (ST-1326). CC-206 contained two SLVs isolated from one skua from Gough Is. (ST-227) and one Western Cape kelp gull (ST-572). Moreover, one king penguin from Marion Is. carried

an isolate differing in two loci (DLV) from that found in a hen from the Falkland Is. (ST-883 and ST-8570, respectively), both belonging to CC-21. However, the only *C. coli* isolated from a hen corresponded to ST-899 (CC-828).

We observed few coincidences between STs and pulsotypes: two for *C. lari* (ST-37 and SK34, ST-33 and SK36) and two for *C. jejuni* (ST-8588 and SK3, ST-2654 and SK13) (Figures 5.2 and 5.3). By comparison, two STs of *C. lari* (ST-43 and ST-27) and four of *C. jejuni* (ST-8570, ST-1268, ST-45 and ST-1223) grouped isolates showing two or three different pulsotypes, some of them with a similarity level <65%. We also detected pulsotypes in common among *C. lari* isolates assigned to different STs (SK41 in ST-46 and ST-55; SK55 in ST-39 and ST-38). However, most *C. jejuni* isolates belonging to the same CC were clustered together by PFGE.

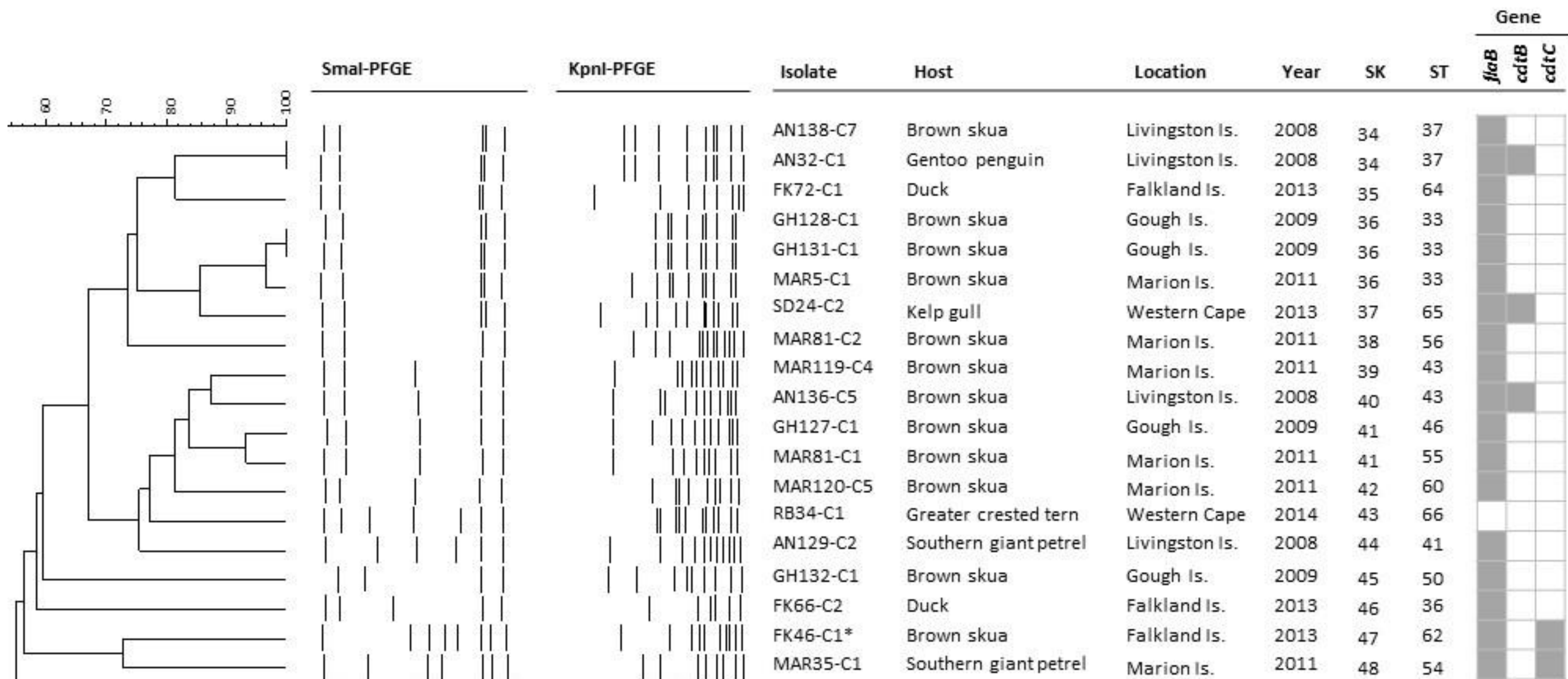
MLST of 23 *Salmonella* isolates showed 14 different STs, two of them with new allele profiles (one of *S. enterica* subsp. *salamae* and one of *S. enterica* subsp. *enterica* serovar Anatum) (Figure 5.4). A low genetic diversity was observed by MLST compared to PFGE since we mostly detected one or two ST per serovar regardless of the pulsotype, whereas several pulsotypes were distinguished within most serovars. Five *S*. Enteritidis isolates from kelp gulls from the Western Cape and Livingston Is. corresponded to ST-11 of eBG4 (eBurst groups are equivalent to *Campylobacter* clonal complexes), while the remaining isolate was assigned to SLV ST-1949. Moreover, two *S*. Typhimurium isolates belonged to ST-34 (eBG1) and one to SLV ST-1952. Three *S*. Anatum isolates (one of them not typeable by BlnI-PFGE) pertained to ST-64 (eBG65) and the other corresponded to a previously undescribed DLV of ST-64. According to the phylogenetic tree generated by concatenated MLST loci, the *S*. Paratyphi B var Java isolate (ST-28, eBG59) was the most divergent of the *Salmonella* isolates, including those belonging to the subspecies *salamae*. This was not perceived in the PFGE dendrogram, although all clusters grouped together isolates of the same serovar (data not shown).

5.4.2. Virulence-associated genes

The presence of virulence-associated genes was determined by PCR in the same selection of 77 *Campylobacter* and 23 *Salmonella* isolates analysed by MLST. In *C. lari* isolates, we detected the motility gene *flaB* (98%), but not *flaA*. The genes encoding for the cytolethal distending toxin (CDT) *cdtB* (7%) and *cdtC* (15%*)* were present at low frequencies, whilst *cdtA* gene was not detected*.* None of the other virulence-associated genes were detected in *C. lari* isolates. Twelve of the 14 tested genes in *Campylobacter* isolates were present in two *C. jejuni* isolates from Western Cape kelp gulls belonging to CC-1275, compared to only four genes in one *C. jejuni* isolate from a skua from Gough Is (Figure 5.3). Almost all *C. jejuni* isolates possessed the *flaB* gene, and 59% carried both motility genes (*flaA* and *flaB*) (Table 5.2, Figure 5.3). However, we only detected the *flaA* gene in one *C. jejuni* from a hen from the Falkland Is*.* The genes *pldA* and *ciaB* involved in invasion of host cells were found in almost all *C. jejuni* isolates. The *virB11* gene was exclusively found in isolates from Western Cape seabirds belonging to CC-1275 and ST-2654. We only detected the *wlaN* gene, associated with Guillain-Barré syndrome, in isolates from Western Cape seabirds from and Falkland Is. hens. Most (74%) *C. jejuni* isolates presented some of the three genes encoding for the CDT, although only 26% of isolates, most of them belonging to CC-45, carried all *cdtABC* genes. The other genes presented a heterogeneous distribution and determined unique combinations of virulence genes (designed as virulotypes) for each *C. jejuni* isolate. The *C. coli* isolate from a hen was only positive for the genes *flaB*, *cadF*, *hcp*, *cdtB* and *cdtC*.

We tested 27 virulence-associated genes in *Salmonella* isolates (Table 5.3). *S. enteritidis* subsp. *salamae* and subsp. *enteritidis* serovar Bovismorbificans isolates lacked most of the SPIs genes, although these genes were rather conserved among the other serovars. Some genes involved in adhesion and invasion (*agfAC, invA*, *orgA* and *tolC)* were present in all *Salmonella* isolates, but the *cdtB* gene encoding for the CDT was not detected in any isolate. The *lpfAC* genes encoding for long polar fimbria showed a high occurrence but were absent in serovars Anatum, Bovismorbificans and Paratyphi B var Java, as well

as in subsp. *salamae*. Other fimbrial genes were only present in specific serovars, such as the *sefC* gene in ser. Enteritidis, and the plasmid-encoded *pefA* gene in serovar Enteritidis, Typhimurium and Bovismorbificans. The *spvBC* plasmid-borne genes involved in intracellular survival were found in all *S*. Enteritidis, *S*. Typhimurium and *S*. Hadar isolates. The genes associated with the P2-like (SopEΦ) and lambda-like (Gifsy) phages, involved in invasion and intracellular survival, respectively, were detected mainly in isolates of *S*. Enteritidis (*sopE, gtgB, gipA* and *gogB*) and *S*. Typhimurium (*sopE, gtgB* and *gipA*), but some were also present in isolates of other serovars, such as Hadar (*sopE*), Muenchen (*sopE*), Saintpaul (*gtgB*) and Anatum (*gtgB*), as well as in subsp. *salamae* (*gipA*). The same virulotype was observed in two isolates of *S*. Anatum ST-64 (VT16B), *S*. Typhimurium ST-34 (VT20A) and *S*. Enteritidis ST-11 (VT18C). The highest number of virulence determinants (25 genes) was detected in *S*. Enteritidis ST-1949 and *S*. Typhimurium ST-1942, (SLVs of ST-11 and ST-34, respectively), both isolated from Western Cape kelp gulls. In addition, one Livingston Is. kelp gull carried a *S*. Enteritidis ST-11 positive for 24 virulence determinants.



60
70
80
90
100
SmaI-PFGE
KpnI-PFGE
Isolate
Host
Location
Year
SK
ST
Gene
flaB
cdtB
cdtC
AN138-C7 Brown skua Livingston Is. 2008 34 37
AN32-C1 Gentoo penguin Livingston Is. 2008 34 37
FK72-C1 Duck Falkland Is. 2013 35 64
GH128-C1 Brown skua Gough Is. 2009 36 33
GH131-C1 Brown skua Gough Is. 2009 36 33
MAR5-C1 Brown skua Marion Is. 2011 36 33
SD24-C2 Kelp gull Western Cape 2013 37 65
MAR81-C2 Brown skua Marion Is. 2011 38 56
MAR119-C4 Brown skua Marion Is. 2011 39 43
AN136-C5 Brown skua Livingston Is. 2008 40 43
GH127-C1 Brown skua Gough Is. 2009 41 46
MAR81-C1 Brown skua Marion Is. 2011 41 55
MAR120-C5 Brown skua Marion Is. 2011 42 60
RB34-C1 Greater crested tern Western Cape 2014 43 66
AN129-C2 Southern giant petrel Livingston Is. 2008 44 41
GH132-C1 Brown skua Gough Is. 2009 45 50
FK66-C2 Duck Falkland Is. 2013 46 36
FK46-C1* Brown skua Falkland Is. 2013 47 62
MAR35-C1 Southern giant petrel Marion Is. 2011 48 54

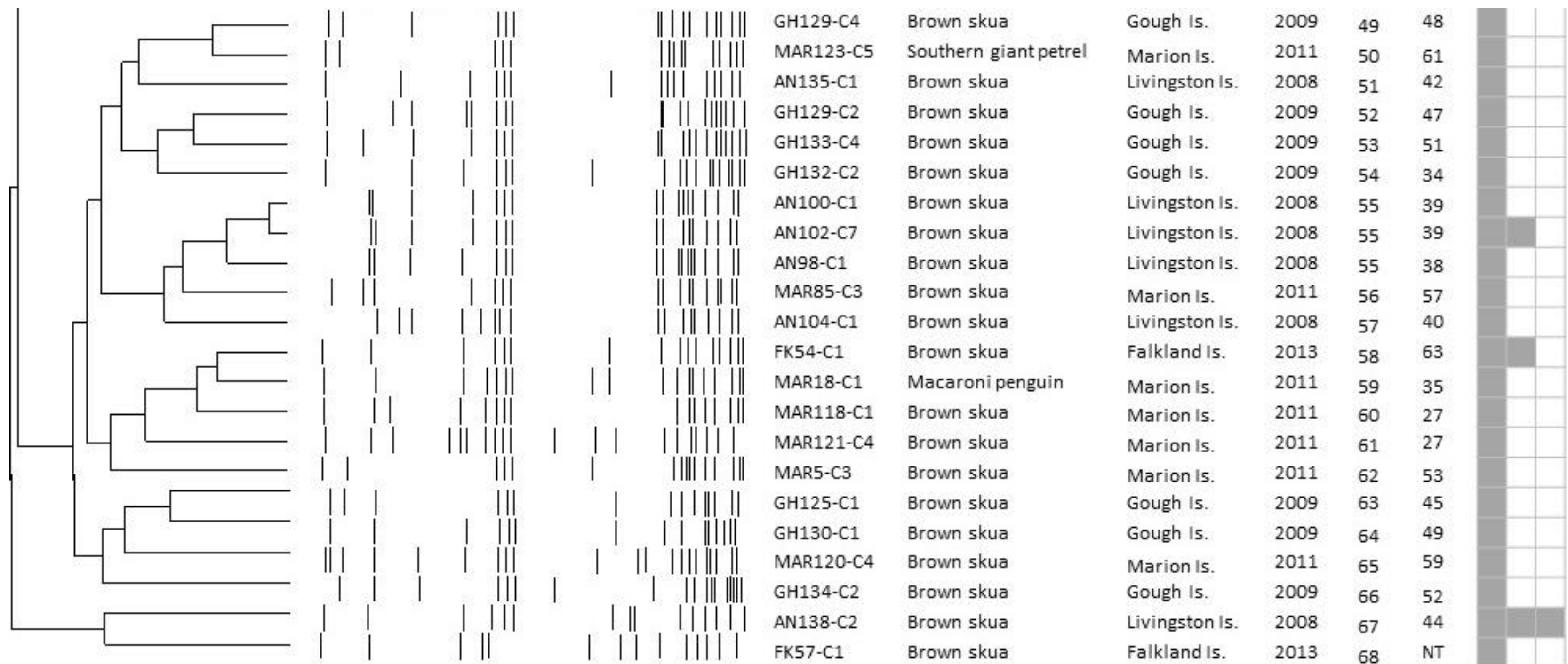


Figure 5.2. Combined dendrogram of SmaI and KpnI PFGE profiles of *C. lari* isolates. The tree was constructed using the UPGMA clustering method and the Dice coefficient for the similarity matrices calculation. Pulsotype (SK) numbers were assigned with a similarity level ≥ 90%. The presence of virulence-associated genes is depicted as grey squares. All isolates were negative for *flaA*, *cadF*, *dnaJ*, *racR*, *hcp*, *pdlA*, *virB11*, *ciaB*, *ceuE*, *wlaN* and *cdtA* genes. NT: non-typeable. **C. lari* subsp. *concheus* isolate.

50 60 70 80 90 100

SmaI-PFGE

KpnI-PFGE

Isolate	Host	Location	Year	SK	ST	CC
MAR86-C2	Brown skua	Marion Is.	2011	1	696	1332
FK361-C1	Hen	Falkland Is.	2013	2	1956	1034
MAR85-C1	Brown skua	Marion Is.	2011	3	8588	NU
MAR119-C1	Brown skua	Marion Is.	2011	3	8588	NU
SD19-C1	Kelp gull	Western Cape	2013	4	637	1275
SD38-C1	Kelp gull	Western Cape	2013	5	4020	1275
SD34-C2	Kelp gull	Western Cape	2013	6	1223	1275
VD11-C1	Kelp gull	Western Cape	2014	7	1275	1275
SD26-C1	Kelp gull	Western Cape	2013	8	1223	1275
RB33-C1	Greater crested tern	Western Cape	2014	9	8575	1275
SD41-C1	Kelp gull	Western Cape	2013	10	1223	1275
RB87-C1	Greater crested tern	Western Cape	2014	11	1268	1275
SD16-C1	Kelp gull	Western Cape	2013	12	1268	1275
SD6-C1	Kelp gull	Western Cape	2013	13	2654	NU
RB94-C1	Greater crested tern	Western Cape	2014	13	2654	NU
RB49-C2	Greater crested tern	Western Cape	2014	14	8577	NU
RB25-C1	Greater crested tern	Western Cape	2014	15	8572	NU
RB18-C1	Greater crested tern	Western Cape	2014	16	1292	1275
VD17-C1	Kelp gull	Western Cape	2014	17	1268	1275
MAR88-C1	Brown skua	Marion Is.	2011	18	8589	45
MAR72-C1	Brown skua	Marion Is.	2011	19	45	45
PD45-C1	Kelp gull	Western Cape	2013	20	267	283
VD10-C1	Kelp gull	Western Cape	2014	21	1326	45
MAR38-C1	Macaroni penguin	Marion Is.	2011	22	137	45
FK44-C1	Brown skua	Falkland Is.	2013	23	45	45
FK72-C2	Duck	Falkland Is.	2013	24	45	45
RB98-C1	Greater crested tern	Western Cape	2014	25	2353	NU

Gene

flaA *flaB* *cadF* *dnaJ* *racR* *hcp* *pldA* *virB11* *ciaB* *ceuE* *wlaN* *cdtA* *cdtB* *cdtC*

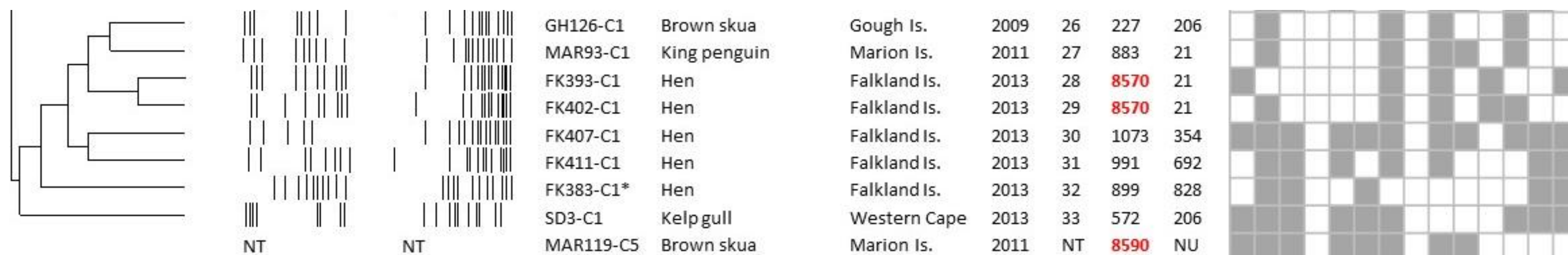


Figure 5.3. Combined dendrogram of SmaI and KpnI PFGE profiles of *C. jejuni* and *C. coli* isolates. The tree was constructed using the UPGMA clustering method and the Dice coefficient for the similarity matrices calculation. Pulsotype (SK) numbers were assigned with a similarity level ≥ 90%. In red, new STs described in this study. The presence of virulence-associated genes is depicted as grey squares. NT: non-typeable; UN: unassigned CC. **C. coli* isolate, all other isolates are *C. jejuni*.

Strain	Subsp. and serovar	XB	ST	eBG	VT
SD3-S1	Enteritidis	17	11	4	21B
VD12-S1	Enteritidis	16	11	4	18C
AN90-S1*	Enteritidis	17	11	4	24A
SD43-S1	Enteritidis	18	11	4	16C
SD42-S1	Enteritidis	15	11	4	18C
SD25-S1	Enteritidis	14	1949	NU	25B
PD7-S1	Saintpaul	10	27	14	19B
PD32-S1	Typhimurium	9	1952	NU	25A
SD42-S4	Typhimurium	8	34	1	20A
SD36-S1	Typhimurium	7	34	1	20A
PD27-S1	Manhattan	6	1540	27	19A
PD40-S1	Muenchen	5	82	8	16A
SD26-S1	Muenchen	4	82	8	18B
YD3-S1	subsp. *salamae*	20	new	NU	6A
SD2-S1	subsp. *salamae*	19	53	214	11A
VD3-S1	Anatum	12	new	NU	9A
SD15-S1	Anatum	11	64	65	16B
PD22-S1	Anatum	NT	64	65	16B
PD4-S1	Anatum	11	64	65	17A
SD7-S1	Hadar	1	473	22	18A
PD31-S1	Hadar	1	473	22	21A
RB13-S1*	Bovismorbificans	3	142	34	7A
PD50-S1	Paratyphi B, var Java	2	28	59	14A

Gene

SPI1-5: *invA*, *orgA*, *prgH*, *sitC*, *avrA*, *spiC*, *mgtC*, *misL*, *orfL*, *pipD*, *sopB*

Islet: *tolC*, *iroN*, *sifA*, *cdtB*

Fimbrial operon: *agfA*, *agfC*, *lpfA*, *lpfC*, *sefC*

Plasmid: *pefA*, *spvB*, *spvC*

Phage: *sopE*, *gtgB*, *gipA*, *gogB*

0.0100

Figure 5.4. Maximum likelihood tree based on concatenated MLST loci of *Salmonella* isolates and presence of virulence-associated genes. The tree is drawn to scale, with branches length measured in the number of substitutions per site. Serovar, pulsotype (XB), sequence type (ST), eBurst Group (eBG), virulotype (VT) and the detailed presence of virulence-associated genes for each isolate are also shown. Pulsotypes were assigned to XbaI and BlnI PFGE profiles with a similarity level ≥ 90%. Virulotypes (VT) are indicated by a number corresponding to the number of positive virulence genes and a capital letter indicating the different virulence profiles detected. The presence of virulence-associated genes is depicted as grey squares. *RB13-S1 isolate from a Western Cape greater crested tern; AN97-S1 isolate from a Livingston Is. kelp gull. All other isolates were recovered from Western Cape kelp gulls. NT: non-typeable; UN: unassigned eBG.

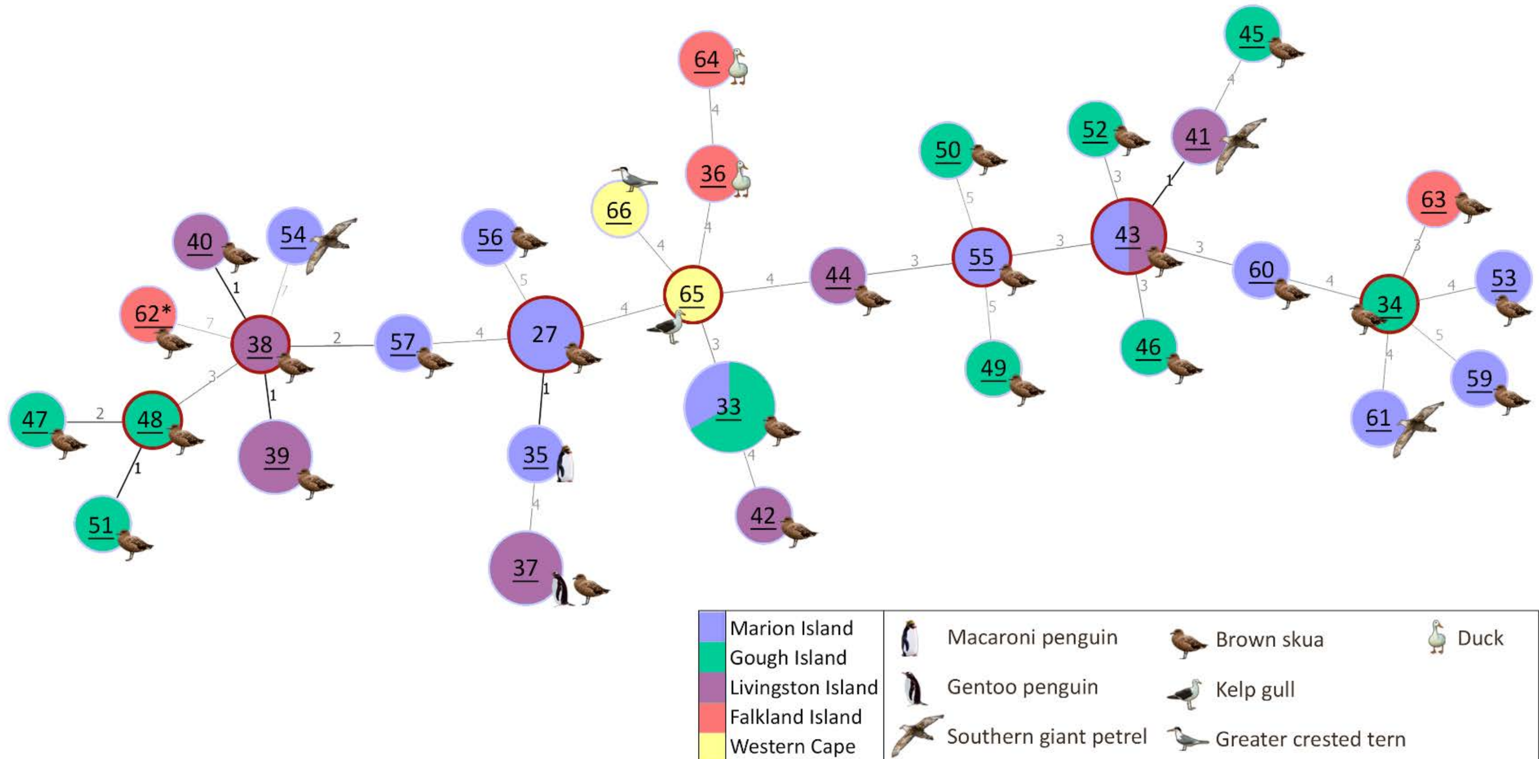


Figure 5.5. Minimum spanning tree showing the distribution of *C. lari* isolates according to bird species and source of isolation. Each circle represents a unique ST; circle size is proportional to the number of isolates within each ST. Underlined ST numbers indicate new STs described in this study. STs with red outlines represent the clonal complex ancestors. Black and grey branches link STs with one or more different loci, respectively, and the number of differences is shown on the branches. * *C. lari* subsp. *concheus* isolate.

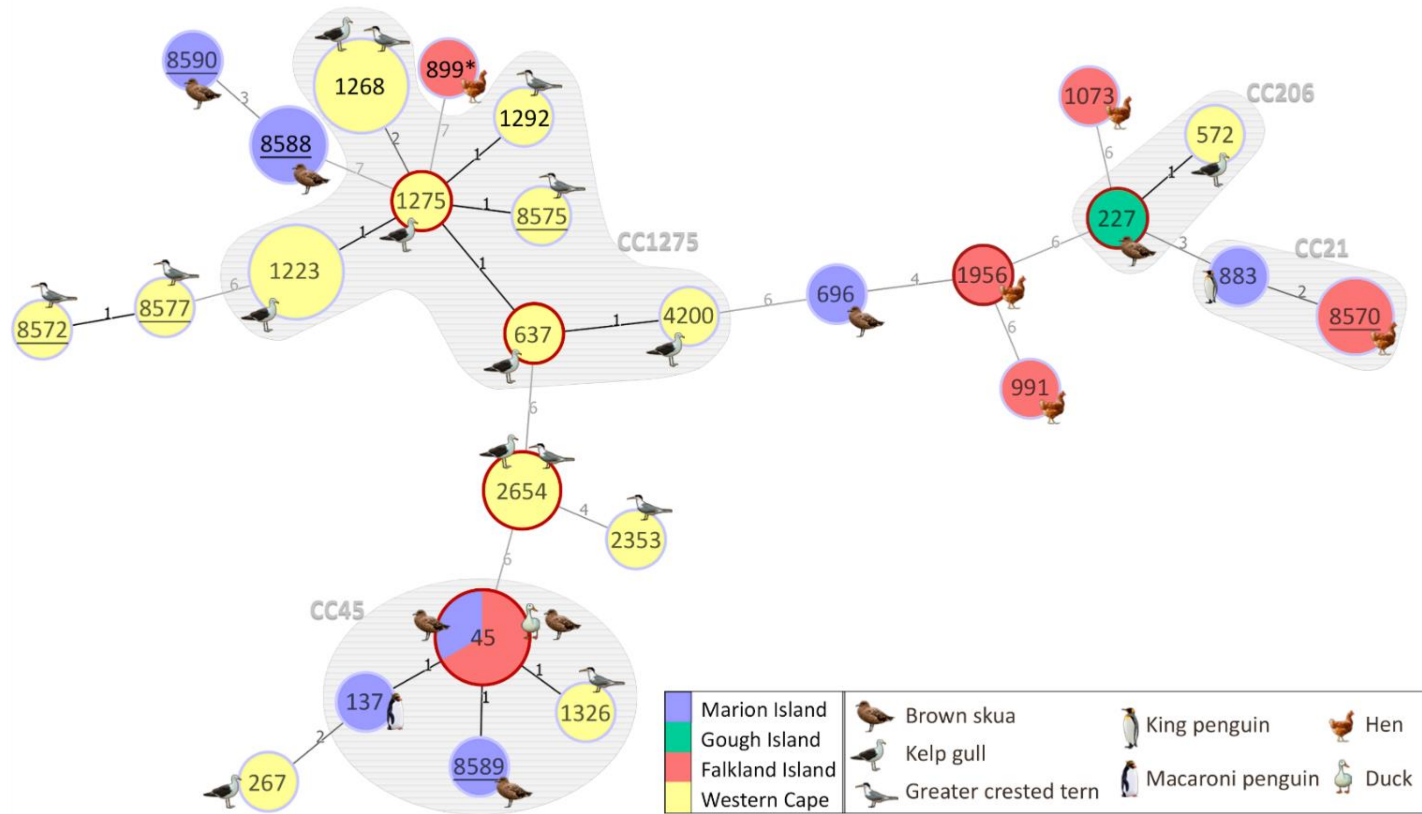


Figure 5.6. Minimum spanning tree showing the distribution of *C. jejuni and C. coli* isolates according to bird species and source of isolation. Each circle represents a unique ST, the circle size is proportional to the number of isolates within each ST. Underlined ST numbers indicate new STs described in this study. The single *C. coli* ST is indicated with an asterisk. STs with red outlines represent the clonal complex ancestors. Black and grey branches link STs with one or more different loci, respectively, and the number of differences is shown on the branches. * *C. coli* isolate, all other isolates are *C. jejuni*.

Table 5.2. Occurrence of virulence-associated genes in *C. jejuni* and *C. coli* isolates from different sources.

Site and host origin	Gene[a]													
	Adhesion and colonization					Invasion					Toxin production			
	flaA	*flaB*	*cadF*	*dnaJ*	*racR*	*hcp*	*pldA*	*virB*	*ciaB*	*ceuE*	*cdtA*	*cdtB*	*cdtC*	*wlaN*
Gough Is.														
Brown skua	0	1/1	0	0	0	0	1/1	0	1/1	0	1/1	0	0	0
Marion Is.														
Penguins[b]	1/2	2/2	1/2	1/2	1/2	0	2/2	0	2/2	2/2	2/2	1/2	1/2	0
Brown skua	6/6	6/6	5/6	2/6	5/6	3/6	6/6	0	6/6	4/6	2/6	2/6	1/6	0
Falkland Is.														
Brown skua	1/1	1/1	1/1	0	1/1	1/1	1/1	0	1/1	0	1/1	1/1	1/1	0
Duck	1/1	1/1	0	0	1/1	0	1/1	0	1/1	0	1/1	1/1	1/1	0
Hen	2/5	4/5	3/5	0	2/5	1/5	5/5	0	5/5	1/5	2/5	3/5	3/5	2/5
Western Cape														
Kelp gull	8/12	12/12	9/12	2/12	9/12	4/12	10/12	9/12	11/12	7/12	3/12	4/12	8/12	4/12
Greater crested tern	2/7	7/7	6/7	2/7	5/7	0	7/7	4/7	7/7	6/7	4/7	2/7	4/7	1/7
Total	21/35	34/35	25/35	7/35	24/35	9/35	33/35	13/35	34/35	20/35	16/35	15/35	19/35	7/35

a) The *C. coli* isolate (n = 1, not included in the table) was postive for *flaB, cadF, hcp, cdtB* and *cdtC* genes. *C. jejuni* , n = 35.

b) King and macaroni penguins.

Table 5.3. Occurrence of virulence-associated genes in *Salmonella* isolates of different subspecies and serovars.

Gene	Virulence role	**Subsp. and serovar**										
		subsp. *salamae*	**Anatum**	**Bovismorbificans**	**Enteritidis**	**Hadar**	**Manhattan**	**Muenchen**	**Paratyphi B, var Java**	**Saintpaul**	**Typhimurium**	**Total**
SPI-1												
invA	Invasion	2/2	4/4	1/1	6/6	2/2	1/1	2/2	1/1	1/1	3/3	23/23
orgA	Invasion	2/2	4/4	1/1	6/6	2/2	1/1	2/2	1/1	1/1	3/3	23/23
prgH	Invasion	1/2	3/4	0	3/6	2/2	1/1	2/2	1/1	1/1	3/3	17/23
sitC	Iron uptake	1/2	3/4	0	5/6	1/2	1/1	2/2	1/1	1/1	3/3	18/23
avrA	Intracel. survival	0	4/4	0	6/6	1/2	1/1	2/2	0	1/1	1/3	16/23
SPI-2												
spiC	Intracel. survival	1/2	4/4	0	5/6	2/2	1/1	2/2	1/1	1/1	3/3	20/23
SPI-3												
mgtC	Intracel. survival	0	3/4	0	3/6	2/2	1/1	1/2	1/1	1/1	3/3	15/23
misL	Colonization and intracel. survival	1/2	3/4	0	3/6	1/2	1/1	2/2	1/1	1/1	3/3	16/23
SPI-4												
orfL	Colonization and intracel. survival	0	3/4	0	6/6	2/2	1/1	2/2	0	1/1	3/3	18/23
SPI-5												
pipD	Invasion	1/2	4/4	0	6/6	2/2	1/1	2/2	1/1	1/1	3/3	21/23
sopB	Invasion and intracel. survival	0	4/4	1/1	6/6	2/2	1/1	2/2	1/1	1/1	3/3	21/23
Islet												
tolC	Colonization and invasion	2/2	4/4	1/1	6/6	2/2	1/1	2/2	1/1	1/1	3/3	23/23

Continued

Table 5.3. Continued.

Gene	Virulence role	Subsp. and serovar										
		subsp. *salamae*	Anatum	Bovismorbificans	Enteritidis	Hadar	Manhattan	Muenchen	Paratyphi B, var Java	Saintpaul	Typhimurium	Total
Islet												
iroN	Iron uptake	1/2	3/4	0	2/6	2/2	1/1	2/2	1/1	1/1	3/3	16/23
sifA	Intracel. survival	0	3/4	0	2/6	2/2	1/1	1/2	1/1	1/1	3/3	14/23
cdtB	Toxicity and intracel. survival	0	0	0	0	0	0	0	0	0	0	0
Fimbrial operon												
agfA	Adhesion and invasion	2/2	4/4	1/1	6/6	2/2	1/1	2/2	1/1	1/1	3/3	23/23
agfC	Adhesion and invasion	2/2	4/4	1/1	6/6	2/2	1/1	2/2	1/1	1/1	3/3	23/23
lpfA	Adhesion and invasion	0	0	0	6/6	2/2	1/1	2/2	0	1/1	3/3	15/23
lpfC	Adhesion and invasion	0	0	0	6/6	2/2	1/1	2/2	0	1/1	3/3	15/23
sefC	Adhesion and intracel. survival	0	0	0	3/6	0	0	0	0	0	0	3/23
Plasmid												
pefA	Adhesion and colonization	0	0	1/1	6/6	0	0	0	0	0	1/3	8/23
spvB	Intracel. survival	0	0	0	6/6	2/2	0	0	0	0	3/3	11/23
spvC	Intracel. survival	0	0	0	6/6	2/2	0	0	0	0	3/3	11/23
Phage												
sopE	Invasion	0	0	0	5/6	2/2	1/1	0	0	0	1/3	9/23
gtgB	Intracel. survival	0	1/4	0	6/6	0	0	0	0	1/1	3/3	11/23
gipA	Intracel. survival	1/2	0	0	1/6	0	0	0	0	0	1/3	3/23
gogB	Intracel. survival	0	0	0	0	0	0	0	0	0	1/3	1/23

5.5. DISCUSSION

Despite the efforts taken to reduce the human incidence of *Campylobacter* and *Salmonella* spp., these pathogens continue to be the most common causes of human bacterial gastroenteritis worldwide. For an effective control of these zoonoses, it is necessary to better understand the global epidemiology of these pathogens, including the environmental reservoir with particular attention to wild animals that may constitute another reservoir of these pathogens. Here, we investigated the diversity, population structure and virulence potential of *Campylobacter* and *Salmonella* isolates from seabirds from remote Southern Ocean islands and the Western Cape coast of South Africa. We also included in the analysis *Campylobacter* isolates from domestic poultry from the Falkland Is. We described 33 new STs of *C. lari* and seven of *C. jejuni,* in addition to 53 and four new alleles, respectively. Novel MLST data, submitted to the corresponding PubMLST database, represented an important contribution in the case of *C. lari,* with only 32 STs described prior to our submission (January 2017). We also found new STs of *S. enterica* subsp. *salamae* and subsp. *enterica* serovar Anatum.

The population structure of *Campylobacter* isolates from Southern Ocean seabirds appeared to be highly diverse and genetically distant, especially in the case of *C. lari* isolates since most of them belonged to new STs with unassigned CCs. Within the genus *Campylobacter*, *C. lari* represent a closely related phylogenetic clade and their members often share similar hosts (e.g. shorebirds, marine mammals and shellfish) and environments (e.g. coastal regions and watersheds) (Miller *et al.*, 2014). Therefore, the genetic heterogenicity observed here may be due to the geographically distinct origin of the isolate collection and the existence of a variety of infection sources. In addition, the relatively high body temperature of birds could provide an optimal growth environment for bacteria and favour their genetic diversification, which may explain the detection of some SLVs and DLVs in brown skuas from the same islands.

Despite the genetic diversity, the same *C. lari* STs were detected repeatedly in brown skuas from different Southern Ocean islands: Marion Is. and Gough Is. (ST-33), Marion Is. and Livingston Is. (ST-43). This suggests some connectivity among Subantarctic and Antarctic islands, probably as a result of direct transmission by skuas or indirectly by some common sources of infection of these seabirds. Moreover, the same STs or SLVs present in skuas were found in other seabird species at the same island: gentoo penguin (ST-37) and southern giant petrel (ST-41, a SLV of ST-43) at Livingston Is., and macaroni penguin (ST-35, a SLV of ST-27) at Marion Is. Brown skuas are predators and scavengers, frequently found close to penguin colonies, where they feed on penguin eggs and, like giant petrels, chicks and sick or wounded adult penguins, which may explain the similarities found among *C. lari* STs carried by these seabirds. No common STs of *C. lari* STs were observed between our study and those previously reported in Antarctic penguins, but it is difficult to compare across studies because authors described many incomplete allelic profiles (García-Peña *et al.*, 2017). According to the MLST database, *C. lari* has been found mainly in aquatic environments, but also in human gastroenteritis cases in some countries (USA, Canada, Belgium and France). An example of this is ST-27 detected in one skua from Marion Is. and also isolated in a human stool in France.

The population structure of *C. jejuni* was less heterogeneous than that of *C. lari,* with most isolates structured into clusters of related lineages. CC-1275 occurred exclusively in kelp gulls and greater crested terns from the Western Cape, and is mainly recorded from environmental waters and wild birds in Europe, USA, Canada, New Zealand and Australia (http://pubmlst.org/campylobacter). Several studies have described that most *C. jejuni* genotypes from wild birds differ from those recovered from food-producing animals and human campylobacteriosis (Broman *et al.*, 2004; Sheppard *et al.*, 2011; Griekspoor *et al.*, 2013). This agrees with what we observed in South African seabirds, where 84% were carriers of *C. jejuni* genotypes mainly linked to aquatic environments, despite inhabiting a populated region in close contact with livestock and human activities.

Surprisingly, we found the opposite scenario in seabirds from remote Southern Ocean islands. Almost all *C. jejuni* genotypes carried by skuas and penguins from Subantarctic regions belong to CCs commonly associated with domestic animals, especially poultry, and human gastroenteritis. One of the most prevalent CCs here is CC-45, widely distributed in the Northern Hemisphere and locally in the Southern Hemisphere (Argentina, Australia and New Zealand), followed by CC-21 and CC-206, usually reported in Europe. These CCs have also been found, albeit less frequently, in natural environments and wild birds, probably as a result of contamination from animal and human waste (Kwan *et al.*, 2008; Levesque *et al.*, 2013; Cody *et al.*, 2015). ST-45, the central genotype of CC-45, is the only *C. jejuni* ST that has been reported previously from the Subantarctic, specifically in a macaroni penguin from Bird Island (South Georgia), suggesting human activities (wastes from scientific bases and passing ships) as possible transmission routes (Griekspoor *et al.*, 2010). Interestingly, we also detected ST-45 in skuas and one domestic duck from the Falkland Is., one of the few areas close to Antarctica (and South Georgia) with a resident human population and free-ranging livestock, which may facilitate the contact between seabirds and domestic animals. Although brown skuas are mainly predatory birds, they scavenge on human food waste, providing a plausible route for *C. jejuni* infection, or they may be infected in wintering and stopover areas where they mix with the local fauna (Shirihai, 2007). Skuas also may spread pathogens around the Southern Ocean as we observed with some *C. lari* genotypes. *C. jejuni* ST-45 and associated SLVs were found in one brown skua and one macaroni penguin from Marion Is. supporting the hypothesis of connectivity among distant islands. However, although several seabirds breeding in Antarctic and Subantarctic islands spend the austral winter in the productive Benguela Upwelling region in the western coast of South Africa (Crawford *et al.*, 1991; Krietsch, 2014), few genetic similarities were found between *C. jejuni* genotypes from Southern Ocean and South African seabirds.

Previous studies have demonstrated that *C. jejuni* genotypes from different bird host species are genetically distinct, although it has been suggested that there is some niche-association in genotypes from wild birds with similar feeding ecology (Sheppard *et al.*, 2011; Griekspoor *et al.*, 2013). We did not observe *C. jejuni* genotypes clearly adapted to Southern Ocean seabirds and closely related *C. jejuni* STs were detected in different host species. For example, *C. jejun*i genotypes of the same CCs were present in brown skuas, greater crested terns and macaroni penguins (CC-45), skuas and kelp gulls (CC-206) and king penguins and domestic hens (CC-21) from different regions. The fact that these seabirds carried *C. jejuni* genotypes commonly found in domestic animals and known to cause human gastroenteritis, strongly suggest that the pathogens were acquired from sporadic infections from anthropogenic sources. These poultry-adapted genotypes probably encounter a similar ecological niche in the gastrointestinal tract of wild birds allowing them to grow and proliferate rapidly.

With regards to *Salmonella* isolates, a single ST per serovar was observed in most cases, except for serovars Typhimurium, Enteritidis and Anatum that displayed two SLVs each. Almost all *S*. Typhimurium isolates corresponded to ST-34, one of the most predominant ST within the eBG1, whilst *S*. Enteritidis isolates belonged to ST-11, the central ST of eBG4 (Achtman *et al.*, 2012; Ashton *et al.*, 2016). Both STs are globally distributed in a broad range of domestic animals and are commonly responsible for human salmonellosis (Enterobase Database, http://enterobase.warwick.ac.uk/). Interestingly, the isolate recovered from an Antarctic kelp gull from Livingston Is. also belonged to ST-11. *S*. Enteritidis has been previously reported in Antarctic and Subantarctic wildlife (seagulls, skuas, penguins, petrels and seals), although the ST was not determined (Olsen *et al.*, 1996; Palmgren *et al.*, 2000; Iveson *et al.*, 2009; Vigo *et al.*, 2011; Dougnac *et al.*, 2015). The presence of this ST in seabirds from Western Cape and even from remote regions of Antarctica reflects the rapid worldwide expansion of *Salmonella* genotypes of public health concern. The remaining *Salmonella* serovar isolates were less frequently reported STs, isolated from diverse origins such as livestock, reptiles, foods,

and humans. The only *S*. Paratyphi B var Java isolated (from a Western Cape kelp gull) was assigned to ST-28 (eBG59), a genotype mainly found in poultry and not as pathogenic for humans as other *S*. Paratyphi B genotypes (Toboldt *et al.*, 2012).

Overall, molecular typing by PFGE allowed a slightly greater discrimination compared to MLST, and isolates with same ST exhibited different pulsotypes in many cases. One drawback of PFGE is determining the degree of relatedness among isolates that have similar but distinguishable macrorestriction profiles. Genomic rearrangements may hinder the interpretation of PFGE data, especially in the case of *Campylobacter* which demonstrates considerable genetic instability (Ridley *et al.*, 2008), and additional sequence-based methods may be required to clarify phylogenetic relationships. However, in our study, PFGE typing clustered *Campylobacter* isolates in the same way as CCs, or eBGs and serovars in the case of *Salmonella,* which is consistent with previous reports (McTavish *et al.*, 2009; Shi *et al.*, 2015). PFGE typing also was a helpful tool for screening closely related genotypes, for instance within individuals or within islands, and for selecting representative isolates for MLST analysis. Although MLST lacks the resolution of PFGE, it provides a good epidemiological concordance in assessing the evolution of bacterial strains, as well as information on source attribution (Barco *et al.*, 2013; Taboada *et al.*, 2013). Nevertheless, it is important to consider that a few *C. lari* isolates with the same pulsotypes turned out to be different STs, unlike what has been observed in other *Campylobacter* species (Yabe *et al.*, 2010; Lucarelli *et al.*, 2016; Cantero, 2017), probably as a result of single nucleotide changes that do not alter the restriction sites and therefore are not reflected in band profiles.

Our study also provides information about the potential virulence of *Campylobacter* and *Salmonella* spp. isolates. *C. lari* isolates were PCR-negative for almost all genes analysed, probably because of significant differences among alleles rather than the absence of most of these genes. Most *C. jejuni* isolates presented both *flaA* and *flaB* genes, while *C. coli* and *C. lari* isolates lacked the *flaB* gene. Experiments with mutants have demonstrated that *flaA* (but not *flaB*) is essential for chicken colonization, although

probably both are needed for full motility (Wassenaar *et al.*, 1993). However, the truncated flagellar filament coded only by *flaB* is enough for secretion of effector proteins such as CiaB required for the invasion of hosts cells (Konkel *et al.*, 2004). While *ciaB* and *pldA* genes were present in almost all *C. jejuni* isolates, other genes involved in adhesion (*cadF, dnaJ* and *racR*) and invasion (*hcp, virB11* and *ceuE*) were less conserved and their heterogeneous distribution gave rise to a unique virulotype for each *C. jejuni* isolate. The *hcp* gene was used as an indicator for the presence of T6SS, a secretion system recently reported in *Campylobacter* that causes lysis of blood cells (Bleumink-Pluym *et al.*, 2013) and associated with severe cases of campilobacteriosis, especially in Asia (Harrison *et al.*, 2014). We found the *hcp* gene in *C. jejuni* from brown skuas and kelp gulls, but also in a *C. jejuni* and a *C. coli* from domestic hens.

We also observed an association between virulence genes and genotypes. The *virB11* gene, located in the virulence plasmid pVir, is involved in adhesion and invasion (Bacon *et al.*, 2000). Several studies have reported a low prevalence of this gene (< 15% or absent) in isolates from humans and domestic animals (Datta *et al.*, 2003; Müller *et al.*, 2006; Talukder *et al.*, 2008; Koolman *et al.*, 2015). We found a higher occurrence of *virB11* (37%), but it was only present in *C. jejuni* CC-1275 and ST-2654 from Western Cape seabirds; these genotypes are mainly related to aquatic environments and wild birds. A recent study found that the absence of *virB11* did not reduce the colonization ability of the bacteria, suggesting that it may not be a relevant virulence factor for human infection (Biswas *et al.*, 2011). Some genotypes of CC-1275, in addition to the novel ST-8570 (CC-21) from the Falkland Is. hens, also possessed the *wlaN* gene which is involved in the Guillian-Barré syndrome (Linton *et al.*, 2000). The prevalence of *wlaN* in isolates from seabirds (20%) was similar to that previously found in chickens and humans (Datta *et al.*, 2003; Cantero, 2017), but substantially higher than reported by (Koolman *et al.*, 2015). These *C. jejuni* genotypes carried by seabirds thus may pose an important risk for human health. In addition, a 23% of *C. jejuni* isolates, mainly belonging to CC-45, exhibited the complete *cdtABC* gene cluster required for producing

functionally active cytolethal distending toxin (CDT) (Asakura *et al.*, 2008). These genotypes were present in different seabird species (skuas, gulls, terns and penguins) from three different regions as well as in backyard poultry, which suggest that the presence of *cdtABC* genes may be associated with certain *C. jejuni* genotypes regardless of host species or geographical location.

In the case of *Salmonella*, SPIs (*invA, orgA, prgH, sitC, arvA, spiC, mgtC, misL, orfL, pipD*, *sopB*) and islet (*tolC, iroN, sifA*) genes were rather conserved, except in subsp. *salamae* and *S*. Bovismorbificans which lacked most of these genes. By comparison, the *cdtB* gene encoding for one of the CDT subunits was not detected in any *Salmonella* isolate. Some variability was observed in the distribution of genes located in fimbrial operons, plasmids and especially prophage regions, which can contribute to the diversity and host adaptation of *Salmonella* serovars. While *agfAC* and *lpfAC* genes encoding for SEF17 and long polar fimbriae, respectively, were present in all or almost all serovars, the *sefC* gene encoding for the SEF14 fimbria was only detected in some *S*. Enteritidis isolates. Also, the plasmid-borne fimbrial gene *pefA* was only found in isolates of serovars Enteritidis, Typhimurium and Bovismorbificans. It seems that the acquisition of different adhesion determinants may expand the host range of the bacteria, including a greater ability to colonize birds and domestic animals. The *pefA* and *spvBC* genes, often located in the same virulence plasmid (Skyberg *et al.*, 2006), were found together in *S*. Enteritidis isolates. However, some *S*. Typhimurium and *S*. Hadar isolates that lacked the *pefA* gene also presented the *spvBC* genes involved in systemic survival (Heithoff *et al.*, 2008). Prophage genes were mainly found in *S*. Enteritidis and *S*. Typhimurium isolates, especially *gtgB* and *sopE*. The existence of efficient mechanisms for horizontal gene transfer between serovars or other Gram-negative bacteria, including lysogenic conversion, may explain the extreme adaptability of *Salmonella* spp. and the wide range of hosts that it can infect. The *sopE* gene is involved in invasion of host cells and is carried by P2-like phages, but also on lambda-like phages that contain the *gtgB* gene (Hoffmann *et al.*, 2014). The lambda-like phage genes encode some

virulence factors responsible for survival in Peyer's patches, and have been reported with high prevalence in *S*. Typhimurium isolates from humans and animals (Drahovská *et al.*, 2007). Other lambda-like phage genes (*gipA* and *gogB*) were present in SLVs of *S*. Enteritidis ST-11 (ST-1949) and *S*. Typhimurium ST-34 (ST-1952). Interestingly, these genotypes found in Western Cape kelp gulls exhibited the highest proportion of virulence-associated genes, followed by *S*. Enteritidis ST-11 carried by a kelp gull from Livingston Is. However, it is important to note that the detection of virulence-associated genes by PCR does not consider possible deletions or insertions in gene sequences, nor does it give information about expression levels. Despite this, the presence of multiple genes potentially involved in pathogenic processes suggest that many *Campylobacter* and *Salmonella* spp. genotypes found in seabirds may be infectious for humans and other animals.

In conclusion, our results highlight that seabirds from Southern Ocean islands and the South African coast can act as carriers of *Campylobacter* and *Salmonella* genotypes associated with human gastroenteritis, some of which have a high virulence potential. Therefore, these seabirds can constitute an important reservoir for *Campylobacter* and *Salmonella* and may play an important role in their dispersion and in the global epidemiology of these pathogens. Although some Southern Ocean seabirds could have acquired these strains during their northward migration, it seems more likely that the strains have been introduced by human activities such as tourism or scientific expeditions.

CHAPTER 6

Study IV: Molecular comparative analysis of nontyphoidal *Salmonella* isolates from humans, poultry and seagulls in Southwestern Europe

6.1. SUMMARY

Salmonellosis, caused by non-typhoidal *Salmonella* (NTS), represents one of the most common human foodborne zoonotic diseases in developed countries. Birds can transmit this pathogen to humans, especially through contaminated poultry products. Seagulls have an elevated risk of exposure to *Salmonella* sources due to their scavenging feeding habits and can contribute to the dissemination of this agent in the environment during their foraging or migrating movements. In this study, 742 isolates of 19 NTS serovars from humans, poultry and seabirds from Southwestern Europe were genotyped using PFGE. Overall, we detected a higher number of exclusive pulsotypes in isolates from gulls (mainly *S*. Typhimurium) and more predominant pulsotypes in isolates from poultry and humans. However, we also found 30 pulsotypes in common among isolates from two or three different host niches (serovars Bredeney, Derby, Enteritidis, Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima, Rissen, Typhimurium and Virchow). Sequence type (ST) and virulotype were further determined in a subset of *Salmonella* isolates from different hosts with pulsotypes in common. *S*. Typhimurium belonged to ST19 and ST34, while *S*. Enteritidis corresponded to ST11. We did not find statistically significant differences in the presence of virulence-associated genes in isolates from a different source, except for the *iroN* gene, which is lacking in most of seagull isolates. These findings further support the role of seagulls as reservoir *Salmonella* strains of public health concern, including less common serovars.

6.2. INTRODUCTION

Non-typhoidal *Salmonella* are the zoonotic agents responsible of one of the main human foodborne gastroenteritis diseases in industrialized countries. Over the last decade the incidence of human salmonellosis in the European Union (EU) has decreased as a result of the interventions put in place for its control in the poultry sector. Despite this, *Salmonella* infection still represents a major public health burden and a considerable economic cost in many countries. In 2016, a total of 94,530 confirmed salmonellosis cases were reported in the EU, being the serovars Enteritidis and Typhimurium the most commonly reported (EFSA and ECDC, 2017b). Transmission of *Salmonella* to humans is mainly due to consumption of contaminated food of animal origin (mostly eggs, poultry meat and milk), even though other foods such as fruits, vegetables or contaminated water have also been implicated in *Salmonella* outbreaks (Pires *et al.*, 2014). Besides, some human salmonellosis can also be attributed to direct or indirect contact with infected animals and contaminated environments.

Salmonella are extensively distributed in the environment where they can survive and persist for a long time (Winfiel and Groisman, 2003), but its natural environment is the intestinal tract of a wide range of domestic and wild animals (Hoelzer *et al.*, 2011; Hilbert *et al.*, 2012). Poultry (chicken and turkey particularly) are frequently colonized with *Salmonella* without apparent clinical symptoms. Therefore, fowls can act as intermittent or persistent healthy carriers and transmit the bacteria vertically and horizontally to all the flock, spreading it also to the environment and farm surroundings (Antunes *et al.*, 2016).

Some wild birds such as seagulls, raptors, pigeons, crows and waterfowl can also be *Salmonella* carriers and faecally excrete these bacteria (Lawson *et al.*, 2010; Ramos *et al.*, 2010; Molina-Lopez *et al.*, 2011; Fresno *et al.*, 2013; Callaway *et al.*, 2014; Gargiulo *et al.*, 2014; Jurado-Tarifa *et al.*, 2016). Seagulls have adapted to an opportunistic lifestyle in close proximity to humans and often complement their diet with leftovers

from animal farms, garbage and waste products. Given their scavenging feeding habits, seagulls have an increased risk of pathogen infection and are one of the most documented carriers of *Salmonella* (Ferns and Mudge, 2000; Palmgren *et al.*, 2006; Ramos *et al.*, 2010; Dolejska *et al.*, 2016; Masarikova *et al.*, 2016). Besides, gulls have the ability to cover long distances and haunt diverse habitats, which may contribute to the pathogen spread into the environment and its transmission to domestic animals or humans via contamination of pastures and agricultural fields, as well as surface waters (Daniels *et al.*, 2003; Reed *et al.*, 2003).

Nonetheless, *Salmonella* epidemiology is still not fully understood due to the multiple infection sources, transmission routes and the wide variety of reservoirs implicated. Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) are powerful tools for establishing the genetic relatedness amongst strains and have been used in numerous studies for linking human infections to specific sources (Barco *et al.*, 2013). However, the implications of *Salmonella* occurrence in wild birds is probably underrated, as few attempts at identifying the role of these animal reservoirs have been conducted successfully (Palomo *et al.*, 2013).

Although some *Salmonella* serovars are adapted to humans (e.g. Typhi and Paratyphi) or non-human hosts (e.g. Gallinarum in poultry, Dublin in cattle and Cholerasuis in pigs), most serovars have a broad-host range and can be infectious to humans (Uzzau *et al.*, 2000). Enteritidis and Typhimurium are the most common zoonotic serovars, but others such as Infantis, Newport, Derby, Kentucky, Virchow and Hadar, are also of public health significance in the EU (EFSA and ECDC, 2017b). While host-adapted serovars produce systemic infection in their natural hosts, generalists serovars commonly cause gastroenteritis in infected hosts (Hoelzer *et al.*, 2011). The severity of infection depends largely on the susceptibility of the host and differs according to the serovar pathogenicity and the virulence potential of the strain itself (Hohmann, 2001; Jones *et al.*, 2008). In humans, nontyphoidal *Salmonella* (NTS) infections are usually self-limiting

but, in some cases, it can become invasive and cause complications such as bacteraemia, focal systemic infections and long-term chronic sequels (Batz *et al.*, 2013).

The virulence of the bacteria is determined by their ability to attach and invade the host intestinal epithelium cells and to survive and replicate within macrophages. Virulence factors involved in these processes are encoded by genes located in *Salmonella* pathogenicity islands (SPIs), *Salmonella* genomic islets (SGIs), fimbrial operons, prophage DNA integrated in the chromosome and virulence plasmids (Van Asten and Van Dijk, 2005; Fàbrega and Vila, 2013). Some of these elements are conserved among serovars, such as SPI-1 and SPI-2 (encoding factors required for invasion and intracellular survival and replication, respectively), while others are adapted to specific serovars. Furthermore, *Salmonella* are capable to acquire new virulence-associated genes by the intra/inter-species horizontal transference of mobile genetic elements which is associated with the emergence of novel pathogenicity phenotypes (Gyles and Boerlin, 2014). Therefore, the expression of certain virulence genes can determine the pathogenicity of *Salmonella* strains and their capacity to infect different hosts as well as the severity of the infection.

Given the need to improve the understanding of the role of domestic and wild birds in the epidemiology of *Salmonella*, we investigated isolates of a wide variety of zoonotic serovars from different hosts niches (human, poultry and seagulls) to assess the existence of common *Salmonella* genotypes circulating among compartments and to determine the presence of virulence-associated genes in isolates from different origins but with highly similar genotypes.

6.3. MATERIALS AND METHODS

6.3.1. Bacterial isolates

A total of 742 *Salmonella* isolates of 19 different serovars from humans (N=155), poultry (N=382) and seagulls (N=205) were characterized in this study (Table 6.1). Clinical human isolates were obtained from gastroenteritis cases occurred during the period 2009-2014 in two hospitals (63 from hospital A and 94 from hospital B) from Catalonia (Spain) (Figure 6.1). Isolates from poultry (laying hens, N= 137; chickens, N= 122; turkeys, N= 49; quails, N=39; partridges, N=18; other domestic birds, N=17) from different farms located in the Northeast of Spain (Catalonia, Valencia and Aragon) were collected at the Poultry Health Centre of Catalonia and Aragon (CESAC) between 2008 and 2012. Additionally, we included in the study a collection of isolates from fledgling seagulls of two different species from nine colonies of the Western Mediterranean and Eastern Atlantic coasts, sampled between 2009 and 2011 (Antilles, 2014). These isolates were obtained from yellow-legged gulls (*Larus michahellis*, N=173) from Ebro Delta, Medes Islands, Columbretes Island, Dragonera Island, Zembra Island, Ons Island, Lanzarote Island and Tenerife Island; and Audouin's gulls (*L. audouinii*, N=32) from Ebro Delta and Alboran Island.

Serotyping of *Salmonella* isolates was carried out according to the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007). *Salmonella* isolates were preserved frozen at -80° C in Brain Heart Infusion broth (BHI, Merck KGaA, Darmstadt, Germany) supplemented with 20% glycerol. Fresh cultures of the isolates were prepared onto TSA (Difco, Madrid, Spain), and plates were incubated at 37° C for 24 h. The extraction of DNA templates from a bacterial suspension in PBS was performed using InstaGene® Matrix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

Table 6.1. Number of *Salmonella* isolates of nontyphoidal serovars included in the study and originating from different hosts and localities.

Host (Code)	**Serovar**																			
	Braenderup	**Bredeney**	**Derby**	**Enteritidis**	**Goldcoast**	**Grumpensis**	**Hadar**	**Indiana**	**Infantis**	**Kentucky**	**Kottbus**	**Mikawasima**	**Newport**	**Rissen**	**Senftenberg**	**Stanley**	**Typhimurium**	**Virchow**	**Wien**	**Total**
Humans																				
Hospital A (HR)	0	0	4	6	0	1	0	0	0	0	0	1	1	2	0	1	44	0	1	61
Hospital B (HT)	0	2	0	2	1	1	5	1	2	2	0	0	0	8	0	0	69	1	0	94
Total humans	0	2	4	8	1	2	5	1	2	2	0	1	1	10	0	1	113	1	1	155
Poultry																				
Chicken (CC)	0	0	0	28	0	0	18	0	0	3	2	0	0	1	0	0	22	48	0	122
Laying hen (CH)	1	2	0	59	2	0	23	0	1	0	2	0	0	0	2	0	23	22	0	137
Turkey (CT)	0	0	0	0	0	0	25	0	0	0	4	0	1	0	0	0	19	0	0	49
Partridge (CP)	0	0	0	1	0	0	4	0	0	0	0	0	0	0	0	0	11	2	0	18
Quail (CQ)	0	1	0	0	0	0	13	1	1	0	0	0	0	0	0	0	19	4	0	39
Other (CO)	0	1	0	4	1	0	3	0	0	2	1	0	0	0	0	0	3	1	1	17
Total poultry	1	4	0	92	3	0	86	1	2	5	9	0	1	1	2	0	97	77	1	382

Continued

Table 6.1. Continued.

Host (Code)	Serovar																			
	Braenderup	**Bredeney**	**Derby**	**Enteritidis**	**Goldcoast**	**Grumpensis**	**Hadar**	**Indiana**	**Infantis**	**Kentucky**	**Kottbus**	**Mikawasima**	**Newport**	**Rissen**	**Senftenberg**	**Stanley**	**Typhimurium**	**Virchow**	**Wien**	**Total**
Seagulls																				
Yellow-legged gulls																				
Columbretes Is. (CM)	2	1	3	3	0	0	1	0	1	4	2	0	2	1	0	0	23	0	0	43
Medes Is. (MM)	0	0	7	1	0	0	3	0	0	0	0	1	0	2	14	0	18	0	1	47
Ebro Delta (DM)	2	2	1	1	0	1	6	0	0	0	0	0	0	1	0	0	10	2	1	27
Dragonera Is. (DGM)	2	0	4	0	0	0	4	0	0	0	0	0	0	0	0	0	7	0	0	17
Ons Is. (GAM)	0	0	1	1	1	0	0	0	3	0	0	0	1	0	0	0	10	0	0	17
Tenerife (GM)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	2
Lanzarote (MCM)	0	1	0	1	0	0	0	0	0	0	0	0	3	0	0	0	2	1	0	8
Zembra Is. (ZM)	0	0	0	3	0	0	1	0	0	3	0	0	0	0	0	0	5	0	0	12
Audouin's gulls																				
Alboran Is. (AA)	0	0	0	2	0	0	1	0	1	3	0	0	2	0	0	0	0	0	0	9
Ebro Delta (DA)	0	0	0	0	0	0	1	0	0	3	3	0	1	0	0	3	11	0	1	23
Total seagulls	6	4	16	12	1	1	17	0	5	13	5	1	9	4	14	3	88	3	3	205
Total	7	10	20	112	5	3	108	2	9	20	14	2	11	16	16	4	298	81	5	742

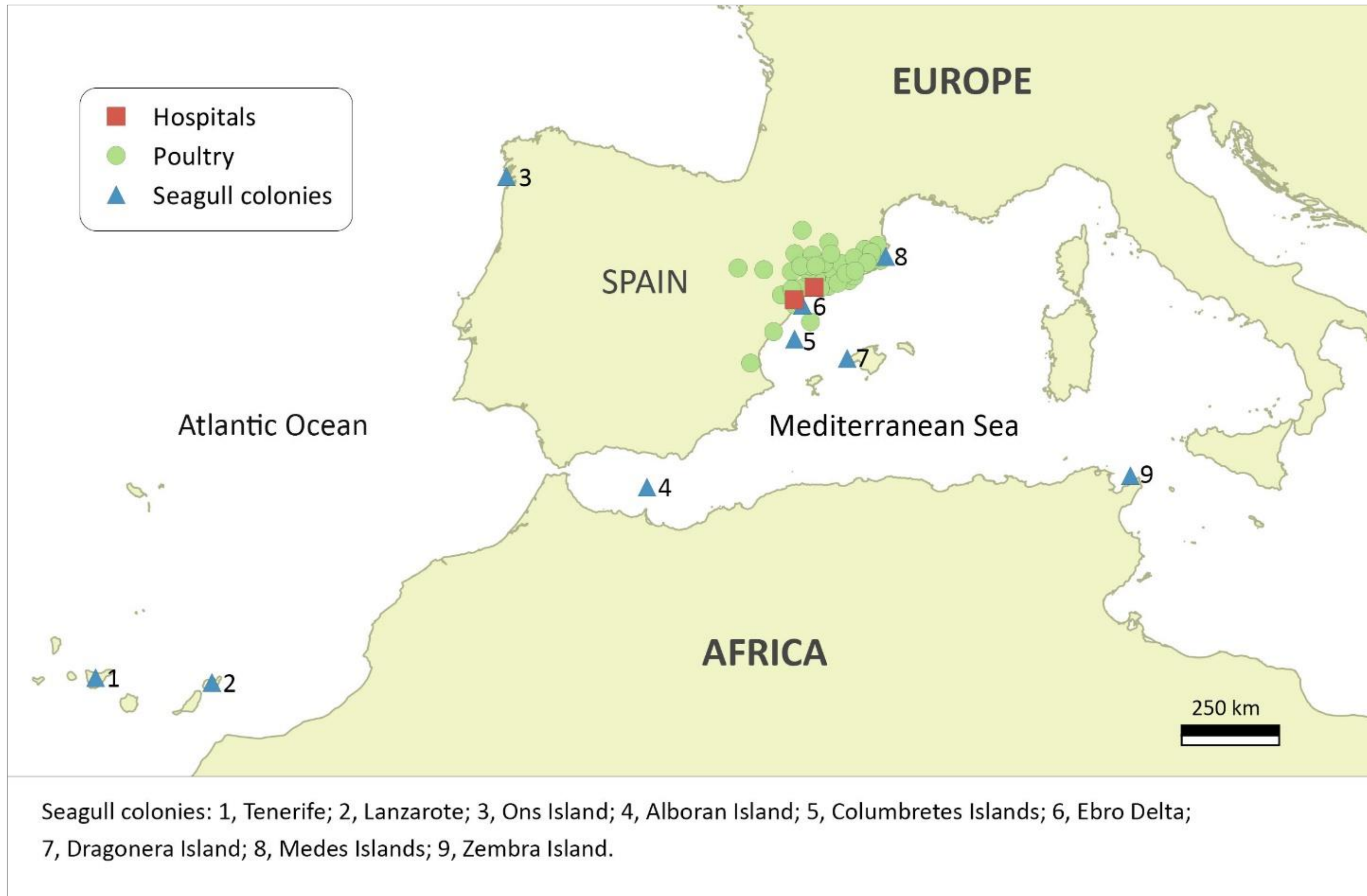


Figure 6.1. Origin of *Salmonella* isolates from different hosts included in this study.

6.3.2. PFGE

Altogether 742 *Salmonella* isolates were analysed by PFGE using the restriction endonuclease XbaI (Roche Applied Science, Indianapolis, IN, USA). A selection of 288 isolates from diverse sources, with same XbaI-PFGE profile, were further genotyped using a secondary restriction enzyme (BlnI) to enhance the discrimination power (Table 6.2). PFGE was carried out according to the standard operating procedure of PulseNet (www.pulsenetinternational.org) and using a CHEF-DR II System (Bio-Rad Laboratories, Hercules, CA). Besides, thiourea was employed during gel electrophoresis when necessary. *Salmonella* Braenderup H9812 restricted with XbaI was used as molecular size standard. The resulting PFGE patterns were transferred to the Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA) for computer analysis. Similarities between banding patterns was determined on the basis of the Dice coefficient with a tolerance and optimization of 1.0%. Cluster analysis was performed by the Unweighted Pair Group Method with Arithmetic averages (UPGMA). Isolates with a minimum level of similarity ≥ 85% were considered as the same pulsotype (XB). Simpson's diversity index (D) of XbaI-PFGE in each compartment (human, domestic and wild) was calculated by the method of Hunter and Gaston (1988). The D-value ranges between 0 and 1; 0 value indicates that all isolates are identical, while a D-value of 1 indicates a maximum diversity.

6.3.3. MLST

A subset of 29 isolates of the most significant clinical serovars (21 of Typhimurium and eight of Enteritidis) and covering human, poultry and seagull origins was chosen for further genotyping by MLST. Fragments of seven housekeeping genes (*aroC, dnaN, hemD, hisD, purE, sucA* and *thrA*) were amplified by PCR and sequenced following the protocol and recommended primers at *S. enterica* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). Sanger sequences were analysed using the Fingerprinting II v3.0 software (Bio-Rad, Hercules, CA, USA). Alleles and sequence

types (STs) were assigned based on the MLST scheme provided on the database at *S. enterica* MLST web site.

6.3.4. Virulence-associated genes

A total of 58 isolates of 12 serovars from different hosts with XbaI and BlnI PFGE pulsotypes in common were screened to characterize their potential pathogenicity. We analysed the presence of 27 virulence-associated genes harboured in *Salmonella* pathogenicity island (SPI)-1 (*orgA, sitC, invA, prgH* and *avrA*), SPI-2 (*spiC*), SPI-3 (*misL, mgtC*), SPI-4 (*orfL*), SPI-5 (*pipD* and *sopB*), pathogenicity islets (*tolC, iroN, sifA* and *cdtB*), fimbrial operons (*agfA, agfC, lpfA, lpfC* and *sefC*), plasmids (*spvB, spvC* and *pefA*) and prophages (*sopE, gipA*, *gogB* and *gtgB*). The primer pairs employed alone or in multiplex PCRs are compiled in Table 6.3. PCR was performed in a 25 µL reaction mixture that included a ready-to-use PCR Master Mix solution (Promega, Wisconsin, USA), 0.4 µM of each primer and and 2.5 µL of DNA. Amplification cycles were the following: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 2 min; and final extension at 72°C for 10 min. Amplicons were detected by gel electrophoresis in a 1.8% agarose gels with 0.2 µg/ml of ethidium bromide. Isolates with the same combination of virulence-associated genes were considered as the same virulotype (VT).

6.3.5. Statistical analyses

Pearson's chi-squared test with Yates's correction for continuity was performed to compare frequencies of virulence-associated genes among different *Salmonella* serovars and host origins. Fisher's exact tests were used when needed. $P < 0.05$ was considered statistically significant. The Deducer GUI of R software (www.R-project.org) was used for the statistical analysis.

Table 6.2. *Salmonella* isolates analysed by PFGE with primary and secondary enzymes (XbaI and BlnI) and the resulting pulsotypes.

Serovar	Humans		Poultry		Gulls		Total[a]	
	XbaI[b]	XbaI&BlnI[c]	XbaI	XbaI&BlnI	XbaI	XbaI&BlnI	XbaI	XbaI&BlnI
Braenderup	0	0	1 (1)	0	6 (3)	0	7 (4)	0
Bredeney	2 (1)	0	4 (2)	3 (3)	4 (4)	2 (2)	10 (7)	5 (4)
Derby	4 (1)	1 (1)	0	0	16 (6)	3 (2)	20 (6)	4 (2)
Enteritidis	8 (2)	5 (2)	92 (4)	20 (3)	12 (2)	8 (2)	112 (4)	33 (3)
Goldcoast	1 (1)	1 (0)[d]	3 (2)	3 (0)[d]	1 (1)	0	5 (3)	4 (0)
Grumpensis	2 (1)	1 (1)	0	0	1 (1)	1 (1)	3 (1)	2 (1)
Hadar	5 (1)	1 (1)	86 (3)	14 (3)	17 (2)	11 (2)	108 (3)	26 (3)
Indiana	1 (1)	0	1 (1)	0	0	0	2 (2)	0
Infantis	2 (1)	1 (1)	2 (1)	2 (2)	5 (1)	3 (2)	9 (2)	6 (3)
Kentucky	2 (1)	1 (1)	5 (3)	2 (1)	13 (2)	5 (3)	20 (4)	8 (3)
Kottbus	0	0	9 (3)	5 (1)	5 (1)	5 (2)	14 (3)	10 (2)
Mikawasima	1 (1)	1 (1)	0	0	1 (1)	1 (1)	2 (1)	2 (1)
Newport	1 (1)	1 (1)	1 (1)	1 (0)[d]	9 (7)	3 (2)	11 (7)	5 (3)
Rissen	10 (3)	4 (4)	1 (1)	1 (1)	5 (3)	2 (2)	15 (5)	7 (6)

Continued

Table 6.2. Continued.

Serovar	Humans		Poultry		Gulls		Total[a]	
	XbaI[b]	XbaI&BlnI[c]	XbaI	XbaI&BlnI	XbaI	XbaI&BlnI	XbaI	XbaI&BlnI
Senftenberg	0	0	2 (2)	0	14 (3)	0	16 (5)	0
Stanley	1 (1)	0	0	0	3 (1)	0	4 (2)	0
Typhimurium	113 (12)	57 (20)[d]	97 (12)	43 (15)[d]	88 (27)	47 (25)	298 (33)	147 (41)
Virchow	1 (1)	1 (1)	77 (3)	22 (6)	3 (1)	2 (2)	81 (3)	25 (7)
Wien	1 (1)	0	1 (1)	1 (1)	3 (3)	3 (3)	5 (4)	4 (4)
Total	155 (30)	75 (34)	382 (40)	117 (36)	206 (68)	96 (51)	742 (99)	288 (83)

a) Total number of isolates genotyped and resulting pulsotypes when analysing all isolates of the three different origins together.

b) Number of isolates genotyped with XbaI enzyme; in brackets, the number of different XbaI-PFGE profiles obtained.

c) Number of isolates genotyped with XbaI and BlnI enzymes; in brackets, the number of different pulsotypes obtained as a result of the combination of XbaI and BlnI PFGE profiles.

d) Four isolates of *S*. Goldcoast, one of *S*. Newport and two of *S*. Typhimurium were not typable with the secondary enzyme BlnI.

Table 6.3. PCR primers used for *Salmonella* virulence-associated genes detection.

Gene	Sequence forward / reverse primers (5' to 3')	PCR	Amplicon size (bp)	Reference
SPI-1[a]				
avrA	CCTGTATTGTTGAGCGTCTGG / AGAAGAGCTTCGTTGAATGTCC	multiplex V	425	Huehn et al., 2010
invA	CTGGCGGTGGGTTTTGTTGTCTTCTCTATT / AGTTTCTCCCCCTCTTCATGCGTTACCC	monoplex	1070	Skyberg et al., 2006
orgA	TTTTTGGCAATGCATCAGGGAACA / GGCGAAAGCGGGGACGGTATT	multiplex VI	255	Skyberg et al., 2006
prgH	GCCCGAGCAGCCTGAGAAGTTAGAAA / TGAAATGAGCGCCCCTTGAGCCAGTC	multiplex VI	657	Skyberg et al., 2006
sitC	CAGTATATGCTCAACGCGATGTGGGTCTCC / CGGGGCGAAAATAAAGGCTGTGATGAAC	multiplex II	768	Skyberg et al., 2006
SPI-2				
spiC	CCTGGATAATGACTATTGAT / AGTTTATGGTGATTGCGTAT	monoplex	301	Hughes et al., 2008
SPI-3				
mgtC	TGACTATCAATGCTCCAGTGAAT / ATTTACTGGCCGCTATGCTGTTG	multiplex IV	655	Soto et al., 2006
misL	GTCGGCGAATGCCGCGAATA / GCGCTGTTAACGCTAATAGT	monoplex	561	Hughes et al., 2008
SPI-4				
orfL	GGAGTATCGATAAAGATGTT / GCGCGTAACGTCAGAATCAA	monoplex	332	Hughes et al., 2008
SPI-5				
pipD	CGGCGATTCATGACTTTGAT / CGTTATCATTCGGATCGTAA	multiplex II	399	Hughes et al., 2008
sopB	CGGACCGGCCAGCAACAAAACAAGAAGAAG / TAGTGATGCCCGTTATGCGTGAGTGTATT	multiplex III	220	Skyberg et al., 2006
Islet				
cdtB	ACAACTGTCGCATCTCGCCCCGTCATT / CAATTTGCGTGGGTTCTGTAGGTGCGAGT	monoplex	268	Skyberg et al., 2006
iroN	ACTGGCACGGCTCGCTGTCGCTCTAT / CGCTTTACCGCCGTTCTGCCACTGC	multiplex IV	1205	Skyberg et al., 2006
sifA	TTTGCCGAACGCGCCCCCACACG / GTTGCCTTTTCTTGCGCTTTCCACCCATCT	multiplex III	449	Skyberg et al., 2006
tolC	TACCCAGGCGCAAAAAGAGGCTATC / CCGCGTTATCCAGGTTGTTGC	multiplex VI	161	Skyberg et al., 2006

Continued

Table 6.3. Continued.

Gene	Sequence forward / reverse primers (5' to 3')	PCR	Amplicon size (bp)	Reference
Fimbrial operon				
agfA	TGCAAAGCGATGCCCGTAAATC / TTAGCGTTCCACTGGTCGATGGTG	multiplex V	151	Bäumler et al., 1997
agfC	CTTTATTGCTCCTTGCCGC / GAAGGCGGCCATTGTTGTGA	multiplex IV	310	Gibson et al., 2007
lpfA	TTGCTCTGTCTGCTCTCGCTGTAG / CATGATTCTCTTCCTGAGCCTCC	multiplex II	250	Bäumler et al., 1995
lpfC	GCCCCGCCTGAAGCCTGTGTTGC / AGGTCGCCGCTGTTTGAGGTTGGATA	multiplex V	641	Skyberg et al., 2006
sefC	GCGAAAACCAATGCGACTGTAG / CCCACCAGAAACATTCATCCC	monoplex	1103	Bäumler et al., 1997
Plasmid				
pefA	GCGCCGCTCAGCCGAACCAG / GCAGCAGAAGCCCAGGAAACAGTG	multiplex III	157	Skyberg et al., 2006
spvB	CTATCAGCCCCGCACGGAGAGCAGTTTTTA / GGAGGAGGCGGTGGCGGTGGCATCATA	multiplex I	717	Skyberg et al., 2006
spvC	CTTGCACAACCAAATGCGGAAGAT / CTCTGCATTTCACCACCATCACG	multiplex I	571	Agron et al., 2001
Phage				
gipA	GCAAGCTGTACATGGCAAAG / GGTATCGGTGACGAACAAAT	multiplex VII	212	Mikasova et al., 2005
gogB	GCTCATCATGTTACCTCTAT / AGGTTGGTATTTCCCATGCA	multiplex VII	598	Drahovska et al., 2007
gtgB	TGCACGGGGAAAACTACTTC / TGATGGGCTGAAACATCAAA	multiplex VII	436	Mikasova et al., 2005
sopE	TCAGTTGGAATTGCTGTGGA / TCCAAAAACAGGAAACCACAC	monoplex	642	Hughes et al. 2008

a) SPI-1: *Salmonella* pathogenicity island 1; SPI-2: *Salmonella* pathogenicity island 2; SPI-3; *Salmonella* pathogenicity island 3; SPI-4: *Salmonella* pathogenicity island 4; SPI-5: *Salmonella* pathogenicity island 5.

6.4. RESULTS

6.4.1. PFGE

All the 742 *Salmonella* isolates, belonging to 19 serovars, from different hosts included in this study were analysed by PFGE using the restriction endonuclease XbaI to determine the genetic relatedness amongst them. Up to 99 different XbaI-PFGE profiles were obtained with a similarity level of 85% (Table 6.2). Overall, a high genetic diversity was detected in *Salmonella* isolates from seagulls (68 profiles; D = 0.970) followed by isolates from human gastroenteritis cases (30 profiles; D = 0.914). However, isolates from poultry presented a lower diversity (40 profiles; D = 0.881) despite coming from different domestic host species and farms distributed in a broad region of the Northeastern Spain.

We found 30 XbaI-PFGE profiles grouping isolates from two or three different origins (human, domestic and wild birds). The 88% of clinical human isolates (136/155) showed profiles also detected in isolates from poultry (six profiles), seagulls (five profiles) or both domestic and wild birds (nine profiles). Twenty-five profiles from poultry (80%, 305/382) overlapped with those from seagulls (ten profiles), humans or both. Conversely, the 48% of isolates (98/205) from gulls exhibited unique profiles not detected in other hosts.

Seventeen unique profiles were detected exclusively in *S*. Typhimurium isolates from seagulls. One of these profiles was found in 14 isolates from six different far apart gull colonies, whereas the others were present in a single or a few isolates from one or more gull species and colonies. In spite of the overall genetic diversity observed in *S*. Typhimurium isolates (33 profiles), five predominant profiles were detected in a high number of isolates (11 to 90) from the three different niches. The most abundant profile included the majority of *S*. Typhimurium isolates (67%, 65/97) from a wide range of domestic birds (laying hens, chickens, turkeys, partridges and quails) in addition to some isolates from humans (15/113) and seagulls (8/88) from different colonies. The other

predominant profiles were mostly detected in *S*. Typhimurium clinical isolates (16 to 26) besides other hosts. On the other hand, isolates of *S*. Enteritidis and *S*. Hadar showed an overall low genetic diversity and predominant profiles were repeatedly observed in a high number of isolates. In fact, most of *S*. Enteritidis isolates from poultry (77/92) showed the same profile as isolates from humans (7/8) and seagulls (11/12) from distinct locations.

Based on the XbaI PFGE results, a representative set of *Salmonella* isolates from different hosts and sites with the same or highly similar profile were selected for further genotyping with a secondary restriction enzyme (BlnI) to increase the discrimination power. Thus, 288 isolates (117 from poultry, 96 from seagulls and 75 from humans) of 15 different serovars were analysed by BlnI-PFGE. Isolates of Braenderup, Indiana, Senftenberg and Stanley serovars were not genotyped with BlnI because the low degree of similarity detected using XbaI. Seven isolates (four of *S*. Goldcoast, two of *S*. Typhimurium and one of *S*. Newport) were non-typable using BlnI. The combined dendrogram with XbaI and BlnI band patterns revealed 83 distinct profiles (PFGE pulsotypes) with a similarity of 85% (Table 6.2). Isolates were grouped according to their serovar, except for serovars Hadar and Kottbus which were clustered together although forming separate branches (Figure 6.2).

We detected 30 XbaI and BlnI PFGE pulsotypes containing isolates from two or three different hosts (Figures 6.2 and 6.3). These pulsotypes in common corresponded mainly to serovar Typhimurium, followed by Enteritidis, Virchow, and other serovars also of public health significance (Bredeney, Derby, Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima, and Rissen). Noteworthy, the XbaI profiles with a high similarity in isolates of *S*. Goldcoast, *S*. Newport and *S*. Wien from different hosts were distinguished using BlnI. At the same time, the restriction with BlnI increased the resolution among isolates of other serovars and allowed to discriminate more pulsotypes whilst maintaining similarities among the different host niches. *S*. Typhimurium isolates from different niches shared a total of 16 pulsotypes; the most

frequent of them (XB20) was detected in 31 isolates from the three different niches, especially from poultry (22 isolates). Other pulsotypes with high occurrence were XB8 and XB1 which were found in 25 *S*. Enteritidis and 21 *S*. Hadar isolates, respectively, from the three different compartments.

6.4.2. Multilocus sequence typing

MLST of 29 isolates of *S*. Typhimurium (N=21) and *S*. Enteritidis (N=8) from different sources with the same or highly related pulsotypes (similarity over 80%) was performed to determine the potential evolutionary relatedness among them. We detected two STs of *S.* Typhimurium differing only by a single locus (*dnaN* gene: alleles 7 and 19), belonging to the same clonal complex, also denominated eBurstGroup (eGB1) (Figure 6.2). Fourteen isolates were classified as ST34 and seven belonged to ST19. These STs grouped five and three different pulsotypes, respectively, represented by isolates from different hosts and sites far apart. All *S*. Enteritidis isolates originating from humans, poultry and seagulls were clustered into a single sequence type ST11 although two different pulsotypes were distinguished by PFGE (XB8 and XB9).

Figure 6.2. Combined dendrogram with XbaI and BlnI profiles of a selection of *Salmonella* isolates from different hosts showing the same or highly similar pulsotypes. The tree was constructed using the UPGMA clustering method and the Dice coefficient for the similarity matrices calculation. Pulsotypes (XB) numbers were assigned to XbaI and BlnI PFGE profiles with a similarity level of 85%. Sequence types (ST) and eBurstGroups (eBG) not analysed are labelled as NA (Non-available). Virulotypes (VT) are indicated by a number corresponding to the number of positive virulence genes and a capital letter regarding to the different profiles detected. The presence of virulence-associated genes is depicted as grey squares. Dark grey squares stand out isolates from different hosts with XB and VT in common. YL gull: yellow-legged gull; AD gull: Audouin's gull.

40 50 60 70 80 90 100

ST11

Isolate	Serovar	Host	Location	Year	XB	ST	eBG	VT
CM104-S1	Hadar	YL gull	Columbretes Is.	2010	1	NA	NA	20A
DA187-S2	Hadar	AD gull	Ebro Delta	2011	1	NA	NA	20A
HT15	Hadar	Human	Catalonia	2009	1	NA	NA	20B
CS77	Hadar	Turkey	Catalonia	2010	1	NA	NA	20B
DA230-S4	Kottbus	AD gull	Ebro Delta	2011	2	NA	NA	18A
CS10	Kottbus	Turkey	Catalonia	2008	2	NA	NA	20B
CM214-S1	Kottbus	YL gull	Columbretes Is.	2011	2	NA	NA	20B
MM141-S1	Hadar	YL gull	Medes Is.	2010	3	NA	NA	16B
CS49	Hadar	Chicken	Catalonia	2009	3	NA	NA	11A
HT9	Kentucky	Human	Catalonia	2011	4	NA	NA	20B
CS382	Kentucky	Chicken	Catalonia	2012	4	NA	NA	20B
DA216-S3	Kentucky	AD gull	Ebro Delta	2011	4	NA	NA	18A
DGM64-S1	Derby	YL gull	Dragonera Is.	2011	5	NA	NA	11B
HR59	Derby	Human	Catalonia	2013	5	NA	NA	16C
HT20	Infantis	Human	Catalonia	2010	6	NA	NA	20B
CS284	Infantis	Hen	Catalonia	2010	6	NA	NA	20B
AA114-S1	Infantis	AD gull	Alboran Is.	2010	6	NA	NA	20B
MM207-S3	Mikawasima	YL gull	Medes Is.	2011	7	NA	NA	21A
HR43	Mikawasima	Human	Catalonia	2013	7	NA	NA	21A
AA145-S1	Enteritidis	AD gull	Alboran Is.	2010	8	11	4	18B
CS218	Enteritidis	Chicken	Catalonia	2010	8	11	4	18C
ZM11-S1	Enteritidis	YL gull	Zembra	2009	8	11	4	20C
HR41	Enteritidis	Human	Catalonia	2013	8	11	4	25A
CS7	Enteritidis	Hen	Catalonia	2008	8	11	4	15A
MM212-S1	Enteritidis	YL gull	Medes Is.	2011	9	11	4	22C
HR6	Enteritidis	Human	Catalonia	2013	9	11	4	16D
CS290	Enteritidis	Hen	Catalonia	2010	9	11	4	20D

Virulence-associated genes

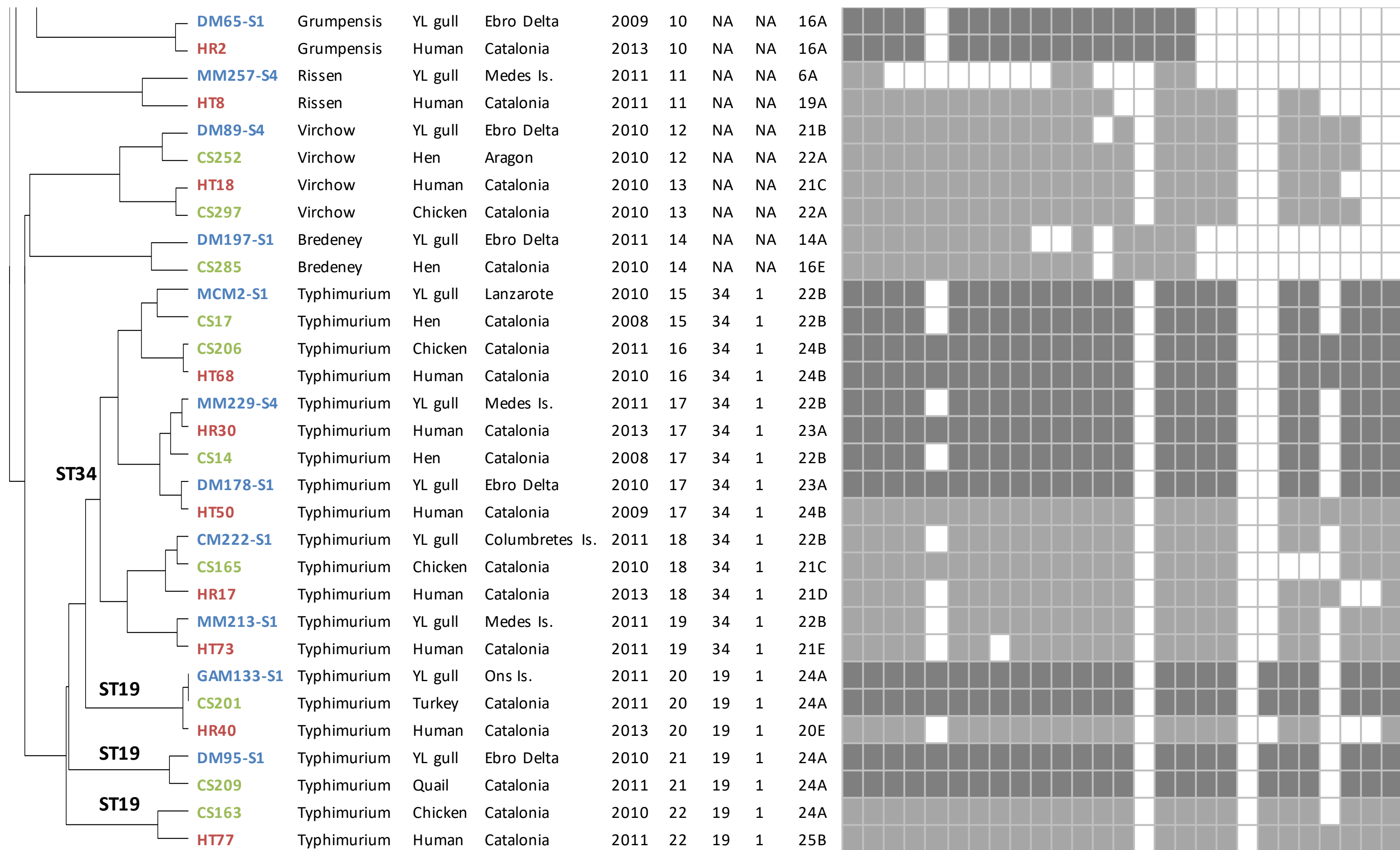
SPI 1-5: *invA*, *orgA*, *prgH*, *sitC*, *avrA*, *spiC*, *mgtC*, *misL*, *orfL*, *pipD*, *sopB*

Islet: *tolC*, *iroN*, *sifA*, *cdtB*

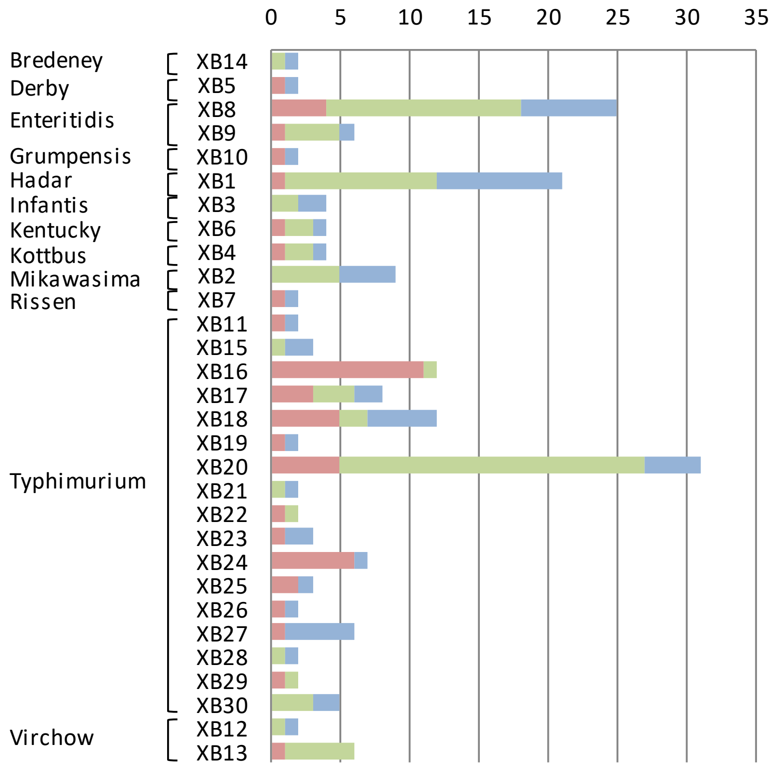
Fimbrial operon: *agfA*, *agfC*, *lpfA*, *lpfC*, *sefC*

Plasmid: *pefA*, *spvB*, *spvC*

Prophage: *sopE*, *gipA*, *gogB*, *gtgB*



DM65-S1 Grumpensis YL gull Ebro Delta 2009 10 NA NA 16A
HR2 Grumpensis Human Catalonia 2013 10 NA NA 16A
MM257-S4 Rissen YL gull Medes Is. 2011 11 NA NA 6A
HT8 Rissen Human Catalonia 2011 11 NA NA 19A
DM89-S4 Virchow YL gull Ebro Delta 2010 12 NA NA 21B
CS252 Virchow Hen Aragon 2010 12 NA NA 22A
HT18 Virchow Human Catalonia 2010 13 NA NA 21C
CS297 Virchow Chicken Catalonia 2010 13 NA NA 22A
DM197-S1 Bredeney YL gull Ebro Delta 2011 14 NA NA 14A
CS285 Bredeney Hen Catalonia 2010 14 NA NA 16E
MCM2-S1 Typhimurium YL gull Lanzarote 2010 15 34 1 22B
CS17 Typhimurium Hen Catalonia 2008 15 34 1 22B
CS206 Typhimurium Chicken Catalonia 2011 16 34 1 24B
HT68 Typhimurium Human Catalonia 2010 16 34 1 24B
MM229-S4 Typhimurium YL gull Medes Is. 2011 17 34 1 22B
HR30 Typhimurium Human Catalonia 2013 17 34 1 23A
CS14 Typhimurium Hen Catalonia 2008 17 34 1 22B
DM178-S1 Typhimurium YL gull Ebro Delta 2010 17 34 1 23A
HT50 Typhimurium Human Catalonia 2009 17 34 1 24B
CM222-S1 Typhimurium YL gull Columbretes Is. 2011 18 34 1 22B
CS165 Typhimurium Chicken Catalonia 2010 18 34 1 21C
HR17 Typhimurium Human Catalonia 2013 18 34 1 21D
MM213-S1 Typhimurium YL gull Medes Is. 2011 19 34 1 22B
HT73 Typhimurium Human Catalonia 2011 19 34 1 21E
GAM133-S1 Typhimurium YL gull Ons Is. 2011 20 19 1 24A
CS201 Typhimurium Turkey Catalonia 2011 20 19 1 24A
HR40 Typhimurium Human Catalonia 2013 20 19 1 20E
DM95-S1 Typhimurium YL gull Ebro Delta 2010 21 19 1 24A
CS209 Typhimurium Quail Catalonia 2011 21 19 1 24A
CS163 Typhimurium Chicken Catalonia 2010 22 19 1 24A
HT77 Typhimurium Human Catalonia 2011 22 19 1 25B
ST34
ST19
ST19
ST19



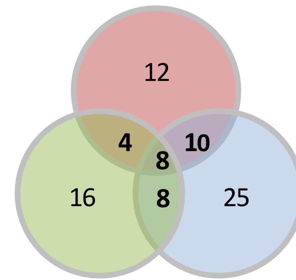


Figure 6.3. *Salmonella* isolates from different hosts with XbaI and BlnI PFGE pulsotypes (XB) in common.

6.4.3. Virulence genes

We examined the presence of 27 virulence-associated genes in 58 NTS isolates from different hosts showing the same or highly similar PFGE pulsotype. All isolates were positive for six or more of the tested genes and at least 20 genes were present in most isolates (78%, 42/58) (Figure 6.2). We observed a high prevalence (>76%) of genes located in SPIs (SPI-1 to SPI-5) which were highly conserved among different NTS serovars (Table 6.4). The *invA* and *orgA* genes were present in all studied isolates. However, the *avrA* gene was absent in *S*. Grumpensis and showed a relative low frequency in *S*. Typhimurium isolates (57%). The 59% of isolates possessed all SPIs genes tested, but one *S*. Rissen isolate (MM257-S4) from a seagull was deficient in many SPI-1 genes (8/11).

On the other hand, the islet gene *tolC* and the fimbrial operon genes *agfA* and *agfC* were detected in all isolates. In contrast, the presence of other virulence-associated genes was more variable. Some genes were found in almost all serovars except for Bredeney (*iroN* and *lpfAC* genes), Grumpensis (*lpfAC* genes) and Rissen (*sifA* gene). However, other genes were only detected in specific serovars, such as the fimbrial gene *sefC* in *S*. Enteritidis. Moreover, the *cdtB* gene encoding for the *Salmonella* cytolethal distending toxin was only found in four isolates of serovars Bredeney and Grumpensis. The target genes for virulence determinants located in plasmids (*spvB* and *spvC*) were observed with a high prevalence (84%) in isolates of many serovars, except for Bredeney, Derby and Grumpensis. Yet the plasmid-located fimbrial gene *pefA* was only present in some *S*. Enteritidis and Typhimurium isolates (75% and 29%, respectively). The prophage genes were detected in a low frequency and only in a few serovars, mainly Enteritidis and Typhimurium. The *sopE* gene involved in invasion of host cells was present in most of *S*. Enteritidis isolates (88%) and in some isolates of *S*. Typhimurium (33%) and *S*. Hadar (24%). The genes associated with the lamboid Gifsy-1 (*gipA* and *gogB*) and Gifsy-2 (*gtgB*) phages were only found in *S*. Typhimurium (*gipA*, *gogB* and *gtgB*), *S*. Enteritidis (*gogB* and *gtgB*) and *S*. Mikawasima (*gtgB*).

We detected a high number of positive virulence-associated genes (21 to 25) in isolates of serovars Enteritidis, Typhimurium and Virchow from the three different niches. No statistically significant differences were detected in virulence gene prevalences or distribution among isolates from humans, poultry and seagulls; except for the *iroN* gene which showed a higher occurrence in isolates from humans compared to those from gulls. The *iroN* gene was present in almost all human clinical isolates (16/17) and in most isolates from poultry (14/18) but only in half of the isolates from seagulls (11/23) (Figure 6.2).

Based on the combination of virulence-associated genes, we observed 33 different virulotypes (VT); ten of them in more than one isolate (two to nine) from different host niches (Figure 6.2). We found the same virulotype (VT20B) in isolates of four serovars (Hadar, Infantis, Kentucky and Kottbus) from different hosts. *S*. Hadar isolates with the same pulsotype (XB1) displayed two different virulotypes: *iroN* gene was present in isolates from human and poultry (VT20B), whilst isolates from seagulls lacked *iroN* and possessed the prophage gene *sopE* (VT20A). Moreover, another virulotype (VT18A), similar to VT20B but lacking genes involved in iron acquisition (*iroN* and *sitC*), was detected in two isolates of different serovars (Kentucky and Kottbus) from the same seagull colony. Noteworthy, *S*. Bredeney and *S*. Grumpensis isolates showed similar virulotypes characterized by the lack of plasmid- and phage-borne virulence genes and lacking also some fimbrial genes; conversely, *cdtB* gene was only detected in those serovars.

We found a unique virulotype in each of the *S*. Enteritidis isolates, despite the low diversity detected by PFGE and MLST, since they were grouped in two different pulsotypes (XB8 and XB9) and a single ST (ST-11). Contrary, four different virulotypes were repeatedly observed in the 76% of *S*. Typhimurium isolates. The same virulotype (VT24A) was observed in five isolates from domestic and wild birds belonging to three pulsotypes (XB20, XB21 and XB22) of the ST19, though it was not detected in closely related isolates of human origin. The remaining three virulotypes (VT22B, VT24B and

VT23A) were detected in isolates corresponding to the five different pulsotypes of the ST34.

Overall, 12 strains with the same pulsotype and virulotype, belonging to serovars Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima and Typhimurium, were found in different host niches (Figure 6.2).

Table 6.4. Occurrence of virulence-associated genes located in different genomic regions in a selection of *Salmonella* isolates of nontyphoidal serovars.

	Serovar												
Gene	**Bredeney**	**Derby**	**Enteritidis**	**Grumpensis**	**Hadar**	**Infantis**	**Kentucky**	**Kottbus**	**Mikawasima**	**Rissen**	**Typhimurium**	**Virchow**	**Total**
SPI-1[a]													
invA	2/2	2/2	8/8	2/2	6/6	3/3	3/3	3/3	2/2	2/2	21/21	4/4	58/58
orgA	2/2	2/2	8/8	2/2	6/6	3/3	3/3	3/3	2/2	2/2	21/21	4/4	58/58
prgH	2/2	1/2	5/8	2/2	4/6	3/3	3/3	3/3	2/2	1/2	21/21	4/4	51/58
sitC	2/2	1/2	4/8	2/2	4/6	3/3	2/3	2/3	2/2	1/2	21/21	4/4	48/58
avrA	2/2	2/2	6/8	0	6/6	3/3	3/3	3/3	2/2	1/2	12/21	4/4	44/58
SPI-2													
spiC	2/2	2/2	6/8	2/2	6/6	3/3	3/3	3/3	2/2	1/2	21/21	4/4	55/58
SPI-3													
mgtC	2/2	1/2	5/8	2/2	4/6	3/3	3/3	3/3	2/2	1/2	21/21	4/4	51/58
misL	2/2	1/2	5/8	2/2	5/6	3/3	3/3	3/3	2/2	1/2	20/21	4/4	51/58
SPI-4													
orfL	2/2	1/2	7/8	2/2	5/6	3/3	3/3	3/3	2/2	1/2	21/21	4/4	54/58
SPI-5													
pipD	1/2	1/2	7/8	2/2	5/6	3/3	3/3	3/3	2/2	1/2	21/21	4/4	53/58
sopB	1/2	2/2	8/8	2/2	6/6	3/3	3/3	3/3	2/2	2/2	21/21	4/4	57/58
Islet													
tolC	2/2	2/2	8/8	2/2	6/6	3/3	3/3	3/3	2/2	2/2	21/21	4/4	58/58
iroN	0	1/2	6/8	2/2	2/6	3/3	2/3	2/3	2/2	1/2	21/21	3/4	41/58
sifA	2/2	2/2	5/8	2/2	6/6	3/3	3/3	3/3	2/2	0	21/21	4/4	51/58
cdtB	2/2	0	0	2/2	0	0	0	0	0	0	0	0	4/58

Continued

Table 6.4. Continued.

Gene	Serovar												
	Bredeney	Derby	Enteritidis	Grumpensis	Hadar	Infantis	Kentucky	Kottbus	Mikawasima	Rissen	Typhimurium	Virchow	Total
Fimbrial operon													
agfA	2/2	2/2	8/8	2/2	6/6	3/3	3/3	3/3	2/2	2/2	21/21	4/4	58/58
agfC	2/2	2/2	8/8	2/2	6/6	3/3	3/3	3/3	2/2	2/2	21/21	4/4	58/58
lpfA	0	1/2	8/8	0	5/6	3/3	3/3	3/3	2/2	1/2	21/21	4/4	51/58
lpfC	0	1/2	8/8	0	5/6	3/3	3/3	3/3	2/2	1/2	21/21	4/4	51/58
sefC	0	0	4/8	0	0	0	0	0	0	0	0	0	4/58
Plasmid													
pefA	0	0	6/8	0	0	0	0	0	0	0	6/21	0	12/58
spvB	0	0	7/8	0	6/6	3/3	3/3	3/3	2/2	1/2	20/21	4/4	49/58
spvC	0	0	7/8	0	6/6	3/3	3/3	3/3	2/2	1/2	20/21	4/4	49/58
Phage													
sopE	0	0	7/8	0	2/6	0	0	0	0	0	5/21	4/4	18/58
gipA	0	0	0	0	0	0	0	0	0	0	19/21	3/4	22/58
gogB	0	0	1/8	0	0	0	0	0	0	0	19/21	0	20/58
gtgB	0	0	8/8	0	0	0	0	0	2/2	0	21/21	0	31/58

a) SPI-1: *Salmonella* pathogenicity island 1; SPI-2: *Salmonella* pathogenicity island 2; SPI-3; *Salmonella* pathogenicity island 3; SPI-4: *Salmonella* pathogenicity island 4; SPI-5: *Salmonella* pathogenicity island 5.

6.5. DISCUSSION

It is well known that domestic and wild birds are important reservoirs of *Salmonella* spp. Antilles (2014) reported that seagulls carried a wide variety of *Salmonella* serovars, most of them associated with human outbreaks, and suggested the contribution of these birds in the transmission of *Salmonella*. In order to investigate the genetic relatedness among NTS present in these seagulls, as well as in poultry and human gastroenteritis cases, we conducted the molecular typing of isolates of 19 serovars from these different host niches.

PFGE revealed a high genetic diversity among *S*. Typhimurium isolates, probably because of the vast number of isolates of this serovar that were analysed. However, this

result may also reflect the heterogenicity of *S*. Typhimurium populations and the different genotypes circulating in the environment. *S*. Typhimurium isolates from seagulls displayed the greatest diversity and almost half of them showed unique profiles not detected neither in poultry nor in humans. However, many of these pulsotypes were found in more than one seagull of both species and colonies far from each other. This may suggest the existence of strains adapted to seagulls, although it could also be due to different infection sources, such as the environment and other livestock apart from poultry. *Salmonella* is present in many animals and has a widespread distribution and survivability in the environment (e.g. soil, animal feed and faeces, and surface waters) (Winfiel and Groisman, 2003; Hoelzer *et al.*, 2011; Levantesi *et al.*, 2012). This, together with the feeding habits and the foraging and migratory movements of seagulls promote their exposure to a wider variety of *Salmonella* infection sources. Thus, these seabirds can act as indicators of the environment *Salmonella* contamination, carrying many different *S*. Typhimurium strains that reflect the genotypic diversity circulating in the habitats where they move (wetlands, swamps and other aquatic ecosystems). Despite yellow-legged gulls and Audouin's gulls are usually resident in a particular region, some juvenile individuals can move long distances, especially when resources are scarce (Christel *et al.*, 2012; Galarza *et al.*, 2012; Arizaga *et al.*, 2014). Also, the movements of seagulls could explain the connectivity among distant colonies which highlight their potential to disseminate the bacteria to other geographic areas.

We detected 17 pulsotypes in common in *S*. Typhimurium isolates from two or all three different host niches. The XB20 and XB18 pulsotypes exhibited a high frequency in *S*. Typhimurium isolates from poultry and humans, respectively, but were also present in seagulls. With regard to *S*. Enteritidis, a low genetic diversity was observed and almost all isolates carried by domestic and wild birds showed the same pulsotypes (XB8 and XB9) as the clinical isolates causing human gastrointestinal cases. Whilst seagulls diet consists mainly of fish, some marine and terrestrial invertebrates, small birds and plants, an important alternative food source comes from human activities (Olsen and Larsson,

2010). Audouin's gulls feed on fisheries discards, marshes and rice fields (Navarro *et al.*, 2010), and yellow-legged gulls frequently scavenge in human waste and sewage that suppose a high risk of becoming infected with pathogens from human and domestic animal origin (Ramos *et al.*, 2010). This behaviour and the occupation of habitats that overlaps with anthropogenic activities, including animal production, could explain the fact that seagulls carried pulsotypes detected with a higher frequency in isolates from humans and poultry.

All *S*. Typhimurium pulsotypes belonged to ST19 and ST34, both predominant in eBG1 (Ashton *et al.*, 2016), while pulsotypes of *S*. Enteritidis corresponded to ST11, the central ST of eBG4 (Achtman *et al.*, 2012). They all are globally spread, largely distributed in animal hosts and their derived products (poultry, pig, cattle, fishmeal) and have been commonly reported to cause human salmonellosis (Enterobase Database, http://enterobase.warwick.ac.uk/). The presence of these ST in seagulls may have contributed to the rapid expansion of these STs in European countries in the last years, which represents a public health concern. Recently, the ST34 has been associated with multidrug-resistant strains (ACSSuT resistance phenotype) carrying transferable plasmids with the mcr-1 mediated colistin resistance gene (Campos *et al.*, 2016; Doumith *et al.*, 2016). Similarly to the *S*. Enteritidis ST11, it has also been associated with *Salmonella* strains producing extended-spectrum β-lactamases (Kim *et al.*, 2011; Yang *et al.*, 2017).

The low diversity observed within the serovar Enteritidis in spite of using two restriction enzymes, and the high genetic similarity detected among hosts may be due to the highly clonal population structure of *S*. Enteritidis circulating in the studied compartments. However, the limited discrimination power of PFGE in isolates of this serovar has been reported in several studies (Boxrud *et al.*, 2007; Ross and Heuzenroeder, 2009; Dewaele *et al.*, 2012). Source-tracking infection of *S*. Enteritidis can be complicated as some pulsotypes may be predominant and widely distributed. In some cases, PFGE can be too discriminatory distinguishing isolates that share a very recent common ancestor or, on

the contrary, less discriminatory grouping isolates with no epidemiological linkages. Therefore, to facilitate the interpretation of PFGE data, it is recommended to use an additional typing method. MLST is a complementary tool that provides a good epidemiological concordance in assessing the evolution of bacterial strains (Barco *et al.*, 2013). In this study, the use of MLST in combination with PFGE did not increase the discriminatory power, supporting the hypothesis that these genotypes are very common and shared among the studied hosts. However, more discriminatory methods, such as whole genome sequence analysis, would be required for a finer genotyping of this highly homogeneous serovar.

Overall, we detected 30 pulsotypes in common in different host niches not only belonging to *S*. Typhimurium and *S*. Enteritidis, but also to other clinical relevant serovars: Bredeney, Derby, Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima and Rissen (EFSA and ECDC, 2017b). Of these, eight pulsotypes were detected in the three host niches, including isolates of serovar Enteritidis, Hadar, Kentucky, Kottbus and Typhimurium. Previous studies have reported same *Salmonella* pulsotypes infecting humans and animals. In different studies conducted in USA, *S*. Enteritidis and *S*. Typhimurium isolates from poultry and bovine, respectively, presented common pulsotypes with clinical human isolates (Oloya *et al.*, 2009; Soyer *et al.*, 2010; Mezal *et al.*, 2014). In the same line, Hauser *et al.* (2011, 2012) identified *S*. Derby and *S*. Infantis pulsotypes frequently isolated in pigs and humans in Germany. They all concluded that these pulsotypes were spread from food-producing animals via the food chain to humans. Moreover, Horton *et al.* (2013) found common *S*. Typhimurium pulsotypes in livestock and different wild birds species and hypothesized that infections in domestic animals could be caused by *Salmonella* strains carried by these birds. Other epidemiological studies described the same pulsotypes of different serovars (Anatum, Enteritidis, Hadar, Infantis, Mikawasima, Rissen and Typhimurium) in humans, domestic birds and/or wild animals (Palomo *et al.*, 2013). Our results are consistent with those previously described and demonstrates an evident association

between NTS strains from humans and animals (either domestic or wild). However, identification of pulsotypes in common in multiple hosts may sometimes not represent a causal relationship and it is not possible to ascertain whether the transmission was from domestic to wild birds, birds to humans or vice-versa. Probably, seagulls get infected when fed on human wastes and contaminated environments. Nevertheless, the fact that seagulls carry NTS strains frequently infecting humans and poultry, suggest that these wild birds may also act as a potential source of infection for livestock and as a reservoir for gastrointestinal infections in humans, which may be regarded as an important threat to public and animal health.

We further characterized the presence of virulence-associated genes in NTS isolates with common pulsotypes in different hosts to assess their pathogenic capacity. As expected, SPIs genes were highly conserved amongst serovars and most isolates presented the complete set of SPIs genes. Nonetheless, we observed a certain degree of variability in gene contents in some serovars, mainly in fimbrial, plasmid and prophage regions.

All isolates possessed the *agfAC* genes encoding for SEF17 fimbriae that enhance adherence to and invasion of eukaryotic cells but also that are involved in biofilm formation and environmental persistence (Austin *et al.*, 1998; Gibson *et al.*, 2007). By contrast, the *sefC* gene encoding for SEF14 fimbriae associated to avian-adapted *Salmonella* serovars, was only present in some *S*. Enteritidis isolates from human and bird origin. Another fimbrial gene, *pefA*, located in a serovar-associated virulence plasmid, was only present in *S*. Enteritids and *S*. Typhimurium. These results are consistent with those reported by Rahman *et al.* (2000) who found both *sefC* and *pefA* genes in *S*. Enteritidis, while *S*. Typhimurium only carried the *pefA* gene. The fimbrial *pefA* gene is often, but not always, harboured in the same virulence plasmid containing the *spv* operon involved in intra-macrophage survival and multiplication (Skyberg *et al.*, 2006). However, we observed that the 76% of isolates positive for *spv* genes lacked the *pefA* gene. The virulence plasmid carrying the *spv* operon has been previously identified

in several serovars (Feng *et al.*, 2012). We detected the *spvBC* genes, in almost all NTS isolates (84%), except for serovars Bredeney, Derby and Grumpensis. These results contrast with the findings of Huehn *et al.* (2010), who detected the *spvC* gene only in *S.* Enteritidis and *S.* Typhimurium.

Regarding the genes of prophage origin, we detected them with a low prevalence and only in *S*. Enteritidis, *S*. Typhimurium and few isolates of serovars Hadar and Virchow (only *gipA* and *sopE* genes) or Mikawasima (only *gtgB* gene). The *sopE* gene encodes for a T3SS effector protein involved in host cell invasion and has been related with some epidemic strains of *S*. Typhimurium in humans (Mirold *et al.*, 1999). However, we detected this gene at a higher prevalence in *S*. Entertidis (88%) compared to *S*. Typhimurium (24%), in agreement with other authors (Huehn *et al.*, 2010; Capuano *et al.*, 2013). Contrary, the gene *gogB* was almost exclusive of *S*. Typhimurium, whilst the *gtgB* gene was found in all tested isolates of both serovars, similarly to what has been reported previously for that gene (Capuano *et al.*, 2013). These genes encoding virulence factors responsible for *Salmonella* survival in Peyer's patches, like the *sopE* gene, can be horizontally transferred by lysogenic conversion among *Salmonella* serovars and also between distantly related Gram-negative bacteria (Figueroa-Bossi *et al.*, 2001). The inclusion and reassortment of phage-associated virulence genes could enable the adaptation of certain *Salmonella* serovars to different environmental conditions and new hosts.

Surprisingly, the presence of *cdtB* gene was detected in *S*. Bredeney and, for the first time, in *S*. Grumpensis isolates from human, poultry and seagull origin, but was absent in other serovars most common in gastroenteritis cases. *Salmonella* cytolethal distending toxin was originally described in *S*. Typhi and cause cell-cycle arrest and severe cell distention (Haghjoo and Galán, 2004). Later, this typhoid-related toxin has also been identified in a variety of NTS serovars, including Bredeney, from humans and poultry (Skyberg *et al.*, 2006; Suez *et al.*, 2013) and it likely induces DNA damage in

eukaryotic cells in a manner similar to how it occurs in *S*. Typhi (Miller and Wiedmann, 2016).

In some cases, the same virulotype was detected in different pulsotypes, but the opposite situation was also observed (isolates with the same pulsotype but different virulotypes). Four different virulotypes were repeatedly observed in the 76% of *S*. Typhimurium isolates. On the contrary, we found a unique virulotype in each *S*. Enteritidis isolate in spite of the low diversity detected by PFGE and MLST. These results indicate that genetic variability in *S*. Enteritidis may be related to the presence of virulence-associated genes mainly located on mobile elements and the characterization of these genes can be a good tool to discriminate isolates of this serovar. However, regardless of the variability of *S*. Enteritidis in gene contents, there was no association between the virulotypes and host origin of isolates.

Overall, we could not detect statistically significant differences in the presence of virulence-associated genes in NTS isolates from different hosts, except for the *iroN* gene that showed a lower frequency in seagulls compared with humans. This gene encodes for a siderophore receptor protein that allows the iron acquisition when bound to the host proteins lactoferrin or transferrin during infection, which is crucial for *Salmonella* survival and proliferation within its host. Acquisition of the *iroN gene along with the iroBCDE* operon by horizontal transfer has been proposed as one of the genetic changes that enables *Salmonella* to benefit from T3SS mediated intestinal inflammation by improving its capability to grow in the gut lumen and to compete with the microbiota (Raffatellu and Bäumler, 2010). However, the existence of redundant iron uptake systems in *Salmonella* suggest that other mechanisms, such as the iron transport system *sitABCD* independent of siderophore (Janakiraman and Slauch, 2000), may be involved in the iron metabolism and compensate for the absence of *iro* gene cluster.

Interestingly, the distribution of virulence-associated genes seems to depend to a greater extent on the serovar than on the host. We did not analyse isolates showing

host-exclusive pulsotypes; in this case we might have found more differences in the virulence determinants repertoire within a serovar. On the other hand, despite the detection of these genes by PCR does not guarantee the expression of the encoding virulence factors, their presence highlights the potential virulence capacity of these *Salmonella* strains. It is therefore tempting to speculate that many NTS strains carried by seagulls may be infectious for humans.

Considering the combination of pulsotypes and virulotypes, we detected 13 overlaps of isolates (including serovars Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima and Typhimurium) from two or three different hosts. Thus, our data support that there is a potential risk of virulent NTS strains transmission between humans and birds. Nevertheless, further research is required to more accurately determine the transmission direction and the relative risk of wild birds spreading the pathogen, as well as to identify other many potential sources of *Salmonella* infection. Meanwhile, the implementation of environmental policies for the management of human wastes, such as refuse, farms and fishery discards, may reduce the source of *Salmonella* infection of seagulls, and help to control the spread of some NTS strains with potential hazard for the public health.

CHAPTER 7

General Discussion

Despite the multiple efforts made to prevent *Campylobacter* and *Salmonella* infections, the incidence of these pathogens in humans is still a major public health concern (EFSA and ECDC, 2017b). Most epidemiological studies on these enterobacteria focus on infections in humans or food-producing animals. However, much less is known about the presence of these pathogens in the environment and wild animals. Due to the complexity of the transmission cycle of these zoonotic agents, with multiple sources of infection and reservoir hosts, a One Health approach involving human, animal and environmental niches is necessary to better understand their epidemiology, paying special attention to wild animals that may constitute an important reservoir of pathogens.

The presence of *Campylobacter* and *Salmonella* spp. has been previously described in several wild birds, especially in seagulls, from Europe, America and Australia (Benskin *et al.*, 2009; Ramos *et al.*, 2010; Molina-Lopez *et al.*, 2011; Antilles, 2014; Gargiulo *et al.*, 2014; Dolejska *et al.*, 2016; Jurado-Tarifa *et al.*, 2016; Konicek *et al.*, 2016). Nevertheless, there is little information about these zoonotic pathogens in wild birds in the African continent and in remote regions such as the Southern Ocean.

Whether wild birds are a source of infection for humans or domestic livestock or are mainly recipients of environmental strains is not fully understood. Several studies have reported that most of the genotypes carried by wild birds differ from those recovered from food-producing animals or causing human campylobacteriosis and salmonellosis, although strains in common also have been found in wild birds and domestic animals or humans (Thorbjorn Refsum *et al.*, 2002; Broman *et al.*, 2004; Griekspoor *et al.*, 2013; Palomo *et al.*, 2013; Retamal *et al.*, 2015). To contribute to understand these issues, there is a need to gain insight into the carriage, the genetic relation, the antimicrobial resistance and the virulence potential of both bacteria in wild birds. In order to solve some of these questions, in this thesis, several studies have been conducted to determine the role of seabirds in the epidemiology of *Campylobacter* and *Salmonella* spp.

Thermophilic *Campylobacter* spp. were isolated in seabirds of the Western Cape coast of South Africa, mainly *C. jejuni*. Similar prevalences were detected in both greater crested terns and kelp gulls. On the contrary, a higher occurrence of *Salmonella* spp. was found in kelp gulls than in greater crested terns, which seems to be related to the scavenging feeding habits of the former. Serovars Anatum, Enteritidis and Hadar were the most frequently isolated, but other serovars of public health concern were also found (Havelaar *et al.*, 2015). Kelp gulls often scavenge at refuse dumps and sewage treatment plants commonly contaminated with pathogens, probably making them more prone to carry this enterobacteria (Hockey *et al.*, 2005). This may explain the high prevalence and diversity of zoonotic serovars detected in gull colonies from urban areas. Thermophilic *Campylobacter* spp. were also found in all sampled Antarctic and Subantarctic islands, mainly *C. lari*, but also *C. jejuni*, specially in brown skuas, one of the main opportunistic seabird species in the Southern Ocean. *Salmonella* was only isolated from a few seabirds at Livingston Is. (Antarctic Peninsula) suggesting this bacterium is not indigenous in the region.

Our results indicate that seabirds feeding habits affects the incidence of *Salmonella* more than *Campylobacter*. This in part may be due to the increased presence and survival of *Salmonella* in gull feeding and breeding areas (Literák *et al.*, 1996). Most birds become asymptomatic carriers of *Salmonella* for some weeks or months and shed it in the faeces sometimes intermittently (Revolledo and Ferreira, 2012). *Campylobacter* is more susceptible to environmental stress and its survival in the environment may be lower, but once within the animal host it may establish a persistent colonization as part of the intestinal microbiota (Murphy *et al.*, 2006).

The frequency of antimicrobial resistant isolates found in seabirds was generally low, especially in the Southern Ocean. However, the presence of at least certain resistance, even in the most remote areas on Earth, is worrying given that wild birds are not directly exposed to antimicrobial agents. On the other hand, the main *Campylobacter* resistance detected in Western Cape seabirds was to tetracyclines and quinolones. The broad use

of tetracyclines in animal production systems in South Africa may account for the high incidence of resistant *Campylobacter* isolates in livestock animals, which in turn may influence the resistance found in wildlife (Eagar *et al.*, 2012). In recent years, there has been a dramatic increase in the proportion of *Campylobacter* clinical isolates resistant to fluoroquinolones in Cape Town, a cause of concern since these are the drug of choice as first line therapy for bacterial gastroenteritis (Lastovica, 2006). Although tetracyclines and fluoroquinolones are highly persistent in the environment, given the absence of a continuous antimicrobial pressure is more likely that seabirds have acquired AMR strains from anthropogenic sources (Wellington *et al.*, 2013). Regarding *Salmonella*, resistance to a wide variety of antimicrobial drugs, mainly tetracyclines and streptomycin, including multi-drug resistance, were detected specially in gull colonies near urban areas, which also may be indicative of anthropogenic contamination. The presence of bacteria harbouring resistance to critically important antimicrobials for human medicine in wildlife evidences the wide degree of expansion of AMR in the natural environment. Wild birds can contribute to the persistence and spread of AMR, also via mobile genetic elements that can be passed to bacteria that are not closely related, with serious implications for ecosystem function, animal disease and public health (Wellington *et al.*, 2013).

The molecular typing of *Campylobacter* and *Salmonella* spp. isolates from Western Cape and Southern Ocean seabirds allowed us to analyse the population structure of these bacteria in regions little investigated to date, establish the possible sources of infection and contribute to the expansion of the MLST Database with 42 new STs described (mainly of *C. lari*).

A major genetic heterogenicity was observed in *C. lari* population in Southern Ocean seabirds, despite *C. lari* represents a highly related phylogenetic clade within the genus *Campylobacter* (Miller *et al.*, 2014). The widespread distribution of *C. lari* among host species and localities and its high genetic diversity may be indicative that it has long been circulating in the region. *C. lari* has been previously found in aquatic environments,

including Southern Ocean penguins, gulls, skuas and seals (Bonnedahl *et al.*, 2005; Leotta *et al.*, 2006; García-Peña *et al.*, 2010, 2017), but some STs (e.g. ST-27) have also been associated with human gastroenteritis cases in some countries (USA, Canada, Belgium and France; MLST Database).

The genetic population of *C. jejuni* was less heterogeneous and was structured into clusters of related lineages. Most of *C. jejuni* isolates from Western Cape seabirds belonged to CCs (mainly CC-1275) related to aquatic environments. Although these seabirds inhabit in a populated region in close contact with livestock and human activities, they carry *C. jejuni* genotypes that differ from those retrieved from food-producing animals and human campylobacteriosis cases, in agreement with previous reports (Broman et al., 2004; Sheppard et al., 2011; Griekspoor et al., 2013). Nevertheless, the opposite scenario was found in seabirds from remote Southern Ocean islands, where almost all *C. jejuni* genotypes belonged to CCs (mainly CC-45, CC-21, CC-206) commonly associated with domestic animals, especially poultry, and human gastroenteritis cases, and widely distributed in the Northern Hemisphere. These CCs have also been found, albeit less frequently, in natural environments and wild birds, probably as a result of contamination from animal and human waste (Kwan *et al.*, 2008; Levesque *et al.*, 2013; Cody *et al.*, 2015). These results strongly suggest that Southern Ocean seabirds have acquired these pathogens as a result of sporadic infections through anthropogenic sources.

The presence of *Campylobacter* STs in common in seabirds from different Antarctic and Subantarctic islands evidences certain degree of connectivity within the Southern Ocean region either directly or indirectly through common contamination sources. In addition, the same STs or SLVs were also found in different Southern Ocean seabird species, similarly to what was also observed in Western Cape seabirds. However, no genetic similarities were found between *Campylobacter* isolates from Western Cape and Southern Ocean seabirds. Several seabirds breeding in Antarctic and Subantarctic islands spend the austral winter in the productive Benguela Upwelling region in the

western coast of South Africa (Crawford *et al.*, 1991; Krietsch, 2014), where they can get in contact with other migrating birds, resident wildlife and even livestock. Southern Ocean seabirds could have acquired these strains in wintering or stopover grounds. However, attending to our results, the presence of *C. jejuni* in seabirds from these remote regions does not seem to be due to their northward migration movements. It seems more plausible that the bacteria have been introduced by human activities such as tourism or scientific expeditions despite the strict biosecurity controls from more than two decades.

With regards to *Salmonella*, almost all *S*. Typhimurium isolates found in Western Cape seabirds corresponded to ST-34, one of the most predominant ST within the eBG1, while *S*. Enteritidis isolates, also detected in a few Southern Ocean seabirds, belonged to ST-11, the central ST of the eBG4 (Achtman *et al.*, 2012; Ashton *et al.*, 2016). Both STs are globally distributed in a broad range of domestic animals and are commonly responsible for human salmonellosis (Enterobase Database, http://enterobase.warwick.ac.uk/). The presence of these STs in seabirds from Western Cape, and even from a remote Antarctic island (Livingston Is.), reflects the rapid worldwide expansion of clonal *Salmonella* genotypes of public health concern.

We observed that the majority of human *Salmonella* isolates, belonging to serovars Anatum, Hadar, Enteritidis and Typhimurium, from Cape Town hospitals causing human salmonellosis analysed in our study had identical or highly similar pulsotypes to those isolates from Western Cape seabirds. In the same line, we also detected a total 30 pulsotypes in common among *Salmonella* isolates, belonging to serovars Bredeney, Derby, Enteritidis, Grumpensis, Hadar, Infantis, Kentucky, Kottbus, Mikawasima, Rissen and Typhimurium, from different hosts (seabirds, poultry and humans) in the southwest of Europe. These results demonstrate an epidemiological link among NTS strains from domestic, wild and human niches as previously reported for serovars Enteritidis and Typhimurium (Palomo *et al.*, 2013; Retamal *et al.*, 2015). However, identification of pulsotypes in common in multiple hosts may sometimes not represent a causal

relationship and it is difficult to ascertain whether seabirds are only mirrors of the strains circulating in the humanised environment or are relevant actors in the transmission.

Disease transmission between wild birds and livestock or humans is often seen as a unidirectional pathway, but, in fact, it should be considered bidirectional. The direct transmission of *Campylobacter* and *Salmonella* from wild birds in general play a limited role in human infectious diseases (Tsiodras *et al.*, 2008). The potential participation of wild birds as a source of infections to domestic animals and humans is mainly indirect, linked to faecal contamination of water supplies, pastures and feed (Benskin *et al.*, 2009). In this context, it is important to underline that, in addition to wild birds, other wild animals (such as insects, reptiles, bats, rodents, foxes, badgers and wild boars) may also be involved in the transmission of these pathogens (Briones *et al.*, 2004; Meerburg and Kijlstra, 2007; Wales *et al.*, 2010; Chiari *et al.*, 2014; Whiley *et al.*, 2016; Ruiz-Fons, 2017; Hazeleger *et al.*, 2018). This may indirectly increase the risk of disease transmission, for example when these bacteria are brought to an environment that is more benign for it or are transmitted to another species that cohabit with poultry and livestock. Several studies highlight the importance of proper on-farm biosecurity and disease surveillance systems to ensure that production animals do not get in contact with, or share pasture or water access with wild animals (Wiethoelter *et al.*, 2015; Sommer *et al.*, 2016). However, currently in the EU animal husbandry is moving from more intensive to more extensive farming systems, increasing interactions in the wildlife-livestock interface.

On the other hand, the presence of multiple virulence-associated genes in *Campylobacter* and *Salmonella* isolates from seabirds highlights their virulent potential and their capacity to infect different hosts. Regardless of the variability in gene contents, in our studies there was no association between the virulotypes and host origin of isolates. Our results suggest that NTS isolates carried by seabirds can be able to infect both poultry and humans, probably because, in fact, they have an anthropogenic origin.

However, the acquisition of virulence determinants by efficient mechanisms of horizontal gene transfer may enable the adaptation of other non-clinical relevant serovars to different environmental conditions and the infection of new hosts. It is also important to mention that the detection of virulence-associated genes by PCR does not guarantee the expression of the encoding virulence factors. Besides, in the case of *Campylobacter,* the role of some virulence genes is only hypothetic and has not been clearly demonstrated, therefore additional studies are needed to elucidate the exact mechanisms of pathogenesis (Bolton, 2015).

Comparing the molecular genotyping techniques used in this thesis, PFGE displayed a slightly greater discrimination than MLST. The main drawback of PFGE is determining the degree of relatedness among isolates that have similar but distinguishable macrorestriction profiles, since some genomic rearrangements may hinder the interpretation of PFGE data. In some cases, PFGE can be too discriminatory distinguishing isolates that share a very recent common ancestor, especially in the case of *Campylobacter* which present a high genetic instability (Ridley *et al.*, 2008). On the contrary, sometimes PFGE may be less discriminatory grouping isolates with no epidemiological linkages, as is the case of highly clonal *Salmonella* serovar Enteritidis (Dewaele *et al.*, 2012). In addition, several factors can alter the similarities detection during the computer analysis of PFGE patterns: subjective differences in the designation of bands position, parameters values of tolerance and optimization; correlation coefficient used for patterns comparison, cut-off established for pulsotypes assignation, etc.

Although PFGE proved to be a helpful tool for screening closely related genotypes in short-term studies, for instance from the same individual or bird colony, an additional sequence-based method may be required to clarify their phylogenetic relationships. MLST does not have such a high resoluteness, but it provides a good epidemiological concordance in assessing the evolution of bacterial strains in long-term studies and brings important information on source attribution (Barco *et al.*, 2013; Taboada *et al.*,

2013). Source-tracking infection of *S*. Enteritidis can be complicated due to their highly clonal population structure, in addition to the fact that some STs present a broad host range and worldwide distribution. Therefore, more discriminatory methods such as whole genome sequence (WGS) analyses would be required for reliable genotyping of highly monomorphic genotypes like *C. jejuni* ST-45, *S*. Enteritidis ST-11 or S. Typhimurium ST-34 (Achtman *et al.*, 2012; Llarena *et al.*, 2016). This technique opens new avenues to differentiate among strains, and provides a much more detailed level for assessing occurrence of particular traits, such as virulence markers or resistance mechanisms, and thus allows a better understanding of disease dynamics and its effects. However, WGS does not provide information about gene expression levels or synergistic effects among different resistance mechanisms or virulence determinants. On the other hand, given that bioinformatic highly-skilled personnel is required for the data analysis and the lack of standardisation of the procedures, WGS implementation for the routine surveillance of foodborne pathogens still remains a challenge (Rantsiou *et al.*, 2017). Nevertheless, the cost of WGS continues to decrease and has become a fast and affordable tool for both reference and diagnostic laboratories, and it is expected that this technology will increasingly be used either alone or in combination with conventional methods.

CHAPTER 8

Conclusions

1. Kelp gulls and greater crested terns in the Western Cape of South Africa are carriers of zoonotic thermophilic *Campylobacter* species and *Salmonella* serovars, some of them resistant to critically important antimicrobials for human medicine.

2. The presence of zoonotic serovars of *Salmonella* and multi-drug resistant strains in seagulls in Western Cape is likely related to their feeding habits and constitute a public health risk, as demonstrated with the finding of same strains recovered from seagulls and human salmonellosis cases in Cape Town.

3. Most of *C. jejuni* genotypes found in Western Cape seabirds are related to aquatic environments, despite these seabirds inhabit in a populated region in close contact with anthropogenic activities.

4. The presence of *C. jejuni* and *S.* Enteritidis genotypes mainly associated with domestic animals and human gastroenteritis cases in seabirds from Antarctica and Southern Ocean islands strongly suggests a reverse zoonosis on most remote regions on Earth.

5. The widespread distribution of *C. lari* among seabird species in Southern Ocean localities and its high genetic diversity may be indicative that it has long been circulating in the region, with certain degree of connectivity among islands.

6. A One Health approach to study the epidemiology of *Salmonella* has allowed us to demonstrate the circulation of a range of serovars and strains with virulence potential among seagulls, poultry and humans in Southwestern Europe.

7. It is necessary to improve the environmental policies for the management of human wastes to reduce the access of seagulls to this source of zoonotic agents, which may help to control the spread of strains with potential hazard for the public health.

REFERENCES

Abbott, S.L., Ni, F.C.Y., and Janda, J.M. (2012) Increase in extraintestinal infections caused by *Salmonella enterica* subspecies II-IV. *Emerg. Infect. Dis.* **18**: 637–639.

Achtman, M., Wain, J., Weill, F.X., Nair, S., Zhou, Z., Sangal, V., et al. (2012) Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. *PLoS Pathog.* **8**: e1002776.

Adzitey, F. and Corry, J. (2011) A comparison between hippurate hydrolysis and multiplex PCR for differentiating *Campylobacter coli* and *Campylobacter jejuni*. *Trop. Life Sci. Res.* **22**: 91–98.

Agersø, Y., Torpdahl, M., Zachariasen, C., Seyfarth, A., Hammerum, A.M., and Nielsen, E.M. (2012) Tentative Colistin Epidemiological Cut-Off Value for *Salmonella* spp. *Foodborne Pathog. Dis.* **9**: 367–369.

Agron, P.G., Walker, R.L., Kinde, H., Sherilyn, J., Hayes, D.C., Wollard, J., et al. (2001) Identification by Subtractive Hybridization of Sequences Specific for *Salmonella enterica* Serovar Enteritidis Identification by Subtractive Hybridization of Sequences Specific for *Salmonella enterica* Serovar Enteritidis. **67**: 4984–4991.

Alix, E. and Blanc-Potard, A.B. (2007) MgtC: a key player in intramacrophage survival. *Trends Microbiol.* **15**: 252–256.

Allos, B.M., Moore, M.R., Griffin, P.M., and Tauxe, R. V (2004) Surveillance for sporadic foodborne disease in the 21st century: the FoodNet perspective. *Clin. Infect. Dis.* **38**: 115–120.

Almeida, F., Medeiros, M.I.C., Rodrigues, D. dos P., and Falcao, J.P. (2015) Genotypic diversity, pathogenic potential and the resistance profile of *Salmonella* Typhimurium strains isolated from humans and food from 1983 to 2013 in Brazil. *J. Med. Microbiol.* **64**: 1395–1407.

Altizer, S., Bartel, R., and Han, B.A. (2011) Animal migration and infectious disease risk. *Science* **331**: 296–302.

Altizer, S., Ostfeld, R.S., Johnson, P.T.J., Kutz, S., and Harvell, C.D. (2013) Climate Change and Infectious Diseases: From Evidence to a Predictive Framework. *Science.* **341**: 514–519.

Álvarez-Ordóñez, A., Begley, M., Prieto, M., Messens, W., López, M., Bernardo, A., and Hill, C. (2011) *Salmonella* spp. survival strategies within the host gastrointestinal tract. *Microbiology* **157**: 3268–3281.

Álvarez-Ordóñez, A., Prieto, M., Bernardo, A., Hill, C., and López, M. (2012) The Acid Tolerance Response of *Salmonella* spp.: An adaptive strategy to survive in stressful environments prevailing in foods and the host. *Food Res. Int.* **45**: 482–492.

Antilles, N. (2014) Epidemiology and antimicrobial resistance of *Salmonella* spp. and *Campylobacter* spp. from wild birds and poultry reared outdoors. *PhD thesis*. Universitat Autònoma de Barcelona, Bellaterra.

Antilles, N., Sanglas, A., and Cerdà-Cuéllar, M. (2015) Free-living Waterfowl as a Source of Zoonotic Bacteria in a Dense Wild Bird Population Area in Northeastern Spain. *Transbound. Emerg. Dis.* **62**: 516–521.

Antunes, P., Mourão, J., Campos, J., and Peixe, L. (2016) Salmonellosis: The role of poultry meat. *Clin. Microbiol. Infect.* **22**: 110–121.

Antunes, P., Mourão, J., Pestana, N., and Peixe, L. (2011) Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms. *J. Antimicrob. Chemother.* **66**: 2028–2032.

Arizaga, J., Aldalur, A., Herrero, A., Cuadrado, J.F., Díez, E., and Crespo, A. (2014) Foraging distances of a resident yellow-legged gull (*Larus michahellis*) population in relation to refuse management on a local scale. *Eur. J. Wildl. Res.* **60**: 171–175.

Asakura, M., Samosornsuk, W., Hinenoya, A., Misawa, N., Nishimura, K., Matsuhisa, A., and Yamasaki, S. (2008) Development of a cytolethal distending toxin (cdt) gene-based species-specific multiplex PCR assay for the detection and identification of *Campylobacter jejuni, Campylobacter coli* and *Campylobacter fetus*. *FEMS Immunol. Med. Microbiol.* **52**: 260–266.

Ashgar, S.S.A., Oldfield, N.J., Wooldridge, K.G., Jones, M.A., Irving, G.J., Turner, D.P.J., and Ala'Aldeen, D.A.A. (2007) CapA, an autotransporter protein of Campylobacter jejuni, mediates association with human epithelial cells and colonization of the chicken gut. *J. Bacteriol.* **189**: 1856–1865.

Ashton, P.M., Nair, S., Peters, T.M., Bale, J.A., Powell, D.G., Painset, A., et al. (2016) Identification of *Salmonella* for public health surveillance using whole genome sequencing. *PeerJ* **4**: e1752.

Van Asten, A.J.A.M. and Van Dijk, J.E. (2005) Distribution of “classic” virulence factors among *Salmonella* spp. *FEMS Immunol. Med. Microbiol.* **44**: 251–259.

Austin, J.W., Sanders, G., Y, W.W.K., and Collinson, S.K. (1998) Thin aggregative fimbriae enhance *Salmonella enteritidis* biofilm formation. **162**: 295–301.

Bachtiar, B.M., Coloe, P.J., and Fry, B.N. (2007) Knockout mutagenesis of the *kpsE* gene of *Campylobacter jejuni* 81116 and its involvement in bacterium-host interactions. *FEMS Immunol. Med. Microbiol.* **49**: 149–154.

Bacon, D.J., Alm, R., Burr, D.H., Hu, L., Kopecko, D.J., Ewing, C.P., et al. (2000) Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infect. Immun.* **68**: 4384–4390.

Bang, D.D., Nielsen, E.M., Scheutz, F., Pedersen, K., Handberg, K., and Madsen, M. (2003) PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. *J. Appl. Microbiol.* **94**: 1003–1014.

Barbosa, A. and Palacios, M.J. (2009) Health of Antarctic birds: A review of their parasites, pathogens and diseases. *Polar Biol.* **32**: 1095–1115.

Barco, L., Barrucci, F., Olsen, J.E., and Ricci, A. (2013) *Salmonella* source attribution based on microbial subtyping. *Int. J. Food Microbiol.* **163**: 193–203.

Barrett, T.J., Gerner-Smidt, P., and Swaminathan, B. (2006) Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. *Foodborne Pathog. Dis.* **3**: 20–31.

Barton, C., Ng, L.K., Tyler, S.D., and Clark, C.G. (2007) Temperate bacteriophages affect pulsed-field gel electrophoresis patterns of *Campylobacter jejuni*. *J. Clin. Microbiol.* **45**: 386–391.

Batz, M.B., Henke, E., and Kowalcyk, B. (2013) Long-term consequences of foodborne infections. *Infect. Dis. Clin. North Am.* **27**: 599–616.

Bäumler, A.J., Gilde, A.J., Tsolis, R.M., Van Der Velden, A.W.M., Ahmer, B.M.M., and Heffron, F. (1997) Contribution of horizontal gene transfer and deletion events to development of distinctive patterns of fimbrial operons during evolution of *Salmonella* serotypes. *J. Bacteriol.* **179**: 317–322.

Bäumler, A.J. and Heffron, F. (1995) Identification and sequence analysis of *lpfABCDE*, a putative fimbrial operon of *Salmonella typhimurium*. *J. Bacteriol.* **177**: 2087–97.

Bäumler, A.J., Norris, T.L., Lasco, T., Voigt, W., Reissbrodt, R., Rabsch, W., and Heffron, F. (1998) IroN, a novel outer membrane siderophore receptor characteristic of *Salmonella enterica*. *J. Bacteriol.* **180**: 1446–1453.

Behringer, M., Miller, W.G., and Oyarzabal, O.A. (2011) Typing of *Campylobacter jejuni* and *Campylobacter coli* isolated from live broilers and retail broiler meat by flaA-RFLP, MLST, PFGE and REP-PCR. *J. Microbiol. Methods* **84**: 194–201.

van Belkum, A., Tassios, P.T., Dijkshoorn, L., Haeggman, S., Cookson, B., Fry, N.K., et al. (2007) Guidelines for the validation and application of typing methods for use in bacterial epidemiology. *Clin. Microbiol. Infect.* **13**: 1–46.

Bell, C. and Kyriakides, A. (2002) *Salmonella*: A practical approach to the organism and its control in foods. Blackwell Science Ltd, Oxford.

Bengis, R.G., Leighton, F. a, Fischer, J.R., Artois, M., Mörner, T., and Tate, C.M. (2004) The role of wildlife in emerging and re-emerging zoonoses. *Rev. Sci. Tech.* **23**: 497–511.

Benjamin, J., Leaper, S., Owen, J., and Skirrow, M.B. (1983) Description of *Campylobacter laridis*, a new species comprising the nalidixic acid resistant thermophilic *Campylobacter* (NARTC) group. *Curr Microbiol.* **8**: 231–238.

Benskin, C.M.H., Wilson, K., Jones, K., and Hartley, I.R. (2009) Bacterial pathogens in wild birds: A review of the frequency and effects of infection. *Biol. Rev.* **84**: 349–373.

Bester, L.A. and Essack, S.Y. (2012) Observational study of the prevalence and antibiotic resistance of *Campylobacter* spp. from different poultry production systems in KwaZulu-Natal, South Africa. *J. Food Prot.* **75**: 154–159.

Beuzon, C.R. (2000) *Salmonella* maintains the integrity of its intracellular vacuole

through the action of SifA. *EMBO J.* **19**: 3235–3249.

Biswas, D., Hannon, S.J., Townsend, H.G.G., Potter, A., and Allan, B.J. (2011) Genes coding for virulence determinants of *Campylobacter jejuni* in human clinical and cattle isolates from Alberta, Canada, and their potential role in colonization of poultry. *Int. Microbiol.* **14**: 25–32.

Blaser, M.J. and Newman, L.S. (1982) A review of human salmonellosis: I. Infective dose. *Rev. Infect. Dis.* **4**: 1096–1106.

Bleumink-Pluym, N.M.C., van Alphen, L.B., Bouwman, L.I., Wösten, M.M.S.M., and van Putten, J.P.M. (2013) Identification of a Functional Type VI Secretion System in *Campylobacter jejuni* Conferring Capsule Polysaccharide Sensitive Cytotoxicity. *PLoS Pathog.* **9**: 16–18.

Van Boeckel, T.P., Brower, C., Gilbert, M., Grenfell, B.T., Levin, S.A., Robinson, T.P., et al. (2015) Global trends in antimicrobial use in food animals. *Proc. Natl. Acad. Sci.* **112**: 5649–5654.

Boer, P. de, Wagenaar, J.A., Achterberg, R.P., Putten, J.P.M. van, Schouls, L.M., and Duim, B. (2002) Generation of *Campylobacter jejuni* genetic diversity in vivo. *Mol. Microbiol.* **44**: 351–359.

Bolton, D.J. (2015) *Campylobacter* virulence and survival factors. *Food Microbiol.* **48**: 99–108.

Bonnedahl, J., Broman, T., Waldenström, J., Palmgren, H., Niskanen, T., Olsen, B., et al. (2005) In search of human-associated bacterial pathogens in Antarctic wildlife: report from six penguin colonies regularly visited by tourists. *Ambio* **34**: 430–432.

Boxrud, D., Pederson-Gulrud, K., Wotton, J., Medus, C., Lyszkowicz, E., Besser, J., and Bartkus, J.M. (2007) Comparison of multiple-locus variable-number tandem repeat analysis, pulsed-field gel electrophoresis, and phage typing for subtype analysis of *Salmonella enterica* serotype enteritidis. *J. Clin. Microbiol.* **45**: 536–543.

Brás, A.M., Chatterjee, S., Wren, B.W., Newell, D.G., and Ketley, J.M. (1999) A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. *J. Bacteriol.* **181**: 3298–3302.

Briones, V., Téllez, S., Goyache, J., Ballesteros, C., Del Pilar Lanzarot, M., Domínguez, L., and Fernández-Garayzábal, J.F. (2004) *Salmonella* diversity associated with wild reptiles and amphibians in Spain. *Environ. Microbiol.* **6**: 868–871.

Broman, T., Bergström, S., On, S.L.W., Palmgren, H., McCafferty, D.J., Sellin, M., and Olsen, B. (2000) Isolation and Characterization of *Campylobacter jejuni* subsp. *jejuni* from Macaroni penguins (*Eudyptes chrysolophus*) in the Subantarctic Region. *Appl. Environ. Microbiol.* **66**: 449–452.

Broman, T., Palmgren, H., Bergström, S., Sellin, M., Waldenström, J., Danielsson-Tham, M.L., and Olsen, B. (2002) *Campylobacter jejuni* in black-headed gulls (*Larus ridibundus*): Prevalence, genotypes, and influence on *C. jejuni* epidemiology. *J. Clin. Microbiol.* **40**: 4594–4602.

Broman, T., Waldenström, J., Dahlgren, D., Carlsson, I., Eliasson, I., and Olsen, B. (2004) Diversities and similarities in PFGE profiles of *Campylobacter jejuni* isolated from migrating birds and humans. *J. Appl. Microbiol.* **96**: 834–843.

Bronowski, C., James, C.E., and Winstanley, C. (2014) Role of environmental survival in transmission of *Campylobacter jejuni*. *FEMS Microbiol. Lett.* **356**: 8–19.

Byrne, C.M., Clyne, M., and Bourke, B. (2007) *Campylobacter jejuni* adhere to and invade chicken intestinal epithelial cells in vitro. *Microbiology* **153**: 561–569.

Callaway, T.R., Edrington, T.S., and Nisbet, D.J. (2014) Isolation of *Escherichia coli* O157:H7 and *Salmonella* from Migratory Brown-Headed Cowbirds (*Molothrus ater*), Common Grackles (*Quiscalus quiscula*), and Cattle Egrets (*Bubulcus ibis*). *Foodborne Pathog. Dis.* **11**: 791–794.

Campos, J., Cristino, L., Peixe, L., and Antunes, P. (2016) MCR-1 in multidrug-resistant and copper-tolerant clinically relevant *Salmonella* 1,4,[5],12:i:- and S. Rissen clones in Portugal, 2011 to 2015. *Eurosurveillance* **21.** doi: 10.2807/1560-7917.ES.2016.21.26.30270.

Cantero, J.G. (2017) *Campylobacter* spp. in broiler farms: genetic diversity, antimicrobial resistance and virulence factors. *PhD thesis.* Universitat Autònoma de Barcelona, Bellaterra.

Capuano, F., Mancusi, A., Capparelli, R., Esposito, S., and Proroga, Y.T.R. (2013) Characterization of drug resistance and virulotypes of *Salmonella* strains isolated from food and humans. *Foodborne Pathog. Dis.* **10**: 963–968.

Carroll, D., Wang, J., Fanning, S., and Mcmahon, B.J. (2015) Antimicrobial Resistance in Wildlife: Implications for Public Health. *Zoonoses Public Health* **62**: 534–542.

Champion, O.L., Gaunt, M.W., Gundogdu, O., Elmi, A., Witney, A.A., Hinds, J., et al. (2005) Comparative phylogenomics of the food-borne pathogen *Campylobacter jejuni* reveals genetic markers predictive of infection source. *Proc. Natl. Acad. Sci.* **102**: 16043–16048.

Chiari, M., Ferrari, N., Giardiello, D., Lanfranchi, P., Zanoni, M., Lavazza, A., and Alborali, L.G. (2014) Isolation and identification of *Salmonella* spp. from red foxes (*Vulpes vulpes*) and badgers (*Meles meles*) in northern Italy. *Acta Vet. Scand.* **56**: 86.

Chmielewski, R., Wieliczko, A., Kuczkowski, M., Mazurkiewicz, M., and Ugorski, M. (2002) Comparison of ITS profiling, REP- and ERIC-PCR of *Salmonella* Enteritidis isolates from Poland. *J. Vet. Med. Ser. B* **49**: 163–168.

Chown, S.L., Huiskes, A.H.L., Gremmen, N.J.M., Lee, J.E., Terauds, A., Crosbie, K., et al. (2012) Continent-wide risk assessment for the establishment of nonindigenous species in Antarctica. *Proc. Natl. Acad. Sci. U. S. A.* **109**: 4938–4943.

Chown, S.L., Lee, J.E., Hughes, K.A., Barnes, J., Barrett, P.J., Bergstrom, D.M., et al. (2012) Challenges to the future conservation of the Antarctic. *Science* **337**: 158–159.

Christel, I., Navarro, J., del Castillo, M., Cama, A., and Ferrer, X. (2012) Foraging movements of Audouin's gull (*Larus audouinii*) in the Ebro Delta, NW Mediterranean: A preliminary satellite-tracking study. *Estuar. Coast. Shelf Sci.* **96**: 257–261.

Chuma, T., Hashimoto, S., and Okamoto, K. (2000) Detection of thermophilic *Campylobacter* from sparrows by multiplex PCR: the role of sparrows as a source of contamination of broilers with *Campylobacter*. *J. Vet. Med. Sci.* **62**: 1291–1295.

Cízek, A., Dolejská, M., Karpíšková, R., Dědičová, D., and Literák, I. (2007) Wild black-headed gulls (*Larus ridibundus*) as an environmental reservoir of *Salmonella*

strains resistant to antimicrobial drugs. *Eur. J. Wildl. Res.* **53**: 55–60.

Ĉíẑek, A., Literák, I., Hejlíćek, K., Treml, F., Smola, J., Cizek, A., et al. (1994) *Salmonella* Contamination of the Environment and its Incidence in Wild Birds. *J. Vet. Med. Ser. B* **41**: 320–327.

Clark, C.G., Bryden, L., Cuff, W.R., Johnson, P.L., Jamieson, F., Ciebin, B., and Wang, G. (2005) Use of the Oxford multilocus sequence typing protocol and sequencing of the flagellin short variable region to characterize isolates from a large outbreak of waterborne *Campylobacter* spp. strains in Walkerton, Ontario, Canada. *J. Clin. Microbiol.* **43**: 2080–2091.

Clouthier, S.C., Muller, K.H., Doran, J.L., Collinson, S.K., and Kay, W.W. (1993) Characterization of three fimbrial genes, sefABC, of *Salmonella enteritidis*. *J. Bacteriol.* **175**: 2523–2533.

CLSI (2016) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-sixth Informational Supplement (M100-S26) CLSI Publication, Wayne, Pennsylvania.

Cody, A.J., Mccarthy, N.D., Bray, J.E., Wimalarathna, H.M.L., Colles, F.M., Jansen van Rensburg, M.J., et al. (2015) Wild bird-associated *Campylobacter jejuni* isolates are a consistent source of human disease, in Oxfordshire, United Kingdom. *Environ. Microbiol. Rep.* **7**: 782–788.

Coker, A.O., Isokpehi, R.D., Thomas, B.N., Amisu, K.O., and Larry Obi, C. (2002) Human campylobacteriosis in developing countries. *Emerg. Infect. Dis.* **8**: 237–243.

Collinson, S.K., Clouthier, S.C., Doran, J.L., Banser, P.A., and Kay, W.W. (1996) *Salmonella enteritidis agfBAC* operon encoding thin aggregative fimbriae. *J. Bacteriol.* **178**: 662–667.

Cooper, J., Crawford, R.J.M., De Villiers, M.S., Dyer, B.M., Hofmeyr, G.J.G., and Jonker, A. (2009) Disease outbreaks among penguins at sub-Antarctic Marion Island: A conservation concern. *Mar. Ornithol.* **37**: 193–196.

Corcoran, D., Quinn, T., Cotter, L., Whyte, P., and Fanning, S. (2006) Antimicrobial resistance profiling and fla-typing of Irish thermophillic *Campylobacter* spp. of

human and poultry origin. *Lett. Appl. Microbiol.* **43**: 560–565.

Crawford, R.J., Ryan, P.G., and Williams, A.J. (1991) Seabird consumption and production in the Benguela and Western Agulhas ecosystems. *South African J. Mar. Sci.* **11**: 357–375.

Crosa, J.H., Brenner, D.J., Ewing, W.H., and Falkow, S. (1973) Molecular Relationships Among the Salmonelleae. *J. Bacteriol.* **115**: 307–315.

Curry, C.H., McCarthy, J.S., Darragh, H.M., Wake, R. a, Todhunter, R., and Terris, J. (2002) Could tourist boots act as vectors for disease transmission in Antarctica? *J. Travel Med.* **9**: 190–193.

Daniels, M.J., Hutchings, M.R., and Greig, A. (2003) The Risk of Disease Transmission to Livestock Posed by Contamination of Farm Stored Feed by Wildlife Excreta. *Epidemiol. Infect.* **130**: 561–568.

Datta, S., Niwa, H., and Itoh, K. (2003) Prevalence of 11 pathogenic genes of *Campylobacter jejuni* by PCR in strains isolated from humans, poultry meat and broiler and bovine faeces. *J. Med. Microbiol.* **52**: 345–348.

Davies, J. and Davies, D. (2010) Origins and Evolution of Antibiotic Resistance. *Microbiol. Mol. Biol. Rev.* **74**: 417–433.

Dearlove, B.L., Cody, A.J., Pascoe, B., Méric, G., Wilson, D.J., and Sheppard, S.K. (2016) Rapid host switching in generalist *Campylobacter* strains erodes the signal for tracing human infections. *ISME J.* **10**: 721–729.

Debruyne, L., Broman, T., Bergström, S., Olsen, B., On, S.L.W., and Vandamme, P. (2010a) *Campylobacter volucris* sp. nov., isolated from black-headed gulls (*Larus ridibundus*). *Int. J. Syst. Evol. Microbiol.* **60**: 1870–1875.

Debruyne, L., Broman, T., Bergström, S., Olsen, B., On, S.L.W., and Vandamme, P. (2010b) *Campylobacter subantarcticus* sp. nov., isolated from birds in the sub-Antarctic region. *Int. J. Syst. Evol. Microbiol.* **60**: 815–819.

Debruyne, L., Gevers, D., and Vandamme, P. (2008) Taxonomy of the Family Campylobacteraceae. In Nachamkin, I., Szymanski, C.M., and Blaser, M.J. (eds), *Campylobacter*. ASM Press, Washington, pp. 3–25.

Debruyne, L., On, S.L.W., De Brandt, E., and Vandamme, P. (2009) Novel *Campylobacter lari* like bacteria from humans and molluscs: Description of *Campylobacter peloridis* sp. nov., *Campylobacter lari* subsp. *concheus* subsp. nov. and *Campylobacter lari* subsp. *lari* subsp. nov. *Int. J. Syst. Evol. Microbiol.* **59**: 1126–1132.

Dekeyser, P., Gossuin-Detrain, M., Butzler, J.P., and Sternon, J. (1972) Acute enteritidis due to related vibrio: First positive stool cultures. *J. Infect. Dis.* **125**: 390–392.

Delord, K., Cherel, Y., Barbraud, C., Chastel, O., and Weimerskirch, H. (2018) High variability in migration and wintering strategies of brown skuas (*Catharacta antarctica lonnbergi*) in the Indian Ocean. *Polar Biol.* **41**: 59–70.

Denis, M., Refrégier-Petton, J., Laisney, M.J., Ermel, G., and Salvat, G. (2001) *Campylobacter* contamination in French chicken production from farm to consumers. Use of a PCR assay for detection and identification of *Campylobacter jejuni* and *Campylobacter coli*. *J. Appl. Microbiol.* **91**: 255–267.

Dewaele, I., Rasschaert, G., Bertrand, S., Wildemauwe, C., Wattiau, P., Imberechts, H., et al. (2012) Enteritidis: Comparison of an Optimized Multi-Locus Variable-Number of Tandem Repeat Analysis (MLVA) and Pulsed-Field Gel Electrophoresis. *Foodborne Pathog. Dis.* **9**: 885–895.

Dingle, K.E., Colles, F.M., Falush, D., Maiden, C.J., and Maiden, M.C.J. (2005) Sequence Typing and Comparison of Population Biology of *Campylobacter coli* and *Campylobacter jejuni* Sequence Typing and Comparison of Population Biology of *Campylobacter coli* and *Campylobacter jejuni*. *Society* **43**: 340–347.

Dingle, K.E., Colles, F.M., Wareing, D.R.A.A., Ure, R., Fox, A.J., Bolton, F.E., et al. (2001) Multilocus Sequence Typing System for *Campylobacter jejuni*. *J. Clin. Microbiol.* **39**: 14–23.

Dolejska, M., Masarikova, M., Dobiasova, H., Jamborova, I., Karpiskova, R., Havlicek, M., et al. (2016) High prevalence of *Salmonella* and IMP-4-producing Enterobacteriaceae in the silver gull on Five Islands, Australia. *J. Antimicrob. Chemother.* **71**: 63–70.

Dominguez, S. a and Schaffner, D.W. (2009) Survival of *Salmonella* in processed chicken

products during frozen storage. *J. Food Prot.* **72**: 2088–2092.

Dorsey, C.W., Laarakker, M.C., Humphries, A.D., Weening, E.H., and Bäumler, A.J. (2005) *Salmonella enterica* serotype Typhimurium MisL is an intestinal colonization factor that binds fibronectin. *Mol. Microbiol.* **57**: 196–211.

Douard, G., Praud, K., Cloeckaert, A., and Doublet, B. (2010) The *Salmonella* genomic island 1 is specifically mobilized in trans by the IncA/C multidrug resistance plasmid family. *PLoS One* **5**: e15302.

Dougnac, C., Pardo, C., Meza, K., Arredondo, C., Blank, O., Abalos, P., et al. (2015) Detection of *Salmonella enterica* in Magellanic penguins (*Spheniscus magellanicus*) of Chilean Patagonia: evidences of inter-species transmission. *Epidemiol. Infect.* **143**: 1187–1193.

Doumith, M., Godbole, G., Ashton, P., Larkin, L., Dallman, T., Day, M., et al. (2016) Detection of the plasmid-mediated mcr-1 gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. *J. Antimicrob. Chemother.* **71**: 2300–2305.

Doyle, L.P. (1944) A vibrio associated with swine dysentery. *Am. J. Vet. Res.* **5**: 3–5.

Drahovská, H., Mikasová, E., Szemes, T., Ficek, A., Sásik, M., Majtán, V., and Turna, J. (2007) Variability in occurrence of multiple prophage genes in *Salmonella* Typhimurium strains isolated in Slovak Republic. *FEMS Microbiol. Lett.* **270**: 237–244.

Eagar, H., Swan, G., and Van Vuuren, M. (2012) A survey of antimicrobial usage in animals in South Africa with specific reference to food animals. *J. S. Afr. Vet. Assoc.* **83**: 16.

EFSA and ECDC (2017a) European union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food 2015. *EFSA Journal 2017* **15**: 4694.

EFSA and ECDC (2017b) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. *EFSA Journal 2017* **15**: 5077.

Egevang, C., Stenhouse, I.J., Phillips, R.A., Petersen, A., Fox, J.W., and Silk, J.R.D. (2010) Tracking of Arctic terns Sterna paradisaea reveals longest animal migration. *Proc. Natl. Acad. Sci.* **107**: 2078–2081.

Engberg, J., Aarestrup, F.M., Taylor, D.E., Gerner-Smidt, P., and Nachamkin, I. (2001) Quinolone and macrolide resistance in *Campylobacter jejuni* and *C. coli*: Resistance mechanisms and trends in human isolates. *Emerg. Infect. Dis.* **7**: 24–34.

Escherich, T. (1886) Articles adding to the knowledge of intestinal bacteria, III. On the existence of vibrios in the intestines and feces of babies. *Munchener Medizinische Wochenschrift* **33**: 815–817. *In German*.

Etoh, Y., Dewhirst, F.E., Paster, B.J., Yamamoto, A., and Goto, N. (1993) *Campylobacter showae* sp. nov., isolated from the human oral cavity. *Int. J. Syst. Bacteriol.* **43**: 631–639.

Ezzati, M., Lopez, A.D., Rodgers, A., Vander Hoorn, S., and Murray, C.J. (2002) Selected major risk factors and global and regional burden of disease. *Lancet* **360**: 1347–1360.

Fàbrega, A. and Vila, J. (2013) *Salmonella enterica* serovar Typhimurium skills to succeed in the host: Virulence and regulation. *Clin. Microbiol. Rev.* **26**: 308–341.

Fakhr, M.K., Nolan, L.K., and Logue, C.M. (2005) Multilocus Sequence Typing Lacks the Discriminatory Ability of Pulsed-Field Gel Electrophoresis for Typing. **43**: 2215–2219.

Feasey, N.A., Dougan, G., Kingsley, R.A., Heyderman, R.S., and Gordon, M.A. (2012) Invasive non-typhoidal salmonella disease: An emerging and neglected tropical disease in Africa. *Lancet* **379**: 2489–2499.

Feil, E.J., Li, B.C., Aanensen, D.M., Hanage, W.P., and Spratt, B.G. (2004) eBURST: Inferring Patterns of Evolutionary Descent among Clusters of Related Bacterial Genotypes from Multilocus Sequence Typing Data. *J. Bacteriol.* **186**: 1518–1530.

Fendri, I., Ben Hassena, A., Grosset, N., Barkallah, M., Khannous, L., Chuat, V., et al. (2013) Genetic diversity of food-isolated Salmonella strains through Pulsed Field Gel Electrophoresis (PFGE) and Enterobacterial Repetitive Intergenic Consensus

(ERIC-PCR). *PLoS One* **8**: e81315.

Feng, Y., Liu, J., Li, Y.G., Cao, F.L., Johnston, R.N., Zhou, J., et al. (2012) Inheritance of the *Salmonella* virulence plasmids: Mostly vertical and rarely horizontal. *Infect. Genet. Evol.* **12**: 1058–1063.

Fermér, C. and Engvall, E.O. (1999) Specific PCR Identification and Differentiation of the Thermophilic Campylobacters, *Campylobacter jejuni, C. coli, C. lari* and *C. upsaliensis*. **37**: 3370–3373.

Ferns, P.N. and Mudge, G.P. (2000) Abundance, diet and *Salmonella* contamination of gulls feeding at sewage outfalls. *Water Res.* **34**: 2653–2660.

Ferrero, R.L. and Lee, A. (1988) Motility of *Campylobacter jejuni* in a viscous environment: comparison with conventional rod-shaped bacteria. *J. Gen. Microbiol.* **134**: 53–59.

Figueroa-Bossi, N., Uzzau, S., Maloriol, D., and Bossi, L. (2001) Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in *Salmonella*. *Mol. Microbiol.* **39**: 260–271.

Fitzgerald, C., Wichard, J., and Nachamkin, I. (2008) Diagnosis and Antimicrobial susceptibility of *Campylobacter* species. In Nachamkin, C.C., Szymanski, C.M., and Blaser, M. (eds), *Campylobacter*. AMS Press, Washington, pp. 227–245.

Foley, S.L., Johnson, T.J., Ricke, S.C., Nayak, R., and Danzeisen, J. (2013) *Salmonella* pathogenicity and host adaptation in chicken-associated serovars. *Microbiol. Mol. Biol. Rev.* **77**: 582–607.

Foley, S.L., Lynne, A.M., and Nayak, R. (2009) Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. *Infect. Genet. Evol.* **9**: 430–440.

Forde, B.M. and O'Toole, P.W. (2013) Next-generation sequencing technologies and their impact on microbial genomics. *Brief. Funct. Genomics* **12**: 440–453.

Frenot, Y., Chown, S.L., Whinam, J., Selkirk, P.M., Convey, P., Skotnicki, M., and Bergstrom, D.M. (2005) Biological invasions in the Antarctic: extent, impacts and implications. *Biol. Rev. Camb. Philos. Soc.* **80**: 45–72.

Fresno, M., Barrera, V., Gornall, V., Lillo, P., Paredes, N., Abalos, P., et al. (2013) Identification of diverse *Salmonella* serotypes, virulotypes, and antimicrobial resistance phenotypes in waterfowl from Chile. *Vector borne zoonotic Dis.* **13**: 884-887.

Friedrich, M.J., Kinsey, N.E., Vila, J., and Kadner, R.J. (1993) Nucleotide sequence of a 13.9 kb segment of the 90 kb virulence plasmid of *Salmonella typhimurium*: the presence of fimbriai biosynthetic genes. *Mol. Microbiol.* **8**: 543–558.

Fuller, T., Bensch, S., Müller, I., Novembre, J., Pérez-Tris, J., Ricklefs, R.E., et al. (2012) The ecology of emerging infectious diseases in migratory birds: An assessment of the role of climate change and priorities for future research. *Ecohealth* **9**: 80–88.

Gaglio, D., Cook, T.R., Connan, M., Ryan, P.G., and Sherley, R.B. (2017) Dietary studies in birds: Testing a non-invasive method using digital photography in seabirds. *Methods Ecol. Evol.* **8**: 214–222.

Gal-Mor, O., Boyle, E.C., and Grassl, G.A. (2014) Same species, different diseases: How and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Front. Microbiol.* **5**: 391.

Galan, J.E., Ginocchio, C., and Costeas, P. (1992) Molecular and functional characterization of the *Salmonella* invasion gene invA: Homology of InvA to members of a new protein family. *J. Bacteriol.* **174**: 4338–4349.

Galarza, A., Herrero, A., Domínguez, J.M., Aldalur, A., and Arizaga, J. (2012) Movements of Mediterranean Yellow-legged Gulls *Larus michahellis* to the Bay of Biscay. *Ringing Migr.* **27**: 26–31.

García-Peña, F.J., Llorente, M.T., Serrano, T., Ruano, M.J., Belliure, J., Benzal, J., et al. (2017) Isolation of *Campylobacter* spp. from Three Species of Antarctic Penguins in Different Geographic Locations. *Ecohealth* **14**: 78–87.

García-Peña, F.J., Pérez-Boto, D., Jiménez, C., San Miguel, E., Echeita, A., Rengifo-Herrera, C., et al. (2010) Isolation and characterization of *Campylobacter* spp. from Antarctic fur seals (*Arctocephalus gazella*) at Deception Island, Antarctica. *Appl. Environ. Microbiol.* **76**: 6013–6016.

Gardner, H., Kerry, K.R., Riddle, M., Brouwer, S., and Gleeson, L. (1997) Poultry virus infection in Antarctic penguins. **387**: 245.

Garénaux, A., Jugiau, F., Rama, F., de Jonge, R., Denis, M., Federighi, M., and Ritz, M. (2008) Survival of *Campylobacter jejuni* strains from different origins under oxidative stress conditions: effect of temperature. *Curr. Microbiol.* **56**: 293–297.

Gargiulo, A., Russo, T.P., Schettini, R., Mallardo, K., Calabria, M., Menna, L.F., et al. (2014) Occurrence of enteropathogenic bacteria in urban pigeons (*Columba livia*) in Italy. *Vector Borne Zoonotic Dis.* **14**: 251–255.

GERMS-SA (2013) GERMS-SA Annual Report 2012. Available at: http://www.nicd.ac.za/assets/files/2012_GERMS-SA_Annual_Report.pdf

GERMS-SA (2014) GERMS-SA Annual Report 2013. Available at: www.nicd.ac.za/assets/files/GERMS-SA%20AR%202013(1).pdf

Gibreel, A. and Taylor, D.E. (2006) Macrolide resistance in *Campylobacter jejuni* and *Campylobacter coli*. *J. Antimicrob. Chemother.* **58**: 243–255.

Gibson, D.L., White, A.P., Rajotte, C.M., and Kay, W.W. (2007) AgfC and AgfE facilitate extracellular thin aggregative fimbriae synthesis in *Salmonella* Enteritidis. *Microbiology* **153**: 1131–1140.

Gilbert, D.N., Eliopoulos, G.M., Chambers, H.F., Saag, M.S., and Pavia, A.T. (2017) Sanford Guide to Antimicrobial Therapy 2017 47th ed. Gilbert, D.N., Eliopoulos, G.M., Chambers, H.F., Saag, M.S. and Pavia, A.T. (eds) Antimicrobial Therapy, Inc., Sperryville, Virginia.

Gilbert, M., Brisson, J., Karwaski, M., Michniewicz, J., Cunningham, A., Wu, Y., et al. (2000) Biosynthesis of Ganglioside Mimics in *Campylobacter jejuni* OH4384. *J. Biol. Chem.* **275**: 3896–3906.

Gilg, O., Moe, B., Hanssen, S.A., Schmidt, N.M., Sittler, B., Hansen, J., et al. (2013) Trans-Equatorial Migration Routes, Staging Sites and Wintering Areas of a High-Arctic Avian Predator: The Long-tailed Skua (*Stercorarius longicaudus*). *PLoS One* **8**: e64614.

Godschalk, P.C.R., Heikema, A.P., Gilbert, M., Komagamine, T., Wim Ang, C., Glerum, J.,

et al. (2004) The crucial role of *Campylobacter jejuni* genes in anti-ganglioside antibody induction in Guillain-Barré syndrome. *J. Clin. Invest.* **114**: 1659–1665.

Goering, R. V. (2010) Pulsed field gel electrophoresis: A review of application and interpretation in the molecular epidemiology of infectious disease. *Infect. Genet. Evol.* **10**: 866–875.

González-Escalona, N., Brown, E.W., and Zhang, G. (2012) Development and evaluation of a multiplex real-time PCR (qPCR) assay targeting ttrRSBCA locus and invA gene for accurate detection of *Salmonella* spp. in fresh produce and eggs. *Food Res. Int.* **48**: 202–208.

Goon, S., Kelly, J.F., Logan, S.M., Ewing, C.P., and Guerry, P. (2003) Pseudaminic acid, the major modification on *Campylobacter* flagellin, is synthesized via the Cj1293 gene. *Mol. Microbiol.* **50**: 659–671.

Grant, K.A., Belandia, I.U., Dekker, N., Richardson, P.T., and Park, S.F. (1997) Molecular characterization of *pldA*, the structural gene for a phospholipase A from *Campylobacter coli*, and its contribution to cell-associated hemolysis. *Infect. Immun.* **65**: 1172–1180.

Greig, J., Rajić, A., Young, I., Mascarenhas, M., Waddell, L., and Lejeune, J. (2015) A scoping review of the role of wildlife in the transmission of bacterial pathogens and antimicrobial resistance to the food chain. *Zoonoses Public Health* **62**: 269–284.

Griekspoor, P., Colles, F.M., McCarthy, N.D., Hansbro, P.M., Ashhurst-Smith, C., Olsen, B., et al. (2013) Marked host specificity and lack of phylogeographic population structure of *Campylobacter jejuni* in wild birds. *Mol. Ecol.* **22**: 1463–1472.

Griekspoor, P., Engvall, E.O., Olsen, B., and Waldenström, J. (2010) Multilocus sequence typing of *Campylobacter jejuni* from broilers. *Vet. Microbiol.* **140**: 180–185.

Grimont, P. and Weill, F.X. (2007) Antigenic formulae of the *Salmonella* servovars 9th ed. *WHO Collab. Cent. Ref. Res. Salmonella.* Institut Pasteur, Paris.

Guerry, P. (2007) *Campylobacter* flagella: not just for motility. *Trends Microbiol.* **15**: 456–461.

Gyles, C. and Boerlin, P. (2014) Horizontally transferred genetic elements and their role in pathogenesis of bacterial disease. *Vet. Pathol.* **51**: 328–340.

Haghjoo, E. and Galán, J.E. (2004) *Salmonella typhi* encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. *Proc. Natl. Acad. Sci. U. S. A.* **101**: 4614–4619.

Hald, B., Skov, M.N., Nielsen, E.M., Rahbek, C., Madsen, J.J., Wainø, M., et al. (2016) *Campylobacter jejuni* and *Campylobacter coli* in wild birds on Danish livestock farms. *Acta Vet. Scand.* **58**: 11.

Hald, B., Skovgård, H., Pedersen, K., and Bunkenborg, H. (2008) Influxed insects as vectors for *Campylobacter jejuni* and *Campylobacter coli* in Danish broiler houses. *Poult. Sci.* **87**: 1428–1434.

Haneda, T., Ishii, Y., Shimizu, H., Ohshima, K., Iida, N., Danbara, H., and Okada, N. (2012) *Salmonella* type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection. *Cell. Microbiol.* **14**: 485–499.

Hänninen, M.L., Hakkinen, M., and Rautelin, H. (1999) Stability of related human and chicken *Campylobacter jejuni* genotypes after passage through chick intestine studied by pulsed-field gel electrophoresis. *Appl. Environ. Microbiol.* **65**: 2272–2275.

Haraga, A., Ohlson, M.B., and Miller, S.I. (2008) Salmonellae interplay with host cells. *Nat. Rev. Microbiol.* **6**: 53–66.

Harrington, C.S., Moran, L., Ridley, A.M., Newell, D.G., and Madden, R.H. (2003) Inter-laboratory evaluation of three flagellin PCR/RFLP methods for typing *Campylobacter jejuni* and *C. coli*: The CAMPYNET experience. *J. Appl. Microbiol.* **95**: 1321–1333.

Harrington, C.S., Thomson-Carter, F.M., and Carter, P.E. (1997) Evidence for recombination in the flagellin locus of *Campylobacter jejuni*: Implications for the flagellin gene typing scheme. *J. Clin. Microbiol.* **35**: 2386–2392.

Harrison, J.W., Dung, T.T.N., Siddiqui, F., Korbrisate, S., Bukhari, H., Vu Tra, M.P., et al.

(2014) Identification of possible virulence marker from *Campylobacter jejuni* isolates. *Emerg. Infect. Dis.* **20**: 1026–1029.

Hatch, J.J. (1996) Threats to public health from gulls (Laridae). *Int. J. Environ. Health Res.* **6**: 5–16.

Hauser, E., Hebner, F., Tietze, E., Helmuth, R., Junker, E., Prager, R., et al. (2011) Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany. *Int. J. Food Microbiol.* **151**: 141–149.

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Prager, R., Schroeter, A., et al. (2012) Clonal dissemination of *Salmonella enterica* serovar Infantis in Germany. *Foodborne Pathog. Dis.* **9**: 352–360.

Havelaar, A.H., Kirk, M.D., Torgerson, P.R., Gibb, H.J., Hald, T., Lake, R.J., et al. (2015) World Health Organization Global Estimates and Regional Comparisons of the Burden of Foodborne Disease in 2010. *PLoS Med.* **12**: e1001923.

Hazeleger, W.C., Jacobs-reitsma, W.F., Lina, P.H.C., Boer, A.G. De, Bosch, T., Hoek, A.H.A.M. Van, and Beumer, R.R. (2018) Wild insectivorous bats might be carriers of *Campylobacter* spp . *PLoS One* **13**: e0190647.

Heithoff, D.M., Shimp, W.R., Lau, P.W., Badie, G., Enioutina, E.Y., Daynes, R.A., et al. (2008) Human *Salmonella* clinical isolates distinct from those of animal origin. *Appl. Environ. Microbiol.* **74**: 1757–1766.

Helms, M., Ethelberg, S., Mølbak, K., and Group, D.S. (2005) Typhimurium DT104 Infections, 1992–2001. *Emerg. Infect. Dis.* **11**: 859–867.

Hendriksen, R.S., Vieira, A.R., Karlsmose, S., Lo Fo Wong, D.M. a, Jensen, A.B., Wegener, H.C., and Aarestrup, F.M. (2011) Global monitoring of *Salmonella* serovar distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: results of quality assured laboratories from 2001 to 2007. *Foodborne Pathog. Dis.* **8**: 887–900.

Hernandez, S.M., Keel, K., Sanchez, S., Trees, E., Gerner-Smidt, P., Adams, J.K., et al. (2012) Epidemiology of a *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain associated with a songbird outbreak. *Appl. Environ. Microbiol.*

78: 7290–7298.

Hernandez, S.M., Welch, C.N., Peters, V.E., Lipp, E.K., Curry, S., Yabsley, M.J., et al. (2016) Urbanized White Ibises (*Eudocimus albus*) as carriers of *Salmonella enterica* of significance to public health and wildlife. *PLoS One* **11**: e0164402.

Heuvelink, A.E., van Heerwaarden, C., Zwartkruis-Nahuis, A., Tilburg, J.J.H.C., Bos, M.H., Heilmann, F.G.C., et al. (2009) Two outbreaks of campylobacteriosis associated with the consumption of raw cows' milk. *Int. J. Food Microbiol.* **134**: 70–74.

Hickey, T.E., McVeigh, A.L., Scott, D.A., Michielutti, R.E., Bixby, A., Carroll, S.A., et al. (2000) *Campylobacter jejuni* cytolethal distending toxin mediates release of interleukin-8 from intestinal epithelial cells. *Infect. Immun.* **68**: 6535–6541.

Higgins, P.J. and Davies, S.J.J.F. (1996) Handbook of Australian, New Zealand & Antarctic Birds. Volume 3, Snipe to pigeons Oxford University Press, Melbourne.

Hilbert, F., Smulders, F.J.M., Chopra-Dewasthaly, R., and Paulsen, P. (2012) *Salmonella* in the wildlife-human interface. *Food Res. Int.* **45**: 603–608.

Hockey, P.A.R., Dean, W.R.J., and Ryan, P.G. (2005) Roberts - Birds of Southern Africa Cape Town: John Voelcker Bird Book Fund, Cape Town.

Hoelzer, K., Switt, A.I.M., and Wiedmann, M. (2011) Animal contact as a source of human non-typhoidal salmonellosis. *Vet. Res.* **42**: 34.

Hoffmann, M., Zhao, S., Pettengill, J., Luo, Y., Monday, S.R., Abbott, J., et al. (2014) Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype heidelberg isolates from humans, retailmeats, and animals. *Genome Biol. Evol.* **6**: 1046–1068.

Hohmann, E.L. (2001) Non-typhoidal salmonellosis. *Clin. Infect. Dis.* **32**: 263–269.

Horiyama, T., Yamaguchi, A., and Nishino, K. (2010) TolC dependency of multidrug efflux systems in *Salmonella enterica* serovar Typhimurium. *J. Antimicrob. Chemother.* **65**: 1372–1376.

Horrocks, S.M., Anderson, R.C., Nisbet, D.J., and Ricke, S.C. (2009) Incidence and ecology of *Campylobacter jejuni* and *coli* in animals. *Anaerobe* **15**: 18–25.

Horton, R.A., Wu, G., Speed, K., Kidd, S., Davies, R., Coldham, N.G., and Duff, J.P. (2013) Wild birds carry similar *Salmonella enterica* serovar Typhimurium strains to those found in domestic animals and livestock. *Res. Vet. Sci.* **95**: 45–48.

Huang, D.B. and DuPont, H.L. (2005) Problem pathogens: Extra-intestinal complications of *Salmonella enterica* serotype Typhi infection. *Lancet Infect. Dis.* **5**: 341–348.

Hudson, C.R., Quist, C., Lee, M.D., Keyes, K., Dodson, S. V., Morales, C., et al. (2000) Genetic relatedness of *Salmonella* isolates from nondomestic birds in southeastern United States. *J. Clin. Microbiol.* **38**: 1860–1865.

Huehn, S., La Ragione, R.M., Anjum, M., Saunders, M., Woodward, M.J., Bunge, C., et al. (2010) Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. *Foodborne Pathog. Dis.* **7**: 523–535.

Hughes, K.A. and Convey, P. (2010) The protection of Antarctic terrestrial ecosystems from inter- and intra-continental transfer of non-indigenous species by human activities: A review of current systems and practices. *Glob. Environ. Chang.* **20**: 96–112.

Hughes, L.A., Shopland, S., Wigley, P., Bradon, H., Leatherbarrow, A.H., Williams, N.J., et al. (2008) Characterisation of *Salmonella enterica* serotype Typhimurium isolates from wild birds in northern England from 2005 – 2006. *BMC Vet. Res.* **4**: 4.

Hughes, L.A., Wigley, P., Bennett, M., Chantrey, J., and Williams, N. (2010) Multi-locus sequence typing of *Salmonella enterica* serovar Typhimurium isolates from wild birds in northern England suggests host-adapted strain. *Lett. Appl. Microbiol.* **51**: 477–479.

Hulton, C.S.J., Higgins, C.F., and Sharp, P.M. (1991) ERIC sequence: a novel family of repetitive elements in the genomes of *Escherichia coli, Salmonella typhimurium* and other enterobacteria. *Molecualar Microbiol.* **5**: 825–834.

Humphrey, S., Chaloner, G., Kemmett, K., Davidson, N., Williams, N., Kipar, A., et al. (2014) *Campylobacter jejuni* is not merely a commensal in commercial broiler chickens and affects bird welfare. *MBio* **5**: e01364-14.

Hunter, P.R. and Gaston, M.A. (1988) Numerical index of the discriminatory ability of

typing systems: An application of Simpson's index of diversity. *J. Clin. Microbiol.* **26**: 2465–2466.

Inglis, G.D., McAllister, T.A., Larney, F.J., and Topp, E. (2010) Prolonged survival of *Campylobacter* species in bovine manure compost. *Appl. Environ. Microbiol.* **76**: 1110–1119.

ISO 10272-1:2017 (2017) Microbiology of the food chain. Horizontal method for detection and enumeration of *Campylobacter* spp. Part 1: Detection Method. Geneva.

ISO 6579-1:2017 (2017) Microbiology of the food chain. Horizontal method for the detection, enumeration and serotyping of *Salmonella*. Part 1: Detection of *Salmonella* spp. Genève.

Issenhuth-Jeanjean, S., Roggentin, P., Mikoleit, M., Guibourdenche, M., De Pinna, E., Nair, S., et al. (2014) Supplement 2008-2010 (no. 48) to the White-Kauffmann-Le Minor scheme. *Res. Microbiol.* **165**: 526–530.

Iveson, J.B., Shellam, G.R., Bradshaw, S.D., Smith, D.W., Mackenzie, J.S., and Mofflin, R.G. (2009) *Salmonella* infections in Antarctic fauna and island populations of wildlife exposed to human activities in coastal areas of Australia. *Epidemiol. Infect.* **137**: 858–870.

Janakiraman, A. and Slauch, J.M. (2000) The putative iron transport system SitABCD encoded on SPI1 is required for full virulence of *Salmonella typhimurium*. *Mol. Microbiol.* **35**: 1146–1155.

Janssen, R., Krogfelt, K.A., Cawthraw, S.A., Van Pelt, W., Wagenaar, J.A., and Owen, R.J. (2008) Host-pathogen interactions in *Campylobacter* infections: The host perspective. *Clin. Microbiol. Rev.* **21**: 505–518.

Jay-Russell, M.T. (2013) What is the risk from wild animals in food-borne pathogen contamination of plants? *CAB Rev.* **8**: 1–16.

Jensenius, M., Han, P. V., Schlagenhauf, P., Schwartz, E., Parola, P., Castelli, F., et al. (2013) Acute and potentially life-threatening tropical diseases in western travelers - A GeoSentinel multicenter study, 1996-2011. *Am. J. Trop. Med. Hyg.* **88**: 397–404.

Jolley, K.A., Bliss, C.M., Bennett, J.S., Bratcher, H.B., Brehony, C., Colles, F.M., et al. (2012) Ribosomal multilocus sequence typing: Universal characterization of bacteria from domain to strain. *Microbiology* **158**: 1005–1015.

Jones, F.S., Orcutt, M., and Little, R.B. (1931) Vibrios (*Vibrio jejuni*, n. sp.) associated with intestinal disorders of cows and calves. *J. Exp. Med.* **53**: 853–864.

Jones, T.F., Ingram, L.A., Cieslak, P.R., Vugia, D.J., Angelo, M.T., Hurd, S., et al. (2008) Salmonellosis outcomes differ substantially by serotype. *J. Infect. Dis.* **198**: 109–114.

Jonker, A. and Picard, J.A. (2010) Antimicrobial susceptibility in thermophilic *Campylobacter* species isolated from pigs and chickens in South Africa. *J. S. Afr. Vet. Assoc.* **81**: 228–236.

Judicial Commission (2005) The type species of the genu Salmonella Lignières 1900 is Salmonella enterica (ex Kauffmann and Edwards 1952) Le Minor and Popoff 1987, with the type strain LT2T, and conservation of the epithet enterica in *Salmonella enterica* over al. *Int. J. Syst. Evol. Microbiol.* **55**: 519–520.

Jurado-Tarifa, E., Torralbo, A., Borge, C., Cerdà-Cuéllar, M., Ayats, T., Carbonero, A., and García-Bocanegra, I. (2016) Genetic diversity and antimicrobial resistance of *Campylobacter* and *Salmonella* strains isolated from decoys and raptors. *Comp. Immunol. Microbiol. Infect. Dis.* **48**: 14–21.

Kaakoush, N.O., Castaño-Rodríguez, N., Mitchell, H.M., and Man, S.M. (2015) Global epidemiology of *Campylobacter* infection. *Clin. Microbiol. Rev.* **28**: 687–720.

Kane, O.J., Uhart, M.M., Rago, V., Pereda, A.J., Smith, J.R., Van Buren, A., et al. (2012) Avian pox in Magellanic Penguins (*Spheniscus magellanicus*). *J. Wildl. Dis.* **48**: 790–794.

Kapperud, G. and Rosef, O. (1983) Avian wildlife reservoir of *Campylobacter fetus* subsp. *jejuni*, *Yersinia* spp., and *Salmonella* spp. in Norway. *Appl. Environ. Microbiol.* **45**: 375–380.

Karesh, W.B., Dobson, A., Lloyd-Smith, J.O., Lubroth, J., Dixon, M.A., Bennett, M., et al. (2012) Ecology of zoonoses: Natural and unnatural histories. *Lancet* **380**: 1936–

1945.

Karlyshev, A. V., Everest, P., Linton, D., Cawthraw, S., Newell, D.G., and Wren, B.W. (2004) The *Campylobacter jejuni* general glycosylation system is important for attachment to human epithelial cells and in the colonization of chicks. *Microbiology* **150**: 1957–1964.

Katzav, M., Isohanni, P., Lund, M., Hakkinen, M., and Lyhs, U. (2008) PCR assay for the detection of *Campylobacter* in marinated and non-marinated poultry products. *Food Microbiol.* **25**: 908–914.

Kauffman, F. (1966) The bacteriology of Enterobacteriaceae. Munksgaard Danmark, Copenhagen.

Keller, J.I. and Shriver, W.G. (2014) Prevalence of three *Campylobacter* species, *C. jejuni*, *C. coli* and *C. lari* using multilocus sequence typing in wild birds of the mid-atlantic region, USA. *J. Wildl. Dis.* **50**: 31–41.

Keller, J.I., Shriver, W.G., Waldenström, J., Griekspoor, P., and Olsen, B. (2011) Prevalence of *Campylobacter* in wild birds of the mid-Atlantic region, USA. *J. Wildl. Dis.* **47**: 750–754.

Kerry, K.R. and Riddle, M. (2009) Health of Antarctic Wildlife. Kerry, K.R. and Riddle, M. (eds). Springer, Berlin.

Kim, Y., Bae, I.K., Jeong, S.H., Lee, C.H., Lee, H.K., Ahn, J., et al. (2011) Occurrence of incfii plasmids carrying the bla CTX-M-15 gene in *Salmonella enterica* serovar enteritidis sequence type 11 in korea. *Diagn. Microbiol. Infect. Dis.* **71**: 171–173.

King, E.O. (1957) Human infections with *Vibrio fetus* and a closely related vibrio. *J. Infect. Dis.* **101**: 119–128.

Klein, J.R., Fahlen, T.F., and Jones, B.D. (2000) Transcriptional organization and function of invasion genes within *Salmonella enterica* serovar Typhimurium pathogenicity island 1, including the *prgH, prgI, prgJ, prgK, orgA, orgB,* and *orgC* genes. *Infect. Immun.* **68**: 3368–3376.

Klena, J.D., Parker, C.T., Knibb, K., Claire Ibbitt, J., Devane, P.M.L., Horn, S.T., et al. (2004) Differentiation of *Campylobacter coli, Campylobacter jejuni, Campylobacter lari,*

and *Campylobacter upsaliensis* by a multiplex PCR developed from the nucleotide sequence of the lipid A gene *lpxA*. *J. Clin. Microbiol.* **42**: 5549–5557.

Konicek, C., Vodrážka, P., Barták, P., Knotek, Z., Hess, C., Račka, K., et al. (2016) Detection of Zoonotic Pathogens in Wild Birds in the Cross-Border Region Austria – Czech Republic. *J. Wildl. Dis.* **52**: 850–861.

Konkel, M.E., Garvis, S.G., Tipton, S.L., Anderson Jr., D.E., and Cieplak Jr., W. (1997) Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol. Microbiol.* **24**: 953–963.

Konkel, M.E., Gray, S.A., Kim, B.J., Garvis, S.G., and Yoon, J. (1999) Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the cadF virulence gene and its product. *J. Clin. Microbiol.* **37**: 510–517.

Konkel, M.E., Kim, B.J., Klena, J.D., and Young, C.R. (1998) Characterization of the Thermal Stress Response of *Campylobacter jejuni*. Infect Immun. **66**: 3666–3672.

Konkel, M.E., Kim, B.J., Rivera-Amill, V., and Garvis, S.G. (1999) Bacterial secreted proteins are required for the internaliztion of *Campylobacter jejuni* into cultured mammalian cells. *Mol. Microbiol.* **32**: 691–701.

Konkel, M.E., Klena, J.D., Rivera-Amill, V., Monteville, M.R., Biswas, D., Raphael, B., and Mickelson, J. (2004) Secretion of Virulence Proteins from *Campylobacter jejuni* is dependent on a funtional flagellar exportus apparatus. *Society* **186**: 3296–3303.

Koolman, L., Whyte, P., Burgess, C., and Bolton, D. (2015) Distribution of virulence-associated genes in a selection of *Campylobacter* isolates. *Foodborne Pathog. Dis.* **12**: 424–432.

Korczak, B.M., Zurfluh, M., Emler, S., Kuhn-Oertli, J., and Kuhnert, P. (2009) Multiplex strategy for multilocus sequence typing, fla typing, and genetic determination of antimicrobial resistance of *Campylobacter jejuni* and *Campylobacter coli* isolates collected in Switzerland. *J. Clin. Microbiol.* **47**: 1996–2007.

Kothary, M.H. and Babu, U.S. (2001) Infective Dose of Foodborne Pathogens in Volunteers: a Review. *J. Food Saf.* **21**: 49–68.

Krietsch, J. (2014) The migration behavior of seabirds from the Antarctic Ocean:

different ways to deal with the winter and how to investigate it in Antarctica.

Kumar, S., Stecher, G., and Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870-1874.

Kuvandik, C., Karaoglan, I., Namiduru, M., and Baydar, I. (2009) Predictive value of clinical and laboratory findings in the diagnosis of the enteric fever. *New Microbiol* **32**: 25–30.

Kwan, P.S.L., Barrigas, M., Bolton, F.J., French, N.P., Gowland, P., Kemp, R., et al. (2008) Molecular epidemiology of *Campylobacter jejuni* populations in dairy cattle, wildlife, and the environment in a farmland area. *Appl. Environ. Microbiol.* **74**: 5130–5138.

Ladely, S.R., Harrison, M.A., Fedorka-Cray, P.J., Berrang, M.E., Englen, M.D., and Meinersmann, R.J. (2007) Development of macrolide-resistant *Campylobacter* in broilers administered subtherapeutic or therapeutic concentrations of tylosin. *J. Food Prot.* **70**: 1945–1951.

Lane, D. (1991) 16S/23S rRNA sequencing. In Stackebrandt, E. and Goodfellow, M. (eds), *Techniques in Bacterial Systematics*. John Wiley and Sons, New York, pp. 115–175.

Lastovica AJ (2006) Antibiotic resistance patterns of *Campylobacter jejuni, C. concisus* and *C. upsaliensis* isolates from paediatric patients in Cape Town, South Africa, 1998–2005. In, *106th General Meeting of the American Society for Microbiology, Orlando FL, 2006. Washington, DC, USA: American Society for Microbiology*., p. Poster presentation C-03.

Lawson, A.J., On, S.L.W., Logan, J.M.J., and Stanley, J. (2001) *Campylobacter hominis* sp. nov., from the human gastrointestinal tract. *Int. J. Syst. Evol. Microbiol.* **51**: 651–660.

Lawson, B., Howard, T., Kirkwood, J.K., MacGregor, S.K., Perkins, M., Robinson, R.A., et al. (2010) Epidemiology of salmonellosis in garden birds in England and Wales, 1993 to 2003. *Ecohealth* **7**: 294–306.

Leatherbarrow, A.J.H., Griffiths, R., Hart, C.A., Kemp, R., Williams, N.J., Diggle, P.J., et al. (2007) *Campylobacter lari*: genotype and antibiotic resistance of isolates from

cattle, wildlife and water in an area of mixed dairy farmland in the United Kingdom. *Environ. Microbiol.* **9**: 1772–1779.

Leblanc-Maridor, M., Beaudeau, F., Seegers, H., Denis, M., and Belloc, C. (2011) Rapid identification and quantification of *Campylobacter coli* and *Campylobacter jejuni* by real-time PCR in pure cultures and in complex samples. *BMC Microbiol.* **11**: 113.

Lee, M.D. and Newell, D.G. (2006) *Campylobacter* in Poultry: Filling an Ecological Niche. *Avian Dis.* **50**: 1–9.

Leotta, G. a., Vigo, G.B., and Giacoboni, G. (2006) Isolation of *Campylobacter lari* from seabirds in Hope Bay, Antarctica. *Polish Polar Res.* **27**: 303–308.

Lesnick, M.L., Reiner, N.E., Fierer, J., and Guiney, D.G. (2001) The *Salmonella spvB* virulence gene encodes an enzyme that ADP-ribosylates actin and destabilizes the cytoskeleton of eukaryotic cells. *Mol. Microbiol.* **39**: 1464–1470.

Levantesi, C., Bonadonna, L., Briancesco, R., Grohmann, E., Toze, S., and Tandoi, V. (2012) *Salmonella* in surface and drinking water: Occurrence and water-mediated transmission. *Food Res. Int.* **45**: 587–602.

Levesque, S., Fournier, E., Carrier, N., Frost, E., D. Arbeit, R., and Michaud, S. (2013) Campylobacteriosis in urban versus rural areas: A case-case study integrated with molecular typing to validate risk factors and to attribute sources of infection. *PLoS One* **8**: 17–20.

Levy, A.J. (1946) A gastro-enteritis cutbreak probably due to a bovine strain of vibrio. *Yale J. Biol. Med.* **18**: 243–258.

Li, H., Bhaskara, A., Megalis, C., and Tortorello, M. Lou (2012) Transcriptomic Analysis of *Salmonella* Desiccation Resistance. *Foodborne Pathog. Dis.* **9**: 1143–1151.

Liakopoulos, A., Mevius, D.J., Olsen, B., and Bonnedahl, J. (2016) The colistin resistance mcr-1 gene is going wild. *J. Antimicrob. Chemother.* **71**: 2335–2336.

Lin, J., Michel, L.O., and Zhang, Q. (2002) CmeABC Functions as a Multidrug Efflux System in Campylobacter jejuni CmeABC Functions as a Multidrug Efflux System in Campylobacter jejuni. *Society* **46**: 2124–2131.

Linton, D., Gilbert, M., Hitchen, P.G., Dell, A., Morris, H.R., Wakarchuk, W.W., et al. (2000) Phase variation of a β-1,3 galactosyltransferase involved in generation of the ganglioside GM1-like lipo-oligosaccharide of *Campylobacter jejuni*. *Mol. Microbiol.* **37**: 501–514.

Linton, D., Owen, R.J., and Stanley, J. (1996) Rapid identification by PCR of the genus *Campylobacter* and of five *Campylobacter* species enteropathogenic for man and animals. *Res. Microbiol.* **147**: 707–718.

Literák, I., Cízek, A., and Smola, J. (1996) Survival of salmonellas in a colony of common black-headed gulls *Larus ridibundus* between two nesting periods. *Colon. Waterbirds* **19**: 268–269.

Liu, Y.Y., Wang, Y., Walsh, T.R., Yi, L.X., Zhang, R., Spencer, J., et al. (2016) Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. *Lancet Infect. Dis.* **16**: 161–168.

Llarena, A.-K., Zhang, J., Vehkala, M., Välimäki, N., Hakkinen, M., Hänninen, M.-L., et al. (2016) Monomorphic genotypes within a generalist lineage of *Campylobacter jejuni* show signs of global dispersion. *Microb. Genomics* **2**: e000088.

Luangtongkum, T., Shen, Z., Seng, V.W., Sahin, O., Jeon, B., Liu, P., and Zhang, Q. (2012) Impaired fitness and transmission of macrolide-resistant *Campylobacter jejuni* in its natural host. *Antimicrob. Agents Chemother.* **56**: 1300–1308.

Lucarelli, C., Dionisi, A.M., Trezzi, L., Farina, C., Passera, M., Kärki, T., et al. (2016) Molecular and Epidemiological Analysis of a *Campylobacter jejuni* Outbreak in Northern Italy in November 2013. *Foodborne Pathog. Dis.* **13**: 490–494.

Magwedere, K., Rauff, D., De Klerk, G., Keddy, K.H., and Dziva, F. (2015) Incidence of nontyphoidal *Salmonella* in food-producing animals, animal feed, and the associated environment in South Africa, 2012-2014. *Clin. Infect. Dis.* **61**: S283–S289.

Malorny, B., Hoorfar, J., Bunge, C., and Helmuth, R. (2003) Multicenter Validation of the Analytical Accuracy of *Salmonella* PCR : towards an International Standard Multicenter Validation of the Analytical Accuracy of Salmonella PCR : towards an

International Standard. *Appl. Environ. Microbiol.* **69**: 290–296.

Maron, D., Smith, T.J., and Nachman, K.E. (2013) Restrictions on antimicrobial use in food animal production: an international regulatory and economic survey. *Global. Health* **9**: 48.

Masarikova, M., Manga, I., Cizek, A., Dolejska, M., Oravcova, V., Myskova, P., et al. (2016) *Salmonella enterica* resistant to antimicrobials in wastewater effluents and black-headed gulls in the Czech Republic, 2012. *Sci. Total Environ.* **542**: 102–107.

Mattick, K.L., Jørgensen, F., Wang, P., Pound, J., Ward, L.R., Legan, J.D., and Humphrey, T.J. (2001) Effect of Challenge Temperature and Solute Type on Heat Tolerance of *Salmonella* Serovars at Low Water Activity Effect of Challenge Temperature and Solute Type on Heat Tolerance of *Salmonella* Serovars at Low Water Activity. *Appl. Environ. Microbiol.* **67**: 4128–4136.

McFadyean, F. and Stockman, S. (1913) Report of the Deparmental Committee Appointed by the Board of Agriculture and Fisheries to Enquire into Epizootic Abortion, Part III: abortation in sheep. His Majesty's Stationery Office, London.

McTavish, S.M., Pope, C.E., Nicol, C., Campbell, D., French, N., and Carter, P.E. (2009) Multilocus sequence typing of *Campylobacter jejuni*, and the correlation between clonal complex and pulsed-field gel electrophoresis macrorestriction profile. *FEMS Microbiol. Lett.* **298**: 149–156.

Meerburg, B.G. and Kijlstra, A. (2007) Role of rodents in transmission of *Salmonella* and *Campylobacter*. *J. Sciece Food Agric.* **87**: 274–2781.

Melero, B., Juntunen, P., Hänninen, M.L., Jaime, I., and Rovira, J. (2012) Tracing *Campylobacter jejuni* strains along the poultry meat production chain from farm to retail by pulsed-field gel electrophoresis, and the antimicrobial resistance of isolates. *Food Microbiol.* **32**: 124–128.

Messenger, A.M., Barnes, A.N., and Gray, G.C. (2014) Reverse zoonotic disease transmission (Zooanthroponosis): A systematic review of seldom-documented human biological threats to animals. *PLoS One* **9**: e89055.

Mezal, E.H., Sabol, A., Khan, M.A., Ali, N., Stefanova, R., and Khan, A.A. (2014) Isolation

and molecular characterization of *Salmonella enterica* serovar Enteritidis from poultry house and clinical samples during 2010. *Food Microbiol.* **38**: 67–74.

Migura-Garcia, L., Ramos, R., and Cerdà-Cuéllar, M. (2017) Antimicrobial Resistance of *Salmonella* Serovars and *Campylobacter* spp. Isolated from an Opportunistic Gull Species, Yellow-legged Gull (*Larus michahellis*). *J. Wildl. Dis.* **53**: 148-152.

Mikasova, E., Drahovska, H., Szemes, T., Kuchta, T., Karpiskova, R., Sasik, M., and Turna, J. (2005) Characterization of *Salmonella enterica* serovar Typhimurium strains of veterinary origin by molecular typing methods. *Vet. Microbiol.* **109**: 113–120.

Miller, R.A. and Wiedmann, M. (2016) The Cytolethal Distending Toxin Produced by Nontyphoidal *Salmonella* Serotypes Javiana, Montevideo, Oranienburg, and Mississippi Induces DNA Damage in a Manner Similar to That of Serotype Typhi. *MBio* **7**: e02109-16.

Miller, W.G., On, S.L.W., Wang, G., Fontanoz, S., Lastovica, A.J., and Mandrell, R.E. (2005) Extended Multilocus Sequence Typing System for *Campylobacter coli , C. lari, C. upsaliensis,* and C. helveticus. *J. Clin. Microbiol.* **43**: 2315–2329.

Miller, W.G., Yee, E., Chapman, M.H., Smith, T.P.L., Bono, J.L., Huynh, S., et al. (2014) Comparative genomics of the *Campylobacter lari* group. *Genome Biol. Evol.* **6**: 3252–3266.

Le Minor, L. and Popoff, M.Y. (1987) Request for an Opinion Designation of *Salmonella enterica* sp. nov., norn. rev., as the Type and Only Species of the Genus *Salmonella*. *Int. J. Syst. Bacteriol.* **37**: 465–468.

Le Minor, L., Verón, M., and Popoff, M. (1982) A proposal for *Salmonella* nomenclature. *Ann. Microbiol. (Paris).* **133**: 245–254. *In French*.

Mirold, S., Rabsch, W., Rohde, M., Stender, S., Tschäpe, H., Rüssmann, H., et al. (1999) Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic *Salmonella typhimurium* strain. *Proc. Natl. Acad. Sci. U. S. A.* **96**: 9845–9850.

Moest, T.P. and Méresse, S. (2013) *Salmonella* T3SSs: Successful mission of the secret(ion) agents. *Curr. Opin. Microbiol.* **16**: 38–44.

Molina-Lopez, R. a, Valverdú, N., Martin, M., Mateu, E., Obon, E., Cerdà-Cuéllar, M., and Darwich, L. (2011) Wild raptors as carriers of antimicrobial-resistant *Salmonella* and *Campylobacter* strains. *Vet. Rec.* **168**: 565.

Moore, J.E., Corcoran, D., Dooley, J.S.G., Fanning, S., Lucey, B., Matsuda, M., et al. (2005) *Campylobacter*. *Vet. Res.* **36**: 351–382.

Moré, E., Ayats, T., Ryan, P.G., Naicker, P.R., Keddy, K.H., Gaglio, D., et al. (2017) Seabirds (Laridae) as a source of *Campylobacter* spp., *Salmonella* spp. and antimicrobial resistance in South Africa. *Environ. Microbiol.* **19:** 4164-4176.

Moriarty, E.M., Sinton, L.W., Mackenzie, M.L., Karki, N., and Wood, D.R. (2008) A survey of enteric bacteria and protozoans in fresh bovine faeces on New Zealand dairy farms. *J. Appl. Microbiol.* **105**: 2015–2025.

Müller, J., Schulze, F., Müller, W., and Hänel, I. (2006) PCR detection of virulence-associated genes in *Campylobacter jejuni* strains with differential ability to invade Caco-2 cells and to colonize the chick gut. *Vet. Microbiol.* **113**: 123–129.

Müllner, P., Collins-Emerson, J.M., Midwinter, A.C., Carter, P., Spencer, S.E.F., Van Der Logt, P., et al. (2010) Molecular epidemiology of *Campylobacter jejuni* in a geographically isolated country with a uniquely structured poultry industry. *Appl. Environ. Microbiol.* **76**: 2145–2154.

Murphy, C., Carroll, C., and Jordan, K.N. (2006) Environmental survival mechanisms of the foodborne pathogen *Campylobacter jejuni*. *J. Appl. Microbiol.* **100**: 623–632.

Murray, C.G. and Hamilton, A.J. (2010) Perspectives on wastewater treatment wetlands and waterbird conservation. *J. Appl. Ecol.* **47**: 976–985.

Nachamkin, I. (2002) Chronic effects of *Campylobacter* infection. *Microbes Infect.* **4**: 399–403.

Nachamkin, I., Bohachick, K., and Patton, C.M. (1993) Flagellin gene typing of *Campylobacter jejuni* by restriction fragment length polymorphism analysis. *J. Clin. Microbiol.* **31**: 1531–1536.

NARMS-FDA (2016) The National Antimicrobial Resistance Monitoring Systems: Enteric Bacteria. NARMS Integrated Report: 2014. Available at:

https://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM528861.pdf

Nascimento, M., Sousa, A., Ramirez, M., Francisco, A.P., Carriço, J.A., and Vaz, C. (2017) PHYLOViZ 2.0: Providing scalable data integration and visualization for multiple phylogenetic inference methods. *Bioinformatics* **33**: 128–129.

Navarro, J., Oro, D., Bertolero, A., Genovart, M., Delgado, A., and Forero, M.G. (2010) Age and sexual differences in the exploitation of two anthropogenic food resources for an opportunistic seabird. *Mar. Biol.* **157**: 2453–2459.

Niedergang, F., Sirard, J.-C., Blanc, C.T., and Kraehenbuhl, J.-P. (2000) Entry and survival of *Salmonella typhimurium* in dendritic cells and presentation of recombinant antigens do not require macrophage-specific virulence factors. *Proc. Natl. Acad. Sci.* **97**: 14650–14655.

Nielsen, L.N., Sheppard, S.K., McCarthy, N.D., Maiden, M.C.J., Ingmer, H., and Krogfelt, K.A. (2010) MLST clustering of *Campylobacter jejuni* isolates from patients with gastroenteritis, reactive arthritis and Guillain-Barré syndrome. *J. Appl. Microbiol.* **108**: 591–599.

O'Neill, J. (2016) Tackling Drug-Resistant Infections Globally: Final Report and Recommendations. The Review on Antimicrobial Resistance. Available at: https://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf

Ó Cróinín, T. and Backert, S. (2012) Host Epithelial Cell Invasion by *Campylobacter jejuni*: Trigger or Zipper Mechanism? *Front. Cell. Infect. Microbiol.* **2**: 25.

Oloya, J., Doetkott, D., and Khaitsa, M.L. (2009) Antimicrobial drug resistance and molecular characterization of *Salmonella* isolated from domestic animals, humans, and meat products. *Foodborne Pathog. Dis.* **6**: 273–284.

Olsen, B., Bergström, S., McCafferty, D., Sellin, M., and Wiström, J. (1996) *Salmonella enteritidis* in Antarctica: zoonosis in man or humanosis in penguins? *Lancet* **348**: 1319–1320.

Olsen, K.M. and Larsson, H. (2010) Gulls of Europe, Asia and North America. Christopher

Helm, London.

Olson, C.K., Ethelberg, S., van Pelt, W., and Tauxe, R.V. (2008) Epidemiology of *Campylobacter jejuni* infections in industrialized nations. In Nachamkin, I., Szymanski, C.M., and Blaser, M.J. (eds), *Campylobacter* 3rd ed. American Society for Microbiology (ASM) Press, Washington DC, pp. 163–189.

Oporto, B., Esteban, J.I., Aduriz, G., Juste, R.A., and Hurtado, A. (2007) Prevalence and strain diversity of thermophilic campylobacters in cattle, sheep and swine farms. *J. Appl. Microbiol.* **103**: 977–984.

Oyarzabal, O.A., Backert, S., Williams, L.L., Lastovica, A.J., Miller, R.S., Pierce, S.J., et al. (2008) Molecular typing, serotyping and cytotoxicity testing of *Campylobacter jejuni* strains isolated from commercial broilers in Puerto Rico. *J. Appl. Microbiol.* **105**: 800–812.

Palmgren, H., Aspán, A., Broman, T., Bengtsson, K., Blomquist, L., Bergström, S., et al. (2006) *Salmonella* in Black-headed gulls (*Larus ridibundus*); prevalence, genotypes and influence on *Salmonella* epidemiology. *Epidemiol. Infect.* **134**: 635–644.

Palmgren, H., McCafferty, D., Aspán, A., Broman, T., Sellin, M., Wollin, R., et al. (2000) *Salmonella* in sub-Antarctica: low heterogeneity in *Salmonella* serotypes in South Georgian seals and birds. *Epidemiol. Infect.* **125**: 257–262.

Palomo, G., Campos, M.J., Ugarte, M., Porrero, M.C., Alonso, J.M., Borge, C., et al. (2013) Dissemination of antimicrobial-resistant clones of *Salmonella enterica* among domestic animals, wild animals, and humans. *Foodborne Pathog. Dis.* **10**: 171–176.

Palyada, K., Threadgill, D., and Stintzi, A. (2004) Iron Acquisition and Regulation in *Campylobacter jejuni*. *J Bacteriol*. **186**: 4714–4729.

Papadopoulos, T., Petridou, E., Zdragas, A., Mandilara, G., Nair, S., and Peters, T. (2016) Comparative study of all *Salmonella enterica* serovar Enteritidis strains isolated from food and food animals in Greece from 2008 to 2010 with clinical isolates. *Eur J Clin Microbiol Infect Dis.* **35**: 741–746.

Patel, S. and McCormick, B.A. (2014) Mucosal inflammatory response to *Salmonella typhimurium* infection. *Front. Immunol.* **5**: 311.

Paxton, E.H., Camp, R.J., Gorresen, P.M., Crampton, L.H., Jr, D.L.L., and Vanderwerf, E.A. (2016) Collapsing avian community on a Hawaiian island. *Sci. Adv.* **2**: e1600029.

Payot, S., Cloeckaert, A., and Chaslus-Dancla, E. (2002) Selection and characterization of fluoroquinolone-resistant mutants of *Campylobacter jejuni* using enrofloxacin. *Microb. Drug Resist. Epidemiol. Dis.* **8**: 335–343.

Pickett, C.L., Pesci, E.C., Cottle, D.L., Russell, G., Erdem, A.N., Pickett, C.L., et al. (1996) Prevalence of cytolethal distending toxin production in *Campylobacter jejuni* and relatedness of *Campylobacter* sp. *cdtB* gene. Prevalence of Cytolethal Distending Toxin Production in *Campylobacter jejuni* and Relatedness of *Campylobacter* sp. *cdtB* Genes. **64**: 2070–2078.

Piddock, L.J. V, Ricci, V., Pumbwe, L., Everett, M.J., and Griggs, D.J. (2003) Fluoroquinolone resistance in *Campylobacter* species from man and animals: detection of mutations in topoisomerase genes. *J. Antimicrob. Chemother.* **51**: 19–26.

Pilar, A.V.C., Reid-Yu, S.A., Cooper, C.A., Mulder, D.T., and Coombes, B.K. (2012) GogB is an anti-inflammatory effector that limits tissue damage during *Salmonella* infection through interaction with human FBXO22 and Skp1. *PLoS Pathog.* **8**: e1002773.

Pires, S.M., Vieira, A.R., Hald, T., and Cole, D. (2014) Source Attribution of Human Salmonellosis: An Overview of Methods and Estimates. *Foodborne Pathog. Dis.* **11**: 667–676.

Pitkänen, T. (2013) Review of *Campylobacter* spp. in drinking and environmental waters. *J. Microbiol. Methods* **95**: 39–47.

Poppert, S., Haas, M., Yildiz, T., Alter, T., Bartel, E., Fricke, U., and Essig, A. (2008) Identification of thermotolerant *Campylobacter* species by fluorescence in situ hybridization. *J. Clin. Microbiol.* **46**: 2133–2136.

Qin, S., Wang, Y., Zhang, Q., Zhang, M., Deng, F., Shen, Z., et al. (2014) Report of ribosomal RNA methylase gene *erm(B)* in multidrug-resistant *Campylobacter coli*. *J. Antimicrob. Chemother.* **69**: 964–968.

Quiñones, B., Miller, W.G., Bates, A.H., and Mandrell, R.E. (2009) Autoinducer-2 production in *Campylobacter jejuni* contributes to chicken colonization. *Appl. Environ. Microbiol.* **75**: 281–285.

Raffatellu, M. and Bäumler, A.J. (2010) *Salmonella*'s iron armor for battling the host and its microbiota. *Gut Microbes* **1**: 70–72.

Raffatellu, M., Wilson, R.P., Winter, S.E., and Bäumler, A.J. (2008) Clinical pathogenesis of typhoid fever. *J. Infect. Dev. Ctries.* **2**: 260–266.

Rahman, H., Prager, R., and Tschape, H. (2000) Ocurrence of *sef* & *pef* genes among different serovars of *Salmonella*. **111**: 40–42.

Ramos, R., Cerdà-Cuéllar, M., Ramírez, F., Jover, L., and Ruiz, X. (2010) Influence of refuse sites on the prevalence of *Campylobacter* spp. and *Salmonella* serovars in seagulls. *Appl. Environ. Microbiol.* **76**: 3052–3056.

Rantsiou, K., Kathariou, S., Winkler, A., Skandamis, P., Saint-Cyr, M.J., Rouzeau-Szynalski, K., and Amézquita, A. (2017) Next generation microbiological risk assessment: Opportunities of whole genome sequencing (WGS) for foodborne pathogen surveillance, source tracking and risk assessment. *Int. J. Food Microbiol.* doi: 10.1016/j.ijfoodmicro.2017.11.007

Raven, S.J. and Coulson, J.C. (2001) Effects of cleaning a tidal river of sewage on gull numbers: A before-and-after study of the River Tyne, northeast England. *Bird Study* **48**: 48–58.

Reed, K.D., Meece, J.K., and Henkel, J.S. (2003) Birds, Migration and Emerging Zoonoses: West Nile Virus, Lyme Disease, Influenza A and Enteropathogens. *Clin. Med. Res.* **1**: 5–12.

Reeves, M.W., Evins, G.M., Heiba, A.A., Plikaytis, B.D., and Farmer, J.J. (1989) Clonal nature of *Salmonella typhi* and its genetic relatedness to other salmonellae as shown by multilocus enzyme electrophoresis, and proposal of *Salmonella bongori* comb. nov. *J. Clin. Microbiol.* **27**: 313–320.

Refsum, T., Handeland, K., Baggesen, D.L., Holstad, G., and Kapperud, G. (2002) Salmonellae in avian wildlife in Norway from 1969 to 2000. *Appl. Environ.*

Microbiol. **68**: 5595–5599.

Refsum, T., Heir, E., Kapperud, G., Vardund, T., and Holstad, G. (2002) Molecular epidemiology of *Salmonella enterica* serovar typhimurium isolates determined by pulsed-field gel electrophoresis: Comparison of isolates from avian wildlife, domestic animals, and the environment in Norway. *Appl. Environ. Microbiol.* **68**: 5600–5606.

Retamal, P., Fresno, M., Dougnac, C., Gutierrez, S., Gornall, V., Vidal, R., et al. (2015) Genetic and phenotypic evidence of the *Salmonella enterica* serotype Enteritidis human-animal interface in Chile. *Front. Microbiol.* **6**: 464.

Retamal, P., Llanos-Soto, S., Salas, L.M., López, J., Vianna, J., Hernández, J., et al. (2017) Isolation of drug-resistant *Salmonella enterica* serovar enteritidis strains in gentoo penguins from Antarctica. *Polar Biol.* **40**: 2531–2536.

Revolledo, L. and Ferreira, A.J.P. (2012) Current perspectives in avian salmonellosis: Vaccines and immune mechanisms of protection. *J. Appl. Poult. Res.* **21**: 418–431.

Ribot, E.M., Fair, M. a, Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B., and Barrett, T.J. (2006) Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathog. Dis.* **3**: 59–67.

Ribot, E.M., Fitzgerald, C., Kubota, K., Swaminathan, B., and Barrett, T.J. (2001) Rapid Pulsed-Field Gel Electrophoresis protocol for subtyping of *Campylobacter jejuni*. *J. Clin. Microbiol.* **39**: 1889–1894.

Ridley, A.M., Toszeghy, M.J., Cawthraw, S.A., Wassenaar, T.M., and Newell, D.G. (2008) Genetic instability is associated with changes in the colonization potential of *Campylobacter jejuni* in the avian intestine. *J. Appl. Microbiol.* **105**: 95–104.

Ring, M., Zychowska, M.A., and Stephan, R. (2005) Dynamics of *Campylobacter* spp. Spread investigated in 14 broiler flocks in Switzerland. *Avian Dis.* **49**: 390–396.

Van Riper, C., Van Riper, S.G., Goff, L.M., and Laird, M. (1986) The Epizootiology and Ecological Significance of Malaria in Hawaiian Land Birds. *Ecol. Monogr.* **56**: 327–344.

Rodriguez-Rivera, L.D., Bowen, B.M., den Bakker, H.C., Duhamel, G.E., and Wiedmann, M. (2015) Characterization of the cytolethal distending toxin (typhoid toxin) in non-typhoidal *Salmonella* serovars. *Gut Pathog.* **7**: 19.

Rollins, D.M. and Colwell, R.R. (1986) Viable but nonculturable stage of *Campylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl. Environ. Microbiol.* **52**: 531–538.

Ross, I.L. and Heuzenroeder, M.W. (2009) A comparison of two PCR-based typing methods with pulsed-field gel electrophoresis in *Salmonella enterica* serovar Enteritidis. *Int. J. Med. Microbiol.* **299**: 410–420.

Rossi, M., Debruyne, L., Zanoni, R.G., Manfreda, G., Revez, J., and Vandamme, P. (2009) *Campylobacter avium* sp. nov., a hippurate-positive species isolated from poultry. *Int. J. Syst. Evol. Microbiol.* **59**: 2364–2369.

Rubinchik, S., Seddon, A., and Karlyshev, A. V (2012) Molecular mechanisms and biological role of *Campylobacter jejuni* attachment to host cells. *Eur. J. Microbiol. Immunol. (Bp).* **2**: 32–40.

Ruby, T., Mclaughlin, L., Gopinath, S., and Monack, D. (2012) *Salmonella*'s long-term relationship with its host. *FEMS Microbiol. Rev.* **36**: 600–615.

Ruiz-Fons, F. (2017) A Review of the Current Status of Relevant Zoonotic Pathogens in Wild Swine (*Sus scrofa*) Populations: Changes Modulating the Risk of Transmission to Humans. *Transbound. Emerg. Dis.* **64**: 68–88.

Ruzauskas, M. and Vaskeviciute, L. (2016) Detection of the *mcr-1* gene in *Escherichia coli* prevalent in the migratory bird species Larus argentatus. *J. Antimicrob. Chemother.* **71**: 2333–2334.

Sabbagh, S.C., Forest, C.G., Lepage, C., Leclerc, J.M., and Daigle, F. (2010) So similar, yet so different: uncovering distinctive features in the genomes of *Salmonella enterica* serovars Typhimurium and Typhi. *FEMS Microbiol. Lett.* **305**: 1–13.

Sahin, O., Morishita, T.Y., and Zhang, Q. (2002) *Campylobacter* colonization in poultry: sources of infection and modes of transmission. *Anim. Heal. Res. Rev.* **3**: 95–105.

Sails, A.D., Swaminathan, B., and Fields, P.I. (2003) Utility of Multilocus Sequence Typing

as an Epidemiological Tool for Investigation of Outbreaks of Gastroenteritis Caused by *Campylobacter jejuni* Utility of Multilocus Sequence Typing as an Epidemiological Tool for Investigation of Outbreaks of Gastroente. *J. Clin. Microbiol.* **41**: 4733–4739.

Sampers, I., Habib, I., De Zutter, L., Dumoulin, A., and Uyttendaele, M. (2010) Survival of *Campylobacter* spp. in poultry meat preparations subjected to freezing, refrigeration, minor salt concentration, and heat treatment. *Int. J. Food Microbiol.* **137**: 147–153.

Sebald, M. and Véron, M. (1963) Base DNA content and classification of Vibrios. *Ann. l'Institut Pasteur* **105**: 897–910. *In French*.

Sensale, M., Cuomo, A., Dipineto, L., Santaniello, A., Calabria, M., Menna, L.F., and Fioretti, A. (2010) Survey of *Campylobacter jejuni* and *Campylobacter coli* in different taxa and ecological guilds of migratory birds. *Ital. J. Anim. Sci.* **5**: 291–294.

Sheppard, S.K., Colles, F.M., McCarthy, N.D., Strachan, N.J.C., Ogden, I.D., Forbes, K.J., et al. (2011) Niche segregation and genetic structure of *Campylobacter jejuni* populations from wild and agricultural host species. *Mol. Ecol.* **20**: 3484–3490.

Sheppard, S.K., Jolley, K.A., and Maiden, M.C.J. (2012) A gene-by-gene approach to bacterial population genomics: Whole genome MLST of *Campylobacter*. *Genes (Basel).* **3**: 261–277.

Shi, C., Singh, P., Ranieri, M.L., Wiedmann, M., and Moreno Switt, A.I. (2015) Molecular methods for serovar determination of *Salmonella*. *Crit. Rev. Microbiol.* **41**: 309–325.

Shirihai, H. (2007) A Complete Guide to Antarctic Wildlife: the birds and marine mammals of the Antarctic Continent and the Southern Ocean 2nd ed. Princeton University Press, Princeton, New Jersey.

Shivaprasad, H.L. and Barrow, P.A. (2008) Pullorum disease and fowl typhoid. In Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., and Swayne, D.E. (eds), *Diseases of Poultry*. Blackwell Publishing, Ames, Iowa, pp. 620–634.

Sippy, R., Sandoval-Green, C.M.J., Sahin, O., Plummer, P., Fairbanks, W.S., Zhang, Q.,

and Blanchong, J.A. (2012) Occurrence and molecular analysis of *Campylobacter* in wildlife on livestock farms. *Vet. Microbiol.* **157**: 369–375.

Skirrow, M.B. (1977) *Campylobacter* enteritis: a “new” disease. *Br. Med. J.* **2**: 9–11.

Skyberg, J., Logue, C.M., and Nolan, L.K. (2006) Virulence genotyping of *Salmonella* spp. with multiplex PCR. *Avian Dis.* **50**: 77–81.

Mughini-Gras, L., Smid, J.H., Wagenaar, J.A., de Boer, A., Havelaar, A.H., Friesema, I.H., French, N.P, Graziani, C., Busani, L., and van Pelt, W. (2014). Campylobacteriosis in returning travellers and potential secondary transmission of exotic strains. *Epidemiol. Infect*. **142**: 1277–1288.

Smith, A.M., Nomsa, T., Arvinda, S., Keddy, K.H., and GERMS-SA Surveillance (2016) Microbiological characterization of *Salmonella enterica* serotype Paratyphi , South Africa , 2003 – 2014. *J Med Microbiol*. **64**: 1450-1453.

Smith, J.J. and Riddle, M.J. (2009) Sewage disposal and wildlife health in Antarctica. In Kerry, K.R. and Riddle, M.J. (eds), *Health of Antarctic Wildlife: A Challenge for Science and Policy*. Springer, Berlin, pp. 271–315.

Smith, T. and Taylor, M.S. (1919) Some Morphological and Biological Characters of the *Spirilla* (*Vibrio fetus*, N. Sp.) Associated With Disease of the Fetal Membranes in Cattle. *J. Exp. Med.* **30**: 299–311.

Sommer, H.M., Høg, B.B., Larsen, L.S., Sørensen, A.I.V., Williams, N., Merga, J.Y., et al. (2016) Analysis of farm specific risk factors for *Campylobacter* colonization of broilers in six European countries. *Microb. Risk Anal.* **2**–**3**: 16–26.

Soto, S.M., Rodríguez, I., Rodicio, M.R., Vila, J., and Mendoza, M.C. (2006) Detection of virulence determinants in clinical strains of *Salmonella enterica* serovar Enteritidis and mapping on macrorestriction profiles. *J. Med. Microbiol.* **55**: 365–373.

Soyer, Y., Alcaine, S.D., Schoonmaker-Bopp, D.J., Root, T.P., Warnick, L.D., McDonough, P.L., et al. (2010) Pulsed-field gel electrophoresis diversity of human and bovine clinical *Salmonella* isolates. *Foodborne Pathog. Dis.* **7**: 707–717.

Spector, M.P. and Kenyon, W.J. (2012) Resistance and survival strategies of *Salmonella enterica* to environmental stresses. *Food Res. Int.* **45**: 455–481.

Sproston, E.L., Ogden, I.D., MacRae, M., Dallas, J.F., Sheppard, S.K., Cody, A.J., et al. (2011) Temporal variation and host association in the *Campylobacter* population in a longitudinal ruminant farm study. *Appl. Environ. Microbiol.* **77**: 6579–6586.

Stanley, T.L., Ellermeier, C.D., and Slauch, J.M. (2000) Tissue-specific gene expression identifies a gene in the lysogenic phage Gifsy-1 that affects *Salmonella enterica* serovar typhimurium survival in Peyer's patches. *J. Bacteriol.* **182**: 4406–4413.

Stenhouse, I.J., Egevan, C., and Phillips, R.A. (2012) Trans-equatorial migration, staging sites and wintering area of Sabine's Gulls Larus sabini in the Atlantic Ocean. *Ibis (Lond. 1859).* **154**: 42–51.

Suez, J., Porwollik, S., Dagan, A., Marzel, A., Schorr, Y.I., Desai, P.T., et al. (2013) Virulence Gene Profiling and Pathogenicity Characterization of Non-Typhoidal *Salmonella* Accounted for Invasive Disease in Humans. *PLoS One* **8**: e58449.

Swanenburg, M., Urlings, H. a P., Keuzenkamp, D. a, and Snijders, J.M. a (1998) Validation of ERIC PCR as a tool in epidemiologic research of *Salmonella* in slaughter pigs. *J. Ind. Microbiol. Biotechnol.* **21**: 141–144.

Taboada, E.N., Clark, C.G., Sproston, E.L., and Carrillo, C.D. (2013) Current methods for molecular typing of *Campylobacter* species. *J. Microbiol. Methods* **95**: 24–31.

Tahoun, A., Mahajan, S., Paxton, E., Malterer, G., Donaldson, D.S., Wang, D., et al. (2012) *Salmonella* transforms follicle-associated epithelial cells into M cells to promote intestinal invasion. *Cell Host Microbe* **12**: 645–656.

Takahashi, T., Ishihara, K., Kojima, A., Asai, T., Harada, K., and Tamura, Y. (2005) Emergence of fluoroquinolone resistance in *Campylobacter jejuni* in chickens exposed to enrofloxacin treatment at the inherent dosage licensed in Japan. *J. Vet. Med. Ser. B Infect. Dis. Vet. Public Heal.* **52**: 460–464.

Talukder, K.A., Aslam, M., Islam, Z., Azmi, I.J., Dutta, D.K., Hossain, S., et al. (2008) Prevalence of virulence genes and cytolethal distending toxin production in *Campylobacter jejuni* isolates from diarrheal patients in Bangladesh. *J. Clin. Microbiol.* **46**: 1485–1488.

Taylor, L.H., Latham, S.M., and woolhouse, M.E.J. (2001) Risk factors for human disease

emergence. *Philos. Trans. R. Soc. B Biol. Sci.* **356**: 983–989.

The Salmonella Subcommittee of the Nomenclature Committee of the International Society for Microbiology (1934) The Genus *Salmonella* Lignières, 1900. *J. Hyg. (Lond).* **34**: 333–350.

Thorns, C.J. (2000) Bacterial food-borne zoonoses. *Rev. Sci. Tech.* **19**: 226–239.

Toboldt, A., Tietze, E., Helmuth, R., Fruth, A., Junker, E., and Malorny, B. (2012) Human infections attributable to the d-tartrate-fermenting variant of *Salmonella enterica* serovar paratyphi B in Germany originate in reptiles and, on rare occasions, poultry. *Appl. Environ. Microbiol.* **78**: 7347–7357.

Toboldt, A., Tietze, E., Helmuth, R., Junker, E., Fruth, A., and Malorny, B. (2014) Molecular epidemiology of *Salmonella enterica* serovar Kottbus isolated in Germany from humans, food and animals. *Vet. Microbiol.* **170**: 97–108.

Tsiodras, S., Kelesidis, T., Kelesidis, I., Bauchinger, U., and Falagas, M.E. (2008) Human infections associated with wild birds. *J. Infect.* **56**: 83–98.

Turki, Y., Mehri, I., Cherif, H., Najjari, A., Ben Aissa, R., Hassen, A., and Ouzari, H. (2012) Epidemiology and antibiotic resistance of *Salmonella enterica* Serovar Kentucky isolates from Tunisia: The new emergent multi-drug resistant serotype. *Food Res. Int.* **45**: 925–930.

Urdaneta, S. (2016) Epidemiología de *Campylobacter* spp. en granas de pollos de engorde: prevalencia, factores de riesgo y dinámica de infección. *PhD thesis*. Universitat Autònoma de Barcelona, Bellaterra.

Urdaneta, S., Dolz, R., and Cerdà-Cuéllar, M. (2015) Assessment of two different types of sample for the early detection and isolation of thermophilic *Campylobacter* in broiler farms. *Avian Pathol.* **44**: 103–105.

Urwin, R. and Maiden, M.C.J. (2003) Multi-locus sequence typing: A tool for global epidemiology. *Trends Microbiol.* **11**: 479–487.

Uzzau, S., Brown, D.J., Wallis, T., Rubino, S., Leori, G., Bernard, S., et al. (2000) Host adapted serotypes of *Salmonella enterica*. *Epidemiol. Infect.* **125**: 229–255.

Valentín-Bon, I.E., Brackett, R.E., Seo, K.H., Hammack, T.S., and Andrews, W.H. (2003) Preenrichment versus direct selective agar plating for the detection of *Salmonella* Enteritidis in shell eggs. *J. Food Prot.* **66**: 1670–1674.

Van, T.T., Elshagmani, E., Gor, M.C., Scott, P.C., and Moore, R.J. (2016) *Campylobacter hepaticus* sp. nov., isolated from chickens with spotty liver disease. *Int. J. Syst. Evol. Microbiol.* **66**: 4518–4524.

Vandamme, P., Daneshvar, M.I., Dewhirst, F.E., Paster, B.J., Kersters, K., Goossens, H., and Moss, C.W. (1995) Chemotaxonomic analysis of *Bacteroides gracilis* and *Bacteroides ureolyticus* and reclassification of *B. gracilis* as *Campylobacter gracilis* comb. nov. *Int. J. Syst. Bacteriol.* **45**: 145–152.

Vandamme, P. and De Ley, J. (1991) Proposal for a New Family. *Int J Syst Bacteriol.* **41**: 451-455.

Véron, M. and Chatelain, R. (1973) Taxonomic Study of the Genus *Campylobacter* Sebald and Veron and Designation of the Neotype Strain for the Type Species, *Campylobacter fetus* (Smith and Taylor) Sebald and Veron. *Int. J. Syst. Bacteriol.* **23**: 122–134.

Versalovic, J., Koeuth, T., and Lupski, J.R. (1991) Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res.* **19**: 6823–6831.

Vigo, G.B., Leotta, G.A., Caffer, M.I., Salve, A., Binsztein, N., and Pichel, M. (2011) Isolation and characterization of *Salmonella enterica* from Antarctic wildlife. *Polar Biol.* **34**: 675–681.

van Vliet, a H. and Ketley, J.M. (2001) Pathogenesis of enteric *Campylobacter* infection. *J. Appl. Microbiol.* **90**: 45S–56S.

Wagner, C. and Hensel, M. (2011) Adhesive Mechanisms of *Salmonella enterica*. *Adv Exp Med Biol.* **715***:* 17–34.

Wales, A.D., Carrique-Mas, J.J., Rankin, M., Bell, B., Thind, B.B., and Davies, R.H. (2010) Review of the carriage of zoonotic bacteria by arthropods, with special reference to salmonella in mites, flies and litter beetles. *Zoonoses Public Health* **57**: 299–314.

Wang, H., Shu, R., Zhao, Y., Zhang, Q., Xu, X., and Zhou, G. (2014) Analysis of ERIC-PCR genomic polymorphism of *Salmonella* isolates from chicken slaughter line. *Eur. Food Res. Technol.* **239**: 543–548.

Wassenaar, T.M. (1997) Toxin production by *Campylobacter* spp. *Clin.Microbiol.Rev.* **10**: 466–476.

Wassenaar, T.M., Geilhausen, B., and Newell, D.G. (1998) Evidence of genomic instability in *Campylobacter jejuni* isolated from poultry. *Appl. Environ. Microbiol.* **64**: 1816–1821.

Wassenaar, T.M., Van Der Zeijst, B.A., Ayling, R., and Newell, D.G. (1993) Colonization of chicks by motility mutants of *Campylobacter jejuni* demonstrates the importance of flagellin A expression. *J. Gen. Microbiol.* **139**: 1171–1175.

Weimerskirch, H. (2004) Diseases threaten Southern Ocean albatrosses. *Polar Biol.* **27**: 374–379.

Wellington, E.M.H., Boxall, A.B.A., Cross, P., Feil, E.J., Gaze, W.H., Hawkey, P.M., et al. (2013) The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria. *Lancet Infect. Dis.* **13**: 155–165.

Weimerskirch, H., Tarroux, A., Chastel, O., Delord, K., Cherel, Y., and Descamps, S. (2015) Population-specific wintering distributions of adult south polar skuas over three oceans. *Mar. Ecol. Prog. Ser.* **538**: 229–237.

Whiley, H., van den Akker, B., Giglio, S., and Bentham, R. (2013) The role of environmental reservoirs in human campylobacteriosis. *Int. J. Environ. Res. Public Health* **10**: 5886–5907.

Whiley, H., McLean, R., and Ross, K. (2016) Detection of *Campylobacter jejuni* in Lizard Faeces from Central Australia Using Quantitative PCR. *Pathogens* **6**: 1.

Whittington, P. a, M Craw ford, R.J., Paul Martin, A., Randall, R.M., Brown, M., Ryan, P.G., et al. (2016) Recent Trends of the Kelp Gull (*Larus dominicanus*) in South Africa. *Waterbirds* **39**: 99–113.

WHO-AGISAR Advisory Group on Integrated Surveillance of Antimicrobial Resistance (2017) Critically Important Antimicrobials for Human Medicine - 5th Revision 2016,

Geneva. Available at: http://apps.who.int/iris/bitstream/10665/255027/1/9789241512220-eng.pdf

WHO (2017a) *Campylobacter*. *Fact sheet*. Available at: http://www.who.int/mediacentre/factsheets/fs255/en/

WHO (2015a) Global action plan on antimicrobial resistance. Geneva. Available at: www.wpro.who.int/entity/drug_resistance/resources/global_action_plan_eng.pdf

WHO (2017b) *Salmonella* (non-typhoidal). *Fact sheet*. Available at: http://www.who.int/mediacentre/factsheets/fs139/en/

WHO (2015b) WHO estimates of the global burden of foodborne diseases: Foodborne Disease Burden Epidemiology Reference Group 2007-2015. WHO Press, Geneva. Available at: http://apps.who.int/iris/bitstream/10665/199350/1/9789241565165_eng.pdf

WHO (2008) Typhoid vaccines: WHO position paper. *Weekly epidemiological record* **83**: 49-60.

Wiethoelter, A.K., Beltrán-Alcrudo, D., Kock, R., and Mor, S.M. (2015) Global trends in infectious diseases at the wildlife–livestock interface. *Proc. Natl. Acad. Sci.* **112**: 9662–9667.

Williams, L.K., Jørgensen, F., Grogono-Thomas, R., and Humphrey, T.J. (2009) Enrichment culture for the isolation of *Campylobacter* spp: Effects of incubation conditions and the inclusion of blood in selective broths. *Int. J. Food Microbiol.* **130**: 131–134.

Winfiel, M.D. and Groisman, E.A. (2003) Role of Nonhost Enviroments in the Lifestyles of *Salmonella* and *E. coli*. *Appl. Environ. Mcrobiology* **69**: 3687–3694.

Winter, S.E., Thiennimitr, P., Winter, M.G., Butler, B.P., Huseby, D.L., Crawford, R.W., et al. (2010) Gut inflammation provides a respiratory electron acceptor for *Salmonella*. *Nature* **467**: 426–429.

Von Wintersdorff, C.J.H., Penders, J., Van Niekerk, J.M., Mills, N.D., Majumder, S., Van Alphen, L.B., et al. (2016) Dissemination of antimicrobial resistance in microbial

ecosystems through horizontal gene transfer. *Front. Microbiol.* **7**: 137.

Witteveen, M., Brown, M., and Ryan, P.G. (2017) Anthropogenic debris in the nests of kelp gulls in South Africa. *Mar. Pollut. Bull.* **114**: 6–11.

Wood, M.W., Jones, M.A., Watson, P.R., Hedges, S., Wallis, T.S., and Galyov, E.E. (1998) Identification of a pathogenicity island required for *Salmonella* enteropathogenicity. *Mol. Microbiol.* **29**: 883–891.

Woodall, C.A., Jones, M.A., Barrow, P.A., Hinds, J., Marsden, G.L., Kelly, D.J., et al. (2005) *Campylobacter jejuni* gene expression in the chick cecum: Evidence for adaptation to a low-oxygen environment. *Infect. Immun.* **73**: 5278–5285.

Wu, H., Jones, R.M., and Neish, A.S. (2013) The *Salmonella* effector AvrA mediates bacterial intracellular survival during infection in vivo Huixia. *Cell Microbiol.* **14**: 28–39.

Yabe, S., Higuchi, W., Iwao, Y., Takano, T., Razvina, O., Reva, I., et al. (2010) Molecular typing of *Campylobacter jejuni* and *C. coli* from chickens and patients with gastritis or guillain-barré syndrome based on multilocus sequence types and pulsed-field gel electrophoresis patterns. *Microbiol. Immunol.* **54**: 362–367.

Yang, L., Hu, X., Xu, X., Yang, C., Xie, J., Hao, R., et al. (2017) *Salmonella enterica* serovar Typhimurium ST34 co-expressing blaNDM-5 and blaCTX-M-55 isolated in China. *Emerg. Microbes Infect.* **6**: e61.

Ye, Y., Wu, Q., Zhang, J., Lu, J., and Lin, L. (2011) Isolation of *Salmonella* from Meat Samples and Characterization by Enterobacterial Repetitive Intergenic Consensus–Polymerase Chain Reaction and Antibiotics Test. *Foodborne Pathog. Dis.* **8**: 935–937.

Young, K.T., Davis, L.M., and Dirita, V.J. (2007) *Campylobacter jejuni*: molecular biology and pathogenesis. *Nat. Rev. Microbiol.* **5**: 665–679.

Yu, X.J., Liu, M., and Holden, D.W. (2004) SsaM and SpiC interact and regulate secretion of *Salmonella* Pathogenicity Island 2 type III secretion system effectors and translocators. *Mol. Microbiol.* **54**: 604–619.

Zaki, S.A. and Karande, S. (2011) Multidrug-resistant typhoid fever: A review. *J. Infect.*

Dev. Ctries. **28**: 324-337.

Zhou, D., Chen, L.M., Hernandez, L., Shears, S.B., and Galán, J.E. (2001) A *Salmonella* inositol polyphosphatase acts in conjunction with other bacterial effectors to promote host cell actin cytoskeleton rearrangements and bacterial internalization. *Mol. Microbiol.* **39**: 248–259.

Zinsstag, J., Schelling, E., Waltner-Toews, D., and Tanner, M. (2011) From “one medicine” to “one health” and systemic approaches to health and well-being. *Prev. Vet. Med.* **101**: 148–156.

ANNEX

Guide of studied wild birds

ORDER SPHENISCIFORMES

King penguin *(Aptenodytes patagonicus)*

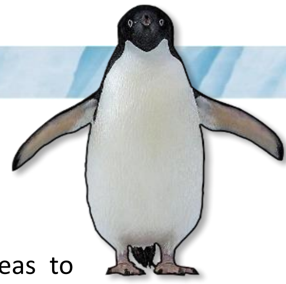
There are two subspecies distributed on sub-Antarctic islands. *A. patagonicus patagonicus* is found at Kerguelen, Crozet, Prince Edward, Marion, Heard and McDonald and Macquarie Islands. *A. patagonicus halli* breeds at South Georgia, at Falkland Islands, and in southern Chile. It spends much of its time near breeding areas since it has a prolonged breeding season (14-15 months).

It forms colonies on flattish beaches free of ice, it does not build a nest but incubates its egg on the feet. Its diet comprises mainly myctophid fish, but ice fish and cephalopods are also taken. It captures prey by means of pursuit-diving mostly during the day, and forages at depths of 200 m.

Magellanic penguin *(Spheniscus magellanicus)*

It breeds on the Atlantic and Pacific coasts of South America, mainly in Argentina, Chile and Falkland Islands. In winter, many Atlantic birds migrate north to Uruguay and southern Brazil, while Pacific birds are less migratory and rarely arrive to Peru.

It nests in a variety of island and mainland coastal habitats, including scrublands and grasslands in Argentina, temperate forests in Chile and tussock grass in Falkland Islands. Individuals show high site fidelity, with nearly all birds returning to the colony in which they were born, and most adults using the same burrow year after year. It forages exclusively in the oceans at hundreds of kilometres from the colony, even during incubation and chick-rearing. It preys cuttlefish, squid, krill, and other crustaceans, diving to depths of 50 m.



Adelie penguin *(Pygoscelis adeliae)*

It is found along the Antarctic coast and at some of its nearby islands. It migrates to more northern latitudes all around Antarctica and stays at the edge of the fast ices during winter.

It nests on ice-free rocky coasts, often in extensive open areas to accommodate typically large colonies which may be far from the open sea. Individuals are dispersive, moving towards areas of persistent sea ice to moult after breeding. It mainly feeds on krill, fish, amphipods and cephalopods. It captures such prey by pursuit-diving to about 150 m.

Chinstrap penguin *(Pygoscelis antarctica)*

It has a circumpolar distribution, being found in Antarctica, South Shetland, South Orkneys, South Sandwich and South Georgia, Bouvet and Balleny Islands. Winter migrations can be extensive, generally at pelagic habitats located north of the sea ice edge.

It breeds on irregular rocky coasts in ice free areas, forming large colonies of hundreds and thousands of birds. Its diet is comprised almost exclusively of Antarctic krill, but it also takes fish and other species of crustaceans when possible. Prey capture is by pursuit-diving up to a depth of 70 m.

Gentoo penguin *(Pygoscelis papua)*

It has a circumpolar breeding distribution ranging from Fish Islands on the Antarctic Peninsula to the Crozet Islands. An 80% of the global population is located in South Georgia, Falkland Islands, and to a lesser extent in the Antarctic Peninsula. Winter movements are more local relative to its congeners with preference for coastal areas.

It nests on flat beaches or among tussock grasses in Falkland and Marion Islands, and South Georgia, and on low lying gravel beaches and dry moraines in the Antarctic Peninsula. It is an opportunistic feeder, preying predominantly on crustaceans, fish, and squid. It prefers foraging inshore, close to the breeding colony.

Macaroni penguin (*Eudyptes chrysolophus*)

It breeds in southern Chile, sub-Antarctic Islands, and very locally on the Antarctic Peninsula. Key populations are found on South Georgia, Marion, Heard and McDonald, Kerguelen and Crozet Islands. Birds from Kerguelen Island spend the winter on the Polar Frontal Zone, while those from South Georgia widely distribute across the Scotia Sea.

It nests on level to steep ground, often walking hundreds of metres across steep scree slopes to nest-sites. It is a pelagic forager, searching for prey at moderate depths, usually less than 50 m. It feeds mainly on small krill, but also euphausiids, crustaceans, amphipods and small amounts of myctophid fish.

Northern rockhopper penguin (*Eudyptes moseleyi)*

It is found in the temperate South Atlantic and Indian Oceans. The 85% of the global population breeds at Tristan da Cunha and Gough Islands. After breeding and moulting, it departs on its winter migration and spends up to six months at sea before returning to its breeding site.

At the Atlantic breeding sites, nests are located in open boulder-strewn beaches and stands of tussock grass. In the Indian Ocean, penguins breed in steep or gently sloping ground up to 170 m above sea-level. During incubation, it forages up to 800 km from the colony, whereas during brooding foraging range is restricted to a maximum distance of 35 km. It is an opportunistic forager, mainly feeding on crustaceans, in particular euphausiids, but also fish and cephalopods.

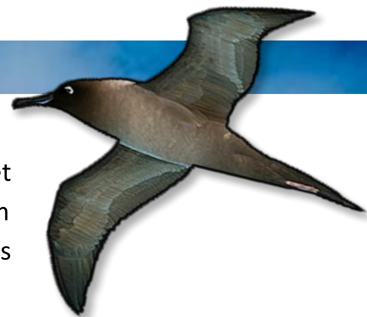
Southern rockhopper penguin (*Eudyptes chrysocome)*

There are two subspecies distributed in islands of the western Pacific and Indian Oceans, as well as around the southern coast of South America. *E. chrysocome chrysocome* is found in the Falkland Islands and other islands off southern Chile and Argentina. *E. chrysocome filholi* is present on several sub-Antarctic islands to the south of New Zealand and South Africa.

Its breeding habits range from sea-level sites to cliff-tops, and sometimes inland. At the Falkland Islands hybridization occurs with Macaroni and Northern Rockhopper penguins. It preys on a variety of fish, crustaceans and cephalopods, but there is individual dietary specialization during part of their annual cycle. It dives to depths of up to 100 m in pursuit of prey.

ORDER PROCELLARIIFORMES

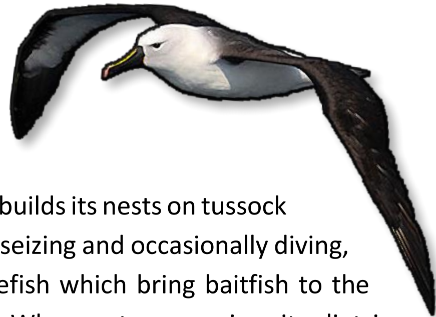
Sooty albatross (*Phoebetria fusca)*



It breeds on islands in the South Atlantic and Indian Oceans, mainly on Tristan da Cunha, Gough, Marion, Crozet and Amsterdam Islands. Adults move north in winter from sub-Antarctic to subtropical seas, whereas immature birds tend to remain in subtropical seas year-round.

It breeds on loose colonies, on cliffs or steep slopes where it can land and take off right next to the nest. It makes a combination of long commuting flights early in the incubation period, looping searching flights later in incubation and linear searching during chick brooding. Its diet consists on squid, fish, crustaceans and carrion; the proportions of each vary between years and locations.

Atlantic yellow-nosed albatross (*Thalassarche chlororhynchos)*



It breeds on islands in the mid-Atlantic, including Tristan da Cunha and Gough Islands. At sea they range across the South Atlantic from South America to Africa.

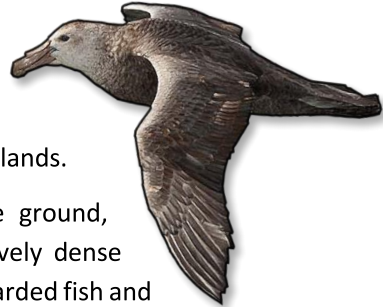
It usually breeds singly or in loose aggregations and builds its nests on tussock grass, on rocks and under trees. It feeds by surface-seizing and occasionally diving, also in association with marine mammals or gamefish which bring baitfish to the surface. It is strongly attracted to fishing vessels. When not scavenging, its diet is largely comprised of fish, but also cephalopods.

Black-browed albatross (*Thalassarche melanophris)*

Atlantic yellow-nosed albatross has a circumpolar distribution ranging from subtropical to polar waters. Most of the population is located at Falkland Islands, but also in Chile and South Georgia. Birds from Falkland Islands winter on the Patagonian Shelf, while birds from South Georgia predominantly migrate to South African waters, spending the first half of the winter in the highly productive Benguela Current.

It nests on steep slopes with tussock grass, sometimes on cliff terraces, but the largest colonies in Falkland Islands are on flat ground along the shore line. During breeding, it tends to remain close to their colonies in shelf, shelf-break and shelf-slope waters. It feeds mainly on crustaceans, fish and squid, and also on carrion and fishery discards.

Southern giant petrel (*Macronectes giganteus)*



Its distribution ranges from Antarctica to the subtropics of Chile, Africa and Australia. It breeds on numerous islands throughout the Southern Ocean. The 42% of the global population is located on the Falkland Islands.

It typically nests in loose colonies on grassy or bare ground, although in Falkland Islands it can nest in large, relatively dense colonies. It feeds on carrion, cephalopods, krill, offal, discarded fish and refuse from ships, often feeding near trawlers and long-liners. Males and females exhibit clearly defined spatial segregation in their foraging ranges.

Northern giant petrel (*Macronectes halli)*

It is found throughout the Southern Ocean north of the Antarctic Convergence Zone, Chile, Argentina, South Africa and half of Australia. It breeds on many sub-Antarctic islands.

It breeds in colonies shared with the southern giant petrel, six weeks earlier than their counterparts. It feeds on penguin and pinniped carrion, cephalopods, krill, offal, discarded fish and refuse from ships, often feeding near trawlers and long-liners. During the breeding season, males exploit scavenging opportunities in and around seal and penguin colonies and are coastal in distribution, whereas females are much more dependent on pelagic resources.

White-chinned petrel (*Procellaria aequinoctialis)*

It is widely distributed throughout the Southern Ocean, as far north as equatorial waters and south to the pack-ice edge off Antarctica. In winter, it migrates from the Antarctic pack ice to the subtropics. Birds from Falkland Islands and South Georgia travel to the Patagonian Shelf waters, birds from Crozet, Marion and Kerguelen Islands fly to South Africa and Namibia over the Benguela Current. Petrels from Auckland and Antipodes Islands winters off the coast of Peru, Ecuador and northern Chile.

It ranges widely when searching for food resources, travelling up to 8,000 km on feeding forays in the breeding season. It feeds on cephalopods, crustaceans and fish, as well as fisheries processing waste or discarded long-line baits.

Soft-plumaged petrel (*Pterodroma mollis)*

It breeds on islands in the Southern Hemisphere, nesting on Tristan da Cunha, Gough, Prince Edward, Crozet and Antipodes Islands. It disperses outside the breeding season, reaching eastern South America north to **Brazil**, South Africa, **Australia** and New Zealand.

It nests in long burrows, occupying steep slopes with tussock grass or ferns, usually along the coast but also inland. It is highly pelagic, rarely approaching land except at colonies. It feeds mostly on cephalopods but will also take crustaceans and fish, which is taken mainly by surface-seizing.

Atlantic petrel (*Pterodroma incerta)*

It breeds only on Tristan da Cunha and Gough Islands. At sea, it is practically restricted to the South Atlantic, occurring off the east coast of South America to the west coast of Africa, occasionally rounding the Cape of Good Hope into the Indian Ocean.

It nests in burrows dug in peaty soils in fern-bush vegetation. It feeds mainly on squid with some fish and crustaceans.

Great shearwater (*Ardenna gravis)*

It breeds mainly in the Tristan da Cunha archipelago and the Gough Island, but also in the Falkland Islands in small numbers. It makes a trans-equatorial migration, moving north-west to South America, up to Canada, past Greenland and onto the north-east Atlantic.

It nests on sloping ground, mainly in areas of tussock grass or Phylica woodland. It feeds mostly on fish, squid and fish offal and some crustaceans.

Sooty shearwater (*Ardenna grisea)*

It breeds on islands in the South Pacific and South Atlantic Oceans off New Zealand, Falkland Islands, Australia and Chile. It is a long-distance migrant following a circular route, travels north up the western side of the Pacific and Atlantic Oceans, reaching subarctic waters and returning south down the eastern side of the oceans.

It nests on islands and headlands in large colonies. It dugs burrows under tussock grass, low scrub and on the Snares Islands under Olearia forest. It feeds on fish, crustacea and cephalopods, caught while diving. During breeding, it can make long provisioning trips along the Antarctic Polar Front, reducing competition close to colonies.

Broad-billed prion (*Pachyptila vittata)*

It is found throughout oceans and coastal areas in the Southern Hemisphere, breeding mainly on Gough and Tristan da Cunha Islands in the South Atlantic, and on Chatham Islands and the south of New Zealand. Adults are thought to remain in waters adjacent to colonies, while young birds occur north of the colonies to Australia and South Africa.

It is strongly colonial, nesting in burrows which are sometimes occupied by more than one pair. It breeds on coastal slopes, flat lava fields, offshore islets and cliffs, dry rocky soil, caves and scree. Its diet is comprised mostly of crustaceans, especially copepods, but also squid and some fish. Prey is obtained usually by hydroplaning and by filtering or surface-seizing.

ORDER PELECANIFORMES

Imperial shag (*Phalacrocorax atriceps)*

It is found on the southern tip of South America, from central **Chile** round to central **Argentina**, and on the **Falkland Islands.** Other subspecies are currently recognized with discrete ranges of distribution. It is basically sedentary.

It breeds in dense colonies, up to hundreds of thousand birds, often shared with other seabirds such as rock shags, southern rockhopper penguins and black-browed albatrosses. It feeds in inshore waters, mainly on benthic fish, crustaceans, squids and sea urchins.

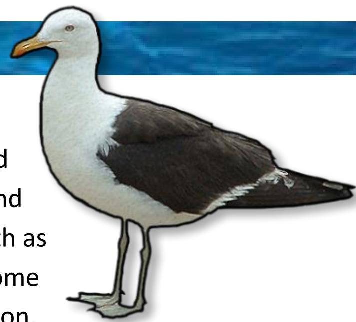
ORDER CHARADRIFORMES

Brown skua (*Stercorarius antarcticus)*

Brown skua is found on the Antarctic Peninsula and sub-Antarctic islands of the Atlantic, Indian and Pacific Oceans. It winters near or slightly dispersed from the breeding area.

It is loosely colonial but highly territorial, nesting on grass, gravel or bare rock. It is found on or around islands populated by burrow-nesting seabirds or penguins. It is highly predatory, feeding mainly on other birds but will also scavenge around fishing boats and ships and feed at sea.

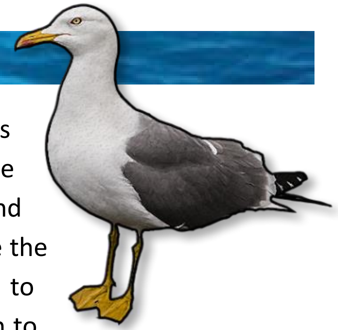
Kelp gull (*Larus dominicanus)*



It is found on a number of sub-Antarctic islands, on the Antarctic peninsula, on the southern coast of Australia and all of New Zealand, on the southern coast of Africa and Madagascar, and on the coast of South America as far north as Ecuador and southern Brazil. It is largely sedentary, but some southern populations migrate north after the breeding season.

Breeding habitats include headlands, sea cliffs, rocky outcrops, stacks, offshore islands, reefs, peninsulas, mudflats, low sandy, pebbly or rocky beaches, sandspits or islands in estuaries and lagoons. Occasionally, also nests on the roofs of coastal buildings, salt and sewage works and guano platforms. It may forage and roost in near-coastal inland habitats including harbours, bays, inlets, lagoons, lakes, swampy basins, rivers, streams, pastures, cultivated land, tussock grassland and scrubland. It often forages around abattoirs, fish- or seafood-factories and at sewage outfalls. Its diet consists of molluscs, echinoderms, sponges, arthropods, macrozooplankton, fish, worms, reptiles, amphibians, small mammals, birds and berries, but it also scavenges on refuse, sewage and carrion.

Yellow-legged gull (*Larus michahellis)*



It 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, and on the Canary Islands. Wintering grounds include the coast of south-west Asia, most of the European coast up to Denmark and the coast of Africa from Western Sahara through to the eastern Mediterranean.

It nests near lakes surrounded by reedbeds, pastures, reservoirs, rivers and on grassy or shrubby river islands, also forming colonies on sea cliffs, rocky and sandy offshore islands, rocky coasts sandy beaches, spits, sand-dunes, and salt-pans. It forages along the coast, in intertidal zones and brackish marshes, around harbours, cultivated fields and rivers, and is especially common at refuse dumps. Its diet consists of fish, insects, molluscs, crabs, reptiles, small mammals and birds (e.g. voles and squirrels, petrels and shearwaters), as well as refuse and offal.

Audouin's gull (*Larus audouinii)*

It is restricted to the Mediterranean Sea, the western coast of Saharan Africa and the Iberian Peninsula. The 67% of the global population is located in Ebro Delta. It spends the winter on the coast of North and West Africa from Libya west to Morocco and south to Gabon, and there is a small wintering population in the east Mediterranean along the Aegean coast of Turkey.

It nests on exposed rocky cliffs and on offshore islands or islets, normally not more than 50 m above sea level. It is a coastal species, rarely occurring inland and generally not travelling far offshore, prefers sheltered bays, either flat and shingly, sandy or with cliffed margins. Its diet consists mostly of epipelagic fish, but also some aquatic and terrestrial invertebrates, small birds and plant material such as the peanut, olive and grain. The Ebro Delta colony feeds largely on fish waste dumped by boats fishing nearby and food discarded at tourist beaches, it also forages in marshes, rice fields and occasionally at refuse tips.

Greater crested tern (*Thalasseus bergii)*

It is found on islands and coastlines of the tropical and subtropical Old World, ranging from the Atlantic Coast of South Africa, around the Indian Ocean to the central Pacific and Australia**.** It remains sedentary in their breeding areas or disperse locally, although some individuals are more migratory.

It nests in bare sand, rock or coral in flat open grounds on offshore islands, low-lying coastal islets, coastal spits, lagoon, mudflats, saltpans and sewage works, within 3 km of the coast. It usually forages in the shallow waters of lagoons, coral reefs, bays, harbours and inlets, along shore, on rocky outcrops in open sea, in mangrove swamps and also far out to sea on open water. Its diet consists predominantly of pelagic fish although it also takes cephalopods, crustaceans, insects and hatchling turtles opportunistically.

Source: The IUCN Red List of Threatened Species. Version 2017-2. (www.iucnredlist.org). Downloaded on 22 November 20