UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

VIRULENCE OF *SALMONELLA* TYPHIMURIUM 1,4,[5],12:i:-, THE NEW EMERGENT STRAIN

RUI EMANUEL ANTUNES DE SEIXAS

Orientadores: Professora Doutora Maria Manuela Castilho Monteiro de Oliveira
Professor Doutor Fernando Manuel d'Almeida Bernardo

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade Sanidade Animal

2017

UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

VIRULENCE OF *SALMONELLA* TYPHIMURIUM 1,4,[5],12:i:-, THE NEW EMERGENT STRAIN

RUI EMANUEL ANTUNES DE SEIXAS

Orientadores: Professora Doutora Maria Manuela Castilho Monteiro de Oliveira
Professor Doutor Fernando Manuel d'Almeida Bernardo

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade Sanidade Animal

Júri:
Presidente: Professor Doutor Rui Manuel de Vasconcelos e Horta Caldeira
Vogais:

- Professor Doutor Virgílio da Silva Almeida
- Professor Doutor Maria Manuela Marin Caniça
- Professor Doutor Maria Madalena Vieira-Pinto
- Professor Doutor Maria João dos Ramos Fraqueza
- Professor Doutor Maria Manuela Castilho Monteiro de Oliveira

2017

“O que hoje não sabemos, amanhã saberemos”
Garcia de Orta, 1563

Acknowledgements

Antes de entrar no mundo da investigação, não pensava que um doutoramento fosse um trabalho solitário mas infelizmente, a realidade encarregou-se de mostrar o contrário. Apesar de tudo, este projecto seria completamente impossível sem a ajuda da família, amigos e colegas que durante todos estes anos celebraram comigo as vitórias, não me deixaram desistir nos momentos sombrios e me aconselharam sabiamente perante novos desafios.

Quero agradecer:

Aos meus pais que durante todo este percurso me guiaram e nunca me deixaram desistir. Quero agradecer do fundo do coração toda a ajuda e apoio, e sei que sem eles nada disto seria possível.

À minha irmã e restante família por todo os conselhos e apoio ao longo de toda a minha vida académica.

À Professora Cristina Vilela, minha orientadora, e que infelizmente nos abandonou prematuramente. Recordo com saudade, todas as pequenas vitórias que celebramos quando os artigos eram aceites, quando as experiências corriam bem e quando recebia comentários simpáticos aos meus trabalhos em forma de "post-it". Guardo todos estes momentos no coração. Obrigado pelo carisma, conhecimento e orientação, características que me conquistaram desde o início, e que foram peças chave no meu desenvolvimento como investigador.

Ao Professor Fernando Bernardo, meu orientador, pela simpatia, conhecimento e orientação. Obrigado pela partilha de conhecimento e disponibilidade mostrada ao longo deste percurso.

À Professora Manuela Oliveira, minha orientadora, pela orientação nestes trabalhos e por ter aceite este desafio.

Ao Dr. Jorge Machado do INSA, pela disponibilidade e colaboração neste projecto de investigação.

Ao Professor Luís Tavares, pela disponibilidade, apoio e por me ter acolhido na sua equipa.

À Professora Gabriela Veloso, por todos os momentos partilhados no corredor, conselhos de vida e até de viagens.

Ao Professor Telmo Nunes, pela preciosa ajuda na área da estatística espacial e por todo o apoio prestado.

Às minhas amigas, sem nenhuma ordem em particular, agradeço-vos do fundo do coração e acreditem que só terminei isto com a vossa ajuda!

Carla, obrigado por seres a minha companheira, amiga e confidente e por me teres apoiado neste percurso, principalmente nos dias mais negros. Recordo com enorme saudade todos os momentos alegres no laboratório, as conversas durante os almoços, os capítulos em

conjunto, assim como as noitadas a trabalhar. Tenho a certeza que as nossas vidas se vão continuar a cruzar no futuro.

Margarida, obrigado por tudo! Apesar de estarmos em ramos diferentes, a nossa vontade de trabalhar em conjunto continuou, e espero que continue por muitos anos. Recordo-me dos nossos pequenos sucessos ao longo deste doutoramento, o nosso "eddie", o insulinoma ou até a leptospira, e vejo que estes "pequenos" êxitos são apenas o espelho de uma enorme amizade. Obrigado por todos os conselhos, apoio, risos, cafés e momentos bons nesta vida da investigação. Sem dúvida que foste um vento que me guiou e guiou este barco a bom porto.

À Joana, a minha "colega do lado", pela sinceridade, apoio, críticas, risadas e todos os momentos que partilhamos no gabinete e laboratório. Repetia-os a todos! Foste uma amiga incrível ao longo deste doutoramento e que me apoiou nos momentos mais tristes. Obrigado por acreditares em mim, e dares-me a confiança que muitas vezes eu próprio não acreditava que tinha.

Ao Hernâni do Carmo, por todo o apoio, conselho e amizade durante este percurso. Obrigado por escutares as minhas tristezas e alegrares os meus dias. Sem ti este trabalho não seria possível e a vida não seria a mesma coisa.

À Carla Carneiro, por todos os risos, conselhos e toda a ajuda na vida microbiológica.

Ao Professor Ricardo Bexiga, pelos conselhos e momentos partilhados no laboratório.

À Pipa Rocha Dias e Marta Gabriel, por toda a ajuda nas "Salmonellas" e momentos divertidos.

Ao Sinclair Owen, pela simpatia, disponibilidade em rever os trabalhos e conversas de corredor.

À Professora Teresa Semedo-Lemsaddek, pela ajuda e apoio no trabalhos que desenvolvemos.

À Professora Lélia Chambel da Faculdade de Ciências da Universidade de Lisboa, pela disponibilidade e conselhos.

À Clara Cartaxeiro, Ju, Gonçalo Frouco e Ferdinando Freitas, por todos os momentos e brincadeiras no corredor.

Ao Dr. Hugo Pissarra pelas conversas e conselhos ao longo destes anos.

À Patrícia Caeiros e Sílvia Cruz, por me ouvirem e apoiarem durante este tempo, principalmente em momentos mais "clínicos". Já nos conhecemos há muitos anos e sei que vamos continuar amigos por muitos mais.

À Ausenda, por me deixares sempre com um sorriso quando estou contigo. Obrigado por todos os momentos!

À Elisabete Silva e Sofia Henriques, por todos os conselhos, risos e momentos partilhados no laboratório do CIISA.

À Doutora Sandra Aguiar, pelos conselhos e momentos divertidos partilhados no gabinete.

À Doutora Salomé Veiga e Professor Miguel Castanho, pela disponibilidade em me acolherem no Laboratório de Bioquímica do IMM e pelos conhecimentos partilhados, e também à Doutora Sandra Pinto, pela ajuda, conhecimento e por me acompanhar na minha estadia no IMM.

À Dra. Lucarelli do Istituto Superiore di Sanità em Itália e Dra. Cristina Solano do Instituto de Agrobiotecnología, Universidad Pública de Navarra, Espanha, pelo o envio de estirpes.

A todas as pessoas que se cruzaram comigo neste projecto e me ajudaram de forma descomprometida.

À FCT, pelo financiamento através da minha bolsa de doutoramento.

À FMV e ao CIISA, por ser a minha 2ª casa, (e as vezes 1ª) durante estes últimos 7 anos, como bolseiro de investigação, aluno de mestrado e bolseiro de doutoramento.

Funding and Support

This work was supported by National Health Institute Doutor Ricardo Jorge (INSA) and funded by Centre for Interdisciplinary Research in Animal Health (CIISA) through project UID/CVT/00276/2013 and by Foundation for Science and Technology (FCT), grant fellowship SFRH/BD/75836/2011 from Ministério da Ciência, Tecnologia e Ensino Superior.

Abstract

Virulence of *Salmonella* Typhimurium 1,4,[5],12:i:-, the new pandemic strain

Salmonella serovar 1,4,[5],12:i:- is presently considered one of the major serovars responsible for human salmonellosis worldwide. A multidisciplinary approach, including the fields of epidemiology, spatial statistics, clinical and applied microbiology was used to perform an extensive characterization of *Salmonella* 1,4,[5],12:i:- isolates obtained by the National Health Institute Dr. Ricardo Jorge, which was lacking due to the recent emergence.

It was observed that cases are reported in most districts, being more frequent in the Portuguese coastland. Spatial statistical analysis showed a significant geographic clustering, pointing out for the importance of evaluating these areas to identify risk factors, in order to establish adequate prevention programs.

The most relevant antimicrobial profile in this serovar is the tetra-resistance pattern (R-type ASSuT), displaying resistance to ampicillin, streptomycin, sulphonamides and tetracyclines. A high occurrence of R-type ASSuT isolates was observed in the isolates under study, with the majority harboring the resistance genes frequently associated with the European clone, namely *bla*TEM*, sul*2*, stra*A-*stra*B, *tet*B. Additionally, resistance to quinolones and 3rd generation cephalosporin was also detected.

In Portugal, the rapid spread of *Salmonella* 1,4,[5],12:i:- R-type ASSuT might be related with the diversity of pulsotypes and also the presence of a core of virulence factors, including biofilm production. Biofilm-forming ability varied between sample locations and collection year, and can be one of the virulence features related with the rise of this serovar. Furthermore, biofilm formation was evaluated *in vitro* using a simulated human intestinal environment. In such conditions was observed an impairment of biofilm production, revealing that conditions mimicking the human intestinal tract can influence the biofilm-forming ability of the isolates under study.

This research highlight the critical importance of close surveillance of *Salmonella* 1,4,[5],12:i:- in Portugal, including R-type ASSuT isolates. Information gathered may unravel *Salmonella* 1,4,[5],12:i:- features, prevent the dissemination to other regions and also benefit the medical community in order to rationalize salmonellosis antimicrobial therapeutics.

Key-words: Biofilm, R-type ASSuT, Virulence factors, Portugal, *Salmonella* 1,4,[5],12:i:-

Resumo

Virulência de *Salmonella* Typhimurium 1,4,[5],12:i:-, a nova estirpe pandémica[1]

Salmonella é uma bactéria Gram-negativa pertencente à família *Enterobacteriaceae*, sendo uma das principais responsáveis pela morbilidade e mortalidade associadas a toxinfecções alimentares. Pode manifestar-se num espectro de sintomatologia variado, incluindo a gastroenterite, a bacteriémia e a infecção focal.

Este género incluí mais de 2600 serovares descritos, distribuídos por apenas duas espécies: *Salmonella enterica* que inclui todos os serovares patogénicos para os humanos e *Salmonella bongori*.

Actualmente, um dos principais serovares responsáveis pela salmonelose humana em todo o mundo é o 1,4,[5],12:i:-. Este serovar é uma variante monofásica de *Salmonella* Typhimurium, muito semelhante a nível molecular, sendo caracterizado pela ausência da expressão do gene *fljB*. Devido à sua recente emergência, estudos que avaliem este serovar são escassos, particularmente em Portugal, o que definiu o âmbito desta investigação, que teve como objectivo a caracterização epidemiológica e microbiológica, tanto do ponto de vista fenotípico e genotípico, de isolados de *Salmonella* 1,4,[5],12:i:- obtidos em Portugal a partir de diferentes origens, incluindo amostras humanas, animais e ambientais.

Numa primeira fase foi realizada uma caracterização demográfica, epidemiológica e espacial de todos os casos de Salmonelose 1,4,[5],12:i:- humana notificados em Portugal pelo Instituto Nacional de Saúde Dr. Ricardo Jorge (INSA), durante um período de 10 anos, desde 2001 a 2011. Foram recolhidos dados sobre a origem, ano e mês de amostragem, género, idade, distrito e município de residência dos pacientes. Foi realizada a análise estatística descritiva, bem como, a análise estatística espacial através do software SaTScan™, combinada com análise através de software de georeferenciação, o QGIS™, de forma a caracterizar a epidemiologia e identificar agrupamentos espaciais de risco superior de infecção por *Salmonella* 1,4,[5],12:i:- em Portugal.

Globalmente, observou-se que em Portugal, a maioria dos distritos tem casos notificados de infecção por *Salmonella* 1,4,[5],12:i:-. Verificou-se também um aumento da incidência durante o intervalo de 2004 a 2011, com um maior número de casos na região litoral do país, incluindo distritos como Porto, Lisboa e Aveiro, o que pode ser explicado pela maior densidade populacional nestas áreas. A maioria das infecções ocorreu durante Maio e Outubro, e o menor número em Fevereiro, afectando principalmente indivíduos jovens. A análise geoespacial revelou 4 aglomerados de risco superior de infecção, três localizados no

1 *O autor escreve segundo o antigo Acordo Ortográfico.

norte de Portugal, incluindo dois no litoral, e um no interior. Foi ainda detectado um aglomerado de maiores dimensões que envolve o centro e sul de Portugal.

Numa segunda fase, e tendo em conta a elevada ocorrência de resistência a antimicrobianos neste serovar, foi realizada a caracterização fenotípica e genotípica dos isolados de *Salmonella* 1,4,[5],12:i:-, em particular nos que apresentavam o perfil de tetraresistência ASSuT, que exibem simultaneamente resistência a ampicilina, estreptomicina, sulfonamidas e tetraciclinas (R-tipo ASSuT). Nesta fase foram caracterizados isolados recolhidos pelo INSA de diferentes fontes, incluindo casos clínicos humanos (n=170), animais (n=10), ambientais (n=6) e alimentares (n=1) obtidos em 15 distritos portugueses, entre 2006 e 2011. A identificação dos isolados foi realizada por serotipificação utilizando o método de aglutinação em lâmina, tendo sido posteriormente confirmada por PCR multiplex (mPCR) para a variante monofásica. Os isolados cuja identificação foi confirmada por mPCR foram seleccionados para detecção do perfil ASSuT através do método de difusão em disco, com posterior determinação da concentração mínima inibitória (CIM) por Etest®. Foi também determinada a resistência a outros antimicrobianos por difusão em disco, nomeadamente amoxicilina/ácido clavulânico, cefotaxima, ceftazidima, ciprofloxacina, cloranfenicol, gentamicina e ácido nalidíxico.

Os isolados de *Salmonella* 1,4,[5],12:i:- com o perfil ASSuT foram submetidos à pesquisa por PCR de genes de virulência (*spv*C, *inv*A, *inv*H, *sop*B, *stn*, *pho*P, *pho*Q, *sly*A,*agf*A, *sef*A, *saf*C, *pef*A, *sdi*A, *gip*A, *lpf*D), assim como de genes de resistência a antimicrobianos associados ao perfil ASSuT (amoxicilina [*bla*TEM], estreptomicina [*str*A-*str*B], sulfametoxazol [*sul*2] e tetraciclina [*tet*B]). Estes isolados foram ainda submetidos a caracterização molecular por electroforese em gel de campo pulsado com restrição por *Xba*I de acordo com o protocolo da PulseNet, e os perfis de macrorestrição obtidos foram analisados através do software Bionumerics®.

Relativamente aos 187 isolados serotipificados, a identificação de 133 foi confirmada como *Salmonella* 1,4,[5],12:i:- por mPCR, com uma ocorrência do perfil de tetraresistência ASSuT de 61,7%. Todos os isolados ASSuT apresentaram valores elevados de CIM para ampicilina (>256 *μ*g/ml) e sulfametoxazol (>1024 *μ*g/ml). A CIM_{50} para a estreptomicina foi de 512 μg/ml e a CIM_{90} de 1024 μg/ml. Os valores de MIC_{50} e MIC_{90} para a tetraciclina foram de 64 *μ*g/ml e 96 *μ*g/ml, respectivamente. Nos isolados R-type ASSuT foram também observados as seguintes co-resistências: amoxicilina (28%, n=23), cloranfenicol (15,9%, n=13), gentamicina (9,8%, n=8), ácido nalidíxico (8,5%, n=7), ceftazidima (4,9%, n=4), cefotaxima (4,9%, n=4) e ciprofloxacina (1,2%, n=1). A análise de clonalidade revelou uma grande diversidade de pulsotipos, indicando que a maioria dos casos de Salmonelose humana possa ser atribuída a eventos esporádicos. Todos os isolados possuíam 14 dos 18 genes de virulência

avaliados e 87,8% apresentavam todos os genes de resistência a antimicrobianos frequentemente associados com o clone europeu: *bla*TEM, *str*A-*str*B, *sul*2 e *tet*B.

Numa terceira e quarta fases, a formação de biofilme pelos isolados de *Salmonella* 1,4,[5],12:i:- foi extensivamente estudada, devido às implicações deste factor de virulência na indústria alimentar e sector médico, além de que não existiam estudos disponíveis sobre a formação de biofilme por *Salmonella* 1,4,[5],12:i:-. Foi avaliada a formação de biofilme pelos 133 isolados serotificados e cuja identificação foi confirmada por mPCR. A detecção de biofilme foi realizada através de métodos fenotípicos e genotípicos, nomeadamente a caracterização do morfotipo em agar e formação de película em meio líquido durante 8 dias, a determinação da densidade óptica através de ensaios em microplacas após 24h, 48h e 72h e observação directa do biofilme por hibridação *in situ* fluorescente após 24h, 48h e 72h. Os genes associados à formação de biofilme (*adr*A, *csg*D e *gcp*A) foram pesquisados por PCR.

Os ensaios de formação de biofilme mostraram que a capacidade de produção deste factor de virulência por *Salmonella* 1,4,[5],12:i:- é elevada, estando presente em todos os isolados estudados. A capacidade de formação de biofilme varia entre isolados obtidos em diferentes regiões, mostrando que os isolados provenientes de alguns distritos, como Lisboa ou Ponta Delgada, tem uma maior capacidade de persistir no ambiente. Esta capacidade também varia de acordo com o ano de colheita da amostra, apresentando um aumento significativo ao longo do tempo, o poderia ser uma das razões para a disseminação deste serovar no país.

Adicionalmente, e uma vez que a maioria dos estudos para avaliar a formação de biofilme são realizados utilizando meios de cultura não relacionados com o ambiente intestinal humano, foi desenvolvida uma metodologia aplicando condições que simulam este ambiente. Assim, foi concebido um ensaio *in vitro* para avaliar a produção de biofilme pelos 133 isolados de *Salmonella* 1,4,[5],12:i:- através da utilização de um meio que mimetiza as condições intestinais. Os isolados foram avaliados em três condições distintas, nomeadamente, meio intestinal com agitação, meio intestinal sem agitação e, por último, meio Mueller Hinton sem agitação, em três tempos diferentes (24h, 48h e 72h).

A utilização da metodologia aplicada permitiu verificar que o meio e as condições dos ensaios originam diferenças significativas nos resultados obtidos. Neste sentido, condições que simulam o stress gastrointestinal *in vivo* a que *Salmonella* é submetida deveriam ser incluídas na avaliação da capacidade de produção de biofilme, permitindo uma melhor correlação entre a capacidade de formação de biofilme *in vitro* e no tracto gastrointestinal.

Embora do ponto de vista epidemiológico os resultados apresentados sejam provenientes de um sistema de vigilância passiva, o que pode subestimar o número de casos, este estudo incluiu a primeira avaliação epidemiológica e análise de distribuição das áreas com maior

risco de infecção em Portugal. Adicionalmente, *Salmonella* 1,4,[5],12:i:- apresentou uma elevada ocorrência de multirresistência, nomeadamente do perfil ASSuT, frequentemente associado a outras resistências antimicrobianas. Estes isolados tetraresistentes estão amplamente distribuídos em Portugal, o que pode estar relacionado com uma vantagem evolutiva associada com o perfil ASSuT, a presença de múltiplos factores de virulência, incluindo a elevada e crescente capacidade de formar biofilmes, e a elevada diversidade de pulsotipos detectados.

As características epidemiológicas e microbiológicas reveladas por este serovar, fazem com que seja recomendável a adaptação de medidas de vigilância apertadas no país, incluindo a avaliação dos perfis de resistência e, em particular, do perfil de tetraresistência ASSuT. A monitorização contínua de *Salmonella* 1,4,[5],12:i:- em Portugal irá contribuir para a prevenção de futuros surtos, assim como fornecer informação relevante aos profissionais de saúde, especialmente na óptica da utilização e prescrição racional de antimicrobianos para tratamento de Salmoneloses.

Palavras-chave: Biofilme, R-type ASSuT, factores de virulência, Portugal, *Salmonella* 1,4,[5],12:i:-

TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF ABBREVIATIONS AND SYMBOLS

aac(3)-IV	aminoglycoside-(3)-N-acetyltransferases IV gene
*aad*A	aminoglycoside-3”-adenylytransferase gene
agf	aggregative fimbriae gene
AHL	acyl-homoserine lactones
AI	autoinducers
ARS	Regional Health Administration
ASSuT	resistance profile to ampicillin, streptomycin, sulphonamides and tetracyclines
AvrA	avirulence factor A
bap	Biofilm associated protein gene
BarA/SirA	bacterial adaptative response/*Salmonella* invasion regulator
BC	before Christ
*bla*TEM	TEM β-Lactamase gene
CDC42	Cell division control protein 42
CLSI	Clinical and Laboratory Standards Institute
cml	chloramphenicol resistance gene
csg	curli subunit gene
dfr	dihydrofolate reductase gene
DGAV	Portuguese National Authority for Animal Health
DNA	Deoxyribonucleic Acid
DT	Definitive Type
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EPS	extracellular polymeric substances
ESBL	Extended-spectrum β-lactamases
*est*X	putative esterase gene
EU	European Union
FISH	Fluorescent *in situ* hybridization
*fli*C	phase 1 flagellin gene
*flj*A	repressor of phage 1 flagellin gene
*flj*B	phase 2 flagellin gene
gip	Phage Gifsy-1 gene
GVB	Gut-Vascular Barrier
hil	hyperinvasion locus gene
HIV	Human Immunodeficiency Virus
ID	Infective Dose
IL	Interleukin

INIAV	National Institute for Agricultural and Veterinary Research
INSA	National Health Institute Doutor Ricardo Jorge
inv	invasion gene
IS	Insertion Sequence
kb	kilobase
kDa	Kilodalton
LAMPs	lysosome-associated membrane proteins of late endosomes/lysosomes
lpf	long polar fimbriae gene
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
mgt	magnesium transporter
MHB	Muller Hinton Broth
MIC	Minimum Inhibitory Concentration
mig-5	macrophage-inducible gene
MLST	Multilocus Sequence Typing
NTS	Non-typhoid *Salmonella*
P.i.	Post Infection
PCR	Polymerase Chain Reaction
pef	plasmid encoded fimbriae gene
PFGE	Pulsed Field Gel Electrophoresis
PhoP/PhoQ	Phosphorylated transcriptional activator/Membrane sensor kinase
PMN	Polymorphonuclear leukocytes
prg	*phoP*-repressed gene
psp	phosphoserine transferase gene
QS	quorum sensing
R-type	Resistance-type
rck	resistance to complement killing gene
RDAR	red, dry and rough
saf	*Salmonella* atypical fiambriae gene
SCV	*Salmonella*-containing vacuole
sdi	Signal receptor of the LuxR family gene
Sifs	*Salmonella*-induced filaments
sii	*Salmonella* intestinal infection gene
sly	Salmolysin gene
sop	*Salmonella* outer protein gene
spa	surface presentation of antigens gene
sip	*Salmonella invasion protein gene*

SPI	*Salmonella* Pathogenicity Island
SptP	*Salmonella* phosphatase tyrosine protein
spv	*Salmonella* plasmid virulence gene
*srg*A	SdiA-regulated gene
ssa	secretion system apparatus
ssc	secretion system chaperone
sse	secretion system effector
ST	Sequence Type
str	streptomycin gene
sul	sulfonamide resistance gene
T1SS	Type I secretion system
T3SS	Type III secretion system
T6SS	Type VI secretion system
Tafi	Thin aggregative fimbriae
TCS	Two component system
tet	tetracycline resistance gene
TSB	Tryptic Soy Broth
USA	United States of America
XLD	Xylose lysine deoxycholate

CHAPTER 1

Bibliographic review and Objectives

1.1. Introduction

Salmonella is widely recognized as a major pathogen causing gastroenteritis with different levels of severity, expressing symptoms like vomit and diarrhea. Although this bacterium usually promotes a self-limiting condition, it's role on food poisoning outbreaks and in many cases of illness in humans raised serious concerns regarding public health safety (Switt, Soyer, Warnick & Wiedmann 2009). Mammals, birds and reptiles are also affected by *Salmonella* infections.

In the European Union (EU), about 100.000 cases of human salmonelosis are annually reported. The European Food Safety Authority (EFSA) has estimated that the overall economic burden of this disease could be as high as 3 billion euros per year. Nowadays, this bacterium is the second most frequent foodborne pathogen after *Campylobacter*, being even the first in several EU countries, as for example, in Italy. However, unlike *Campylobacter*, *Salmonella* often causes very large multistate foodborne outbreaks which proves its greater dispersion in the environment (EFSA 2010b).

In 2010, the EFSA Panel on Biological Hazards published a Scientific Opinion alerting for the increasing number of outbreaks in the EU member states caused by "*Salmonella Typhimurium*-like" strains. The Panel has recommended that these strains should be further typed and characterized. However, only a few studies to date have focused on their phenotypic or genotypic traits (Bugarel, Vignaud, Moury, Fach & Brisabois 2012, Mandilara, Lambiri, Polemis, Passiotou & Vatopoulos 2013). As a result, only limited evidence is available on their virulent features, with few data available regarding biofilm formation or even information regarding the epidemiology of Portuguese isolates. The scarce research available on *Salmonella* 1,4,[5],12:i:- and its high resemblance in virulence with *Salmonella* Typhimurium, influenced the focus of this review aiming at the comparison between both serovars.

This first chapter of this thesis includes a general review on *Salmonella*, with particular focus on the characterization of the monophasic variant 1,4,[5],12:i:-, followed by a description of the infection pathogenesis by non-typhoid *Salmonella.* Subsequently, the different mechanisms of virulence are analysed according to the updated literature. A detailed description about the importance of *Salmonella* Pathogenicity Islands (SPI) during host infection, the most common antimicrobial resistance profiles and their genetic determinants and the mechanisms of biofilm formation are described. Finally, this chapter includes a description of the surveillance measures of human salmonellosis in Portugal, followed by the identification of the main objectives of this thesis.

1.2. *Salmonella* through History: from its discover to the early outbreaks

Salmonella was isolated for the first time from pig's intestine in 1885 by Theobald Smith, a research laboratory assistant at the Veterinary Division of the United States Department of Agriculture. The research was performed under the guidance of Daniel Elmer Salmon, an American veterinary pathologist, responsible for the origin of the genus name.

However, the history of *Salmonella* did not begin at the 19th century. In ancient times, some important historical celebrities are believed to have died due to infections caused by this bacterium. In 2001, a group of researchers at the University of Maryland in the United States of America (USA) suggested that an infection by *Salmonella* Typhi was the cause of death of Alexander the Great in 323 BC, based on a description of Alexander's symptoms written by the Greek author Arrian of Nicomedia (Moulopoulos 1998).

In more recent times, Prince Albert, the husband of Queen Victoria, died in 1861 of Typhoid fever. In fact, during the Victorian era, 50.000 cases of Typhoid fever per year were estimated to occur in England (Morser, Puskoor & Zubay 2005).

Even at important wars, *Salmonella* caused more deaths than battle wounds. During the South African War (1899-1902), a Typhoid outbreak in British camps mainly attributed to unsanitary conditions (1899-1902) killed 13.000 soldiers, as compared to 8.000 battle deaths (Cirillo 2006).

In the early 1900's, Mary Mallon, also known as "Typhoid Mary", was responsible for several Typhoid outbreaks, becoming the first famous carrier of Typhoid fever in USA (Soper 1907). Mary Mallon was hired as a cook at several private houses, working in the New York area for wealthy families. She caused several Typhoid outbreaks, by moving from house to house and always disappearing before an epidemic could be traced back to her. In the end, she had worked for eight families, with 22 cases of Typhoid and at least 3 deaths directly attributed to her cooking job (Soper 1907, Marineli, Tsoucalas, Karamanou & Androutsos 2013). She was finally overtaken by the authorities in 1907 and committed to an isolation center on North Brother Island in New York. Mary Mallon was released in 1910, on the condition that she never again accepted employment involving food handling. She changed her name and was found working as a cook, after causing further typhoid outbreaks. She was admitted back to North Brother Island, where she lived until her death in 1938 (Marineli et al. 2013).

These events showed that some individuals have a natural immunity to *Salmonella* and Mary Mallon was the first asymptomatic Typhoid carrier to be identified (Soper 1907). Only recently, medical science was able to clarify that *Salmonella enterica* serovar Typhi can cause a chronic and asymptomatic infection, persisting primarily in the gallbladder, mainly due to biofilm formation (Gonzalez-Escobedo & Gunn 2013).

1.3. Characterization of *Salmonella* 1,4,[5],12:i:-, the new emergent strain

Bacteria classification is of major importance, creating order in the complex world of microbiology. Below the subspecies level, *Salmonella* isolates are discriminated using the White-Kauffmann-Le Minor serotyping scheme, established in the middle of the last century and still recognized as the reference method to discriminate between *Salmonella* varieties. The *Salmonella* genus includes two species, *S. enterica* and *S. bongori*. *S. enterica* is divided into 6 subspecies, I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*) and VI (*indica*). The White-Kauffmann-Le Minor scheme, published by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, France, uses serotyping to distinguish between *Salmonella* serovars, allowing the identification of more than 2.600 different serovars belonging (Grimont & Weill 2007).

1.3.1. *Microbiological characterization*

Salmonellae are facultative anaerobic, Gram-negative rod shaped bacteria belonging to the *Enterobacteriaceae* family. Although most members of this genus are motile by peritrichous flagella, a few non-flagellated variants, such as *Salmonella* Gallinarum and *Salmonella* Pullorum are non-motile. *Salmonellae* are chemoorganotrophic, with the ability to metabolize nutrients by both respiratory and fermentative pathways (Gyles, Prescott, Songer & Thoen 2010). *Salmonella* is oxidase negative, catalase positive, indole and Voges Proskauer negative, methyl red and Simmons citrate positive, hydrogen sulfide producing and urea negative. Some of these characteristics are used for biochemical identification of *Salmonella* isolates (Grimont & Weill 2007).

1.3.2. *Antigenic characterization*

Most human and food-producing animal serovars (more than 1.500) belong to the first subspecies, *Salmonella enterica* subspecies *enterica* (EFSA 2010b, Ranieri, Shi, Moreno Switt, den Bakker & Wiedmann 2013). While *S. enterica* normally includes all major serovars that are pathogenic to humans and animals, *S. bongori* is considered a bacterium of cold-blooded animals, most frequently associated with reptiles (Gyles et al. 2010). *Salmonella enterica* subsp. *enterica* serovars are usually designated by a name, frequently related to the geographical place where the serovar was first isolated (for example, *Salmonella* Havana) or to the animal from which it was isolated (for example, *Salmonella* Gallinarum) (Backer et al. 2000, Shah et al. 2005). Serovars belonging to other *S. enterica* subspecies and to *S. bongori* are designated by their antigenic formula (EFSA 2010b).

Serotyping is based on the antigenic characterization of surface structures, such as lipopolysaccharides (O-antigen), flagellar proteins (H-antigen), and capsular polysaccharides

(Vi-antigen), through agglutination with polyvalent and monovalent antisera (Grimont & Weill 2007, Switt et al. 2009).

The O-antigen is the external component of the lipopolysaccharide located on the cell wall, consisting of a long linear polysaccharide containing 50 to 100 repeating saccharide units, with four to seven sugars per unit (EFSA 2010b). Different sugars and different linkages between sugars give origin to different antigens (Reeves & Wang 2013).

The H-antigen corresponds to flagellin, the major component of flagella (Switt et al. 2009). The Vi-antigen is a surface polysaccharide, which only occurs in three *Salmonella* serovars, namely *Salmonella* Typhi, *Salmonella* Paratyphi C and *Salmonella* Dublin (Johnson, Krauskopf & Baron 1965, Snellings NJ 1977).

The full antigenic formula system determined by the White-Kauffmann-Le Minor scheme is represented as follows: first, O-antigens, followed by H-antigens of first phase and H-antigens of second phase; the three antigen designations are separated by colons (EFSA 2010b). According to this scheme, *Salmonella* Typhimurium would be described as I 1,4,[5],12:i:1,2, indicating that this serotype belongs to subspecies I and carries the ‘‘1,4,[5],12’’ O antigens, the ‘‘i’’ phase 1 H-antigen, and the ‘‘1,2’’ phase 2 H-antigens. The underlined O factor 1 means that this factor is determined by phage conversion, being present only if the culture is lysogenized by a particular converting phage. The factor 5 between square brackets means that the presence of the antigen is not related to phage conversion.

Salmonella 1,4,[5],12:i:– thus shares all O antigens and phase 1 H-antigens with *Salmonella* Typhimurium. Antigenic variants like the monophasic *Salmonella* Typhimurium 1,4,[5],12:i:- that lack the second phase H-antigen have been described in the literature as “*Salmonella* Typhimurium-like” strains (EFSA 2010b, Bugarel et al. 2012).

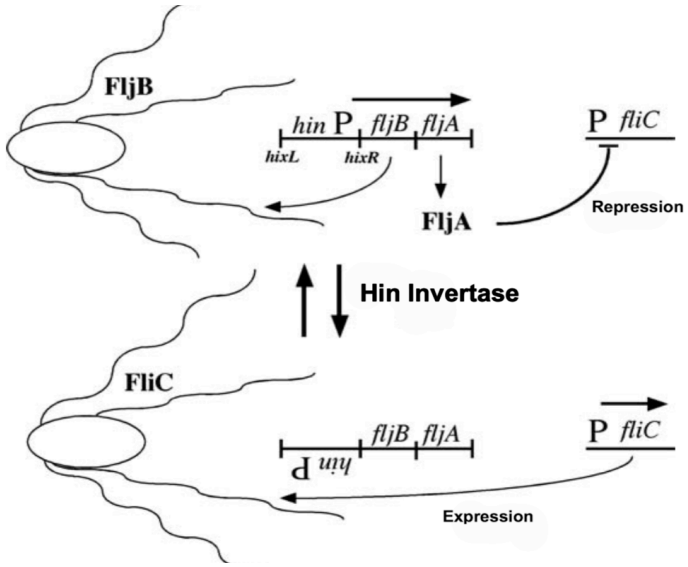
1.3.3. *Molecular characterization*

As mentioned, most *Salmonella* strains are motile due to the presence of peritrichous flagella, which are encoded by two different flagellin genes located on the bacterial chromosome, *fli*C and *flj*B. The biphasic character of the flagella antigen consists of its ability to modify its composition by switching the expression of two loci encoding the major flagellar protein, between FliC for phase 1 flagella and FljB for phase 2 (Ranieri et al. 2013). Before flagella composition was even known, the expression of these two separate antigens was identified and described as "phases", through a mechanism called “phase variation” (EFSA 2010b, Bugarel et al. 2012). Flagellar phase variation is caused by the reversible inversion of a deoxyribonucleic acid (DNA) segment, designated the H segment, containing the *flj*B promoter (Kutsukake, Nakashima, Tominaga & Abo 2006). The H segment is flanked by inverted repeat sequences, *hix*L and *hix*R (Fig. 1), between which site-specific recombination

phenomenon occurs, leading to inversion of the H segment (Aldridge et al. 2006, Bugarel et al. 2012).

A DNA invertase encoded by *hin*, which is located within the H segment, catalyzes this recombination event. The gene *flj*A, which encodes a negative regulator for *fli*C expression, is located downstream of *flj*B. When the H segment is in the "on" orientation, it results in the transcription of both *flj*B and *flj*A, expressing only the second flagellar phase, since *flj*A represses *fli*C expression (Yamamoto & Kutsukake 2006, Bugarel et al. 2012).

Figure 1 - Flagellar phase variation in *Salmonella*. Adapted from Aldridge et al., 2006



When the H segment is on the "off" orientation, neither *flj*B nor *flj*A are transcribed and the *fli*C gene is not inhibited, resulting in the expression of the first flagellar phase (Kutsukake et al. 2006, EFSA 2010b). This switch mechanism only allows the expression of one flagellin variety at a time (Bugarel et al. 2012).

Most serovars are biphasic, meaning that they can express both phase 1 and phase 2 genes (Barco et al. 2011). However, some *Salmonella* strains are monophasic, producing only one type of flagellin, and may lack the expression of phase 1 or phase 2 genes. For example, *Salmonella* 1,4,[5],12:i:- isolates lack expression of phase 2 flagella (EFSA 2010b, Barco et al. 2011).

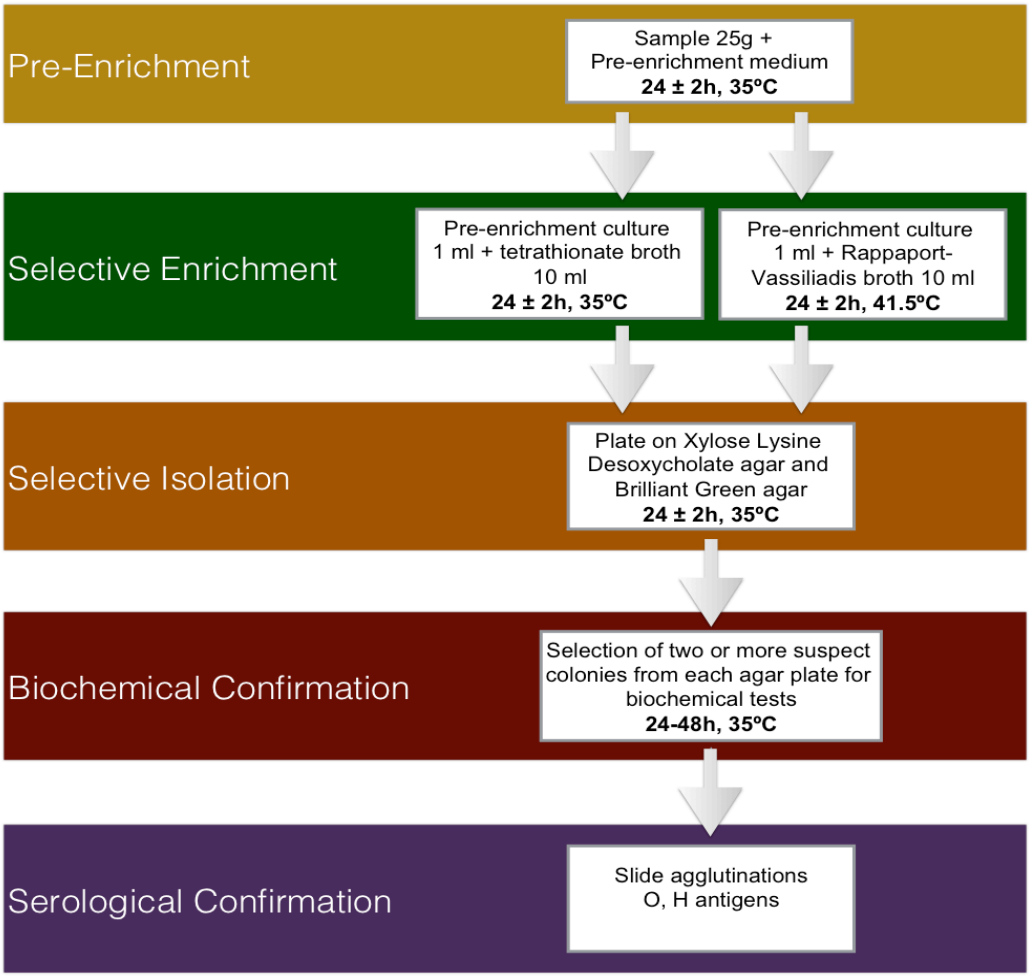
1.4. Laboratory methods for identification and characterization of *Salmonella*

Several laboratory techniques are used for identification and typing *Salmonella* in epidemiological order to track changes in epidemiology and to trace sources of foodborne outbreaks (Wattiau, Boland & Bertrand 2011, Achtman et al. 2012).

Isolation of *Salmonella* from faeces and food samples may be difficult due to several factors, including the occurrence of subclinical infections or intermittent shedding of small numbers of *Salmonellae* in faeces. Additionally, *Salmonella* populations in food samples may be stressed due to unfavourable storage and processing conditions, including high or low

temperatures, pH, or salt content. In order to insure the isolation of *Salmonella* and avoiding false negative results, several steps must be performed, including the use of a large sample volume, inoculation in a non-selective pre-enrichment medium, such as buffered peptone water, followed by the use of a combination of two selective enrichments broths such as Müller-Kauffmann Tetrathionate broth and Rappaport Vassiliadis Soy and plating on two selective media, such as Xylose Lysine Desoxycholate agar and Brilliant Green agar, or another selective agar media like Hektoen or *Salmonella-Shigella* agar. Subsequently, colonies with a typical *Salmonella* morphology are confirmed by further culturing in Triple Sugar Iron agar and Urea agar and using biochemical tests, including L-lysine decarboxylase, β-galactosidase, Voges Proskauer and Indole tests. Then, *Salmonella* colonies are serotyped classified at the subspecies level and eventually serotyped (Fig. 2) (WHO Global Foodborne Infections Network 2010).

Figure 2 - *Salmonella* isolation protocol from food and animal samples.
Adapted from WHO Global Foodborne Infections Network (2010). Available at:
http://www.antimicrobialresistance.dk/232-169-215-protocols.htm



Serotyping is a relatively easy technique to perform, however it requires skilled technicians to interpret the results, being an expensive and prolonged procedure since hundreds of antisera are required (Grimont & Weill 2007, Guibourdenche et al. 2010). In fact, the replacement of

serotyping by molecular techniques is being tested. Through the years, several molecular techniques have been used to identify *Salmonella* serotypes based on microarrays (Fang et al. 2010), real-time polymerase chain reaction (PCR) (Maurischat, Baumann, Martin & Malorny 2015), repetitive sequence-based PCR (Wise et al. 2009, Ranieri et al. 2013) and multilocus sequence typing (Ranieri et al. 2013). Each of these methods has advantages and drawbacks in terms of costs, speed and sensitivity (Ranieri et al. 2013). Although considered improvements in several aspects, none has been considered as the ideal method to be conducted as routine in microbiology laboratories (Wattiau et al. 2011) and serotyping still remains an indispensable tool to discriminate *Salmonella* serovars. This technique provide valuable information regarding potential sources, as several serovars are correlated with specific hosts (subspecies IIIb serovars are common in reptiles) or geographical regions (Backer et al. 2000), type of disease and severity (EFSA 2010b) and potential multidrug resistance profile, since some serovars may be linked to certain antimicrobial resistance profiles (Clemente et al. 2014). Additionally, serotyping information is also essential to achieve its main purpose, which is to allow for an internationally accepted nomenclature (Grimont & Weill 2007).

Bacteriophages, or more commonly phages, are viruses that can only replicate within specific bacteria. Phage typing can differ between strains of the same serovar. This technique is based on the principle that specific phages will only lyse particular strains of a specific serovar, currently allowing the identification of over 200 definitive types (DT) (EFSA 2010b). The lysis pattern can be compared to a standard scheme to identify the strain phage type (De Lappe, Doran, O'Connor, O'Hare & Cormican 2009).

Typing schemes were conceived for particular *Salmonella* serovars and showed to be valuable epidemiological tools to investigate outbreaks. Information regarding the typing schemes in combination with antimicrobial susceptibility testing has led to identification of many large international outbreaks (O'Mahony et al. 1990, Mahon et al. 1997, Backer et al. 2000, Mossong et al. 2007).

Nowadays, molecular methods are critical for outbreak detection, investigation and control. Pulsed Field Gel Electrophoresis (PFGE) is a molecular typing method, considered to be the "Gold Standard" for supporting the identification of epidemiological links between isolates (Wattiau et al. 2011), However, multilocus sequence typing (MLST) and whole genome sequencing are becoming more favoured options (Achtman et al. 2012).

1.5. Pathogenesis of non-typhoid *Salmonella*

Among *Salmonella* serovars associated with gastroenteritis, the non-typhoid *Salmonella* (NTS), trigger an intestinal inflammatory response, whereas typhoid serovars cause enteric fever by their ability to persist and multiply inside mononuclear phagocytes promoting systemic infections (Ohl & Miller, 2001). Most NTS enter the host via ingestion of

contaminated water or food (Crum-Cianflone 2008), but person-to-person spread may also occur. NTS must survive the passage through the stomach, in order to invade the mucosa of the small and large intestine, usually the distal ileum and colon, and may produce toxins (Crum-Cianflone 2008). The infectious dose varies among *Salmonella* serovars but for non-typhoid strains, the infectious dose is approximately 10^3 bacilli (Blaser & Newman 1982).

After overcoming the gastric of the stomach acid environment and evading intestinal defences such as the action of the specific Immunoglobulin A, NTS infection is characterized by bacterial attachment to enterocytes by fimbriae or pili, which also selectively attach to specialized epithelial cells located in the ileal Peyer patches, the M cells (Carrie, Sheila, Fedorka-Cray & Isaacson 2003). Bacterial invasion occurs by inducing the "ruffling" of the enterocyte membrane and subsequent internalization by receptor-mediated endocytosis, followed by transportation within phagosomes to the *lamina propria*, where they are released. This process is an important pathway for allowing invasive *Salmonella* to reach deeper tissues (Drecktrah, Knodler, Ireland & Steele-Mortimer 2006).

Invasion of the intestinal epithelial barrier by NTS allows bacteria to interact with macrophages and lymphocytes in Peyer's patches, where *Salmonella* multiplication occurs, resulting in marked enlargement and necrosis of the lymphoid tissue (Hackett, Kotlarski, Mathan, Francki & Rowley 1986). NTS invasion usually precipitates a localized immune response by the stimulation and release of pro-inflammatory cytokines, including interleukin (IL)-1, IL-6, IL-8, Tumor Necrosis Factor-2, Interferon-U, Monocyte Chemoattractant Protein-1 and Granulocyte Macrophage Colony-Stimulating Factor, which induce an inflammatory reaction, promoting a large influx of polymorphonuclear leukocytes (PMN) to the intestinal lumen (Dougan, John, Palmer & Mastroeni 2011). The acute inflammatory response causes diarrhoea and may lead to mucosa ulceration and necrosis after which, bacteria can disseminate from the intestines and cause systemic disease.

In order to express the ability to invade animal tissues, NTS must possess and express a variety of virulence factors. *Salmonella* virulence attributes are complex and encoded both on the bacterial chromosome and/or on large plasmids (Suez et al. 2013). For example, attachment and invasion mechanisms are under distinct genetic control and involve multiple genes located in both the chromosome and plasmids.

Illness severity in individuals with salmonellosis is not only determined by the presence of virulence factors but also by other features, in particular host susceptibility and defences, infective dose and also the interaction between the pathogen and the host (Hohmann 2001, Jones-Carson & Vazquez-Torres 2007, Borriello et al. 2012).

1.5.1. *Host susceptibility*

Host factors predisposing to severe NTS infection include reduced gastric acidity, pernicious anemia, antacids and H_2 blockers administration (Hohmann 2001). Impaired cell mediated

and humoral immunity and decreased phagocytic function were also described as related with severe disease (Gondwe et al. 2010, Dougan et al. 2011). *Salmonellae* are unable to survive at a gastric pH inferior to 2.5 and patients with anatomical or functional achlorhydria are at increased risk of developing infection (Tennant et al. 2008). Other risk factors for salmonellosis include extremes of age, intestinal microbiota variation, diabetes, malignancy, rheumatic disorders, reticuloendothelial blockade as a result of malaria, Human Immunodeficiency Virus (HIV) infection, and therapeutic immunosuppression (Hohmann 2001, Crum-Cianflone 2008). Also, co-infection with *Schistosoma* has been reported to cause prolonged and severe illness due to altered macrophage function and *Salmonella* replication and survival ability of *Salmonella* within the parasite (Abruzzi & Fried 2011).

1.5.2. *Host defences*

Once in the gastrointestinal tract, *Salmonellae* faces a series of nonspecific host defenses and innate immune mechanisms that impair its ability to colonize the intestinal surface, to translocate across the epithelial barrier and to disseminate systemically. Briefly, non-specific defences consist of gastric acidity, intestinal mucus, intestinal peristalsis, host microbiota and the presence of lactoferrin and lysozyme (Bearson, Bearson & Rasmussen 2006, Jones-Carson & Vazquez-Torres 2007). Specific defences consist of mucosal intestine immunoglobulin A, systemic antibodies and genetic resistance to invasion (Jones-Carson & Vazquez-Torres 2007).

1.5.3. *Infectious Dose*

Salmonella infective dose 50 (ID_{50}), which is the number of viable cells required to cause infection in 50% of the experimental subjects, was determined in human volunteers. Those experiments pointed to an ID_{50} of 10^3 to 10^6 cells, which can be considered a relatively large dose when compared to other foodborne pathogens like *Campylobacter jejuni* (500 microorganisms) or *Shigella* spp. (10 microorganisms) (Hara-Kudo & Takatori 2011). However, other reports regarding salmonellosis outbreaks investigations suggest that the infective dose is often lower, being calculated to be inferior to 10^3 bacilli (Blaser & Newman 1982).

Higher doses are associated with higher rates of illness and shorter incubation periods (Hara-Kudo & Takatori 2011). Lower infective doses may promote and cause infection, if *Salmonella* is co-ingested with foods that rapidly pass the stomach, such as liquids, or that includes a high content of fat and protein, like ice cream, cheese and chocolate, which may play a role in protecting *Salmonella* from gastric acidity (Kothary & Babu 2001). Antacids administration or defective immune systems are conditions that may require lower ID to cause infection.

Even within the genus, the infective dose is variable among different serovars. For example, it was reported that *Salmonella* Pullorum infective dose is 10^9 to 10^{10} bacilli, while *Salmonella* Newport infective dose is estimated to be 10^5 to 10^6 microorganisms (Kothary & Babu 2001).

1.6. Emergence of monophasic *Salmonella* Typhimurium 1,4,[5],12:i:-

In the 90s was reported in Europe the first isolation of *S*. *enterica* subsp. *enterica* serovar 1,4,[5],12:i:-, presently considered one of the major serovars responsible for human salmonellosis worldwide (Machado & Bernardo 1990). This study documented the serovar isolation from a chicken carcass in Portugal in 1986/87 (Machado & Bernardo 1990). However, scarce reports of *Salmonella* 1,4,[5],12:i:- isolation may reflect the difficulties in serotyping this serovar, with many isolates probably incorrectly designated as *Salmonella* Typhimurium. Additionally, reporting *Salmonella* 1,4,[5],12:i:- isolates may also be underestimated due to inconsistent ways used by different countries to report this serovar since it was common to report some *Salmonella* serovars as "Group B" or "subspecies I" (Switt et al. 2009).

A few years later, a high number of cases related with the 1,4,[5],12:i:- serovar have been documented in Spain, with the first isolation reported in 1997 (Echeita, Aladueña, Cruchaga & Usera 1999). In this country, monophasic *Salmonella* 1,4,[5],12:i:- rapidly became the most common serovar in swine and the second most frequent in pork products, which lead to the hypothesis that pigs may be an important reservoir for these strains (de la Torre et al. 2003). Isolation of serovar 1,4,[5],12:i:– has also been reported in other European countries, including Luxemburg (Mossong et al. 2007), Germany (Guerra, Junker, Miko, Helmuth & Mendoza 2004), Greece (Mandilara et al. 2013), Italy (Dionisi et al. 2009), Poland (Wasyl & Hoszowski 2012), as well as in Austria, France, Ireland, the Netherlands (EFSA 2010b), Denmark, Bulgaria and Slovakia (Switt et al. 2009, Majtan, Majtanova & Majtan 2011). Outside Europe, it was also detected in the American and Asian continents (EFSA 2010b).

In several countries, different varieties of monophasic *Salmonella* Typhimurium have emerged, showing different phage types, genotypes and antimicrobial resistance profiles. The phenotypic and genotypic diversity of *Salmonella* 1,4,[5],12:i:- isolates is probably linked to multiple clones which might have emerged through independent deletion events (Switt et al. 2009). In Europe, the clonal evolution of this serovar promoted the emergence of two distinct lines over the last two decades. In the late 90s, one clonal line appeared in Spain, expressing plasmid-mediated resistance to a wide range of antimicrobials compounds. In 2000, a second clone showing co-resistance to ampicillin (A), streptomycin (S), sulphonamides (Su) and tetracyclines (T) [Resistance-type (R-type) ASSuT] emerged in Italy, being now frequently detected in many European Countries, such as Spain, Germany, Denmark and the United Kingdom (Hopkins et al. 2010, Hopkins, de Pinna & Wain 2012, Mandilara et al. 2013).

The emergence of *Salmonella* 1,4,[5],12:i:- is strongly linked to *Samonella* Typhimurium. Few years later after the firsts reports, two hypothesis emerged regarding the origin of this serovar: the first considered it to be a single-phase variation of previously identified serovars, such as *Salmonella* Typhimurium (with an antigenic formula 4,[5],12;i;1,2) and *Salmonella* Lagos (with an antigenic formula 4,[5],12;i;1,5), and the second considered it to be a whole new serovar (Switt et al. 2009). However, several studies (Echeita et al. 1999, Echeita, Herrera & Usera 2001, de la Torre et al. 2003) showed the close link between *Samonella* Typhimurium and *Salmonella* 1,4,[5],12:i:-, and due to the relation serovar 1,4,[5],12:i:- is also known as monophasic variant of *Salmonella* Typhimurium. In one study (Echeita et al. 2001), an Insertion Sequence IS200*,* serovar specific of *Salmonella* Typhimurium (Sanderson, Sciore, Liu & Hessel 1993), present in the intergenic *fli*B-*fli*A flagellin cluster region, was detected in all *Salmonella* serovar 1,4,[5],12:i:- isolates in the same position and with the same sequence as *Salmonella* Typhimurium, suggesting that this is a monophasic variant of this serovar. In another study (de la Torre et al. 2003), pulsed-field gel electrophoresis comparing both serovars showed similarities superior to 78%, and also a common plasmid profile and identical antimicrobial resistance patterns; in particular, a pattern of multidrug resistance frequently found in monophasic isolates, including resistance to ampicillin, chloramphenicol, streptomycin, sulfonamide, tetracycline, gentamicin, and trimethoprim-sulfamethoxazole.

Microarrays studies comparing the first *Salmonella* 1,4,[5],12:i:- isolates identified in Spain in the late 1990s and *Salmonella* Typhimurium isolates, detected only minor genetic deletions, homology in virulence plasmid genes and identical invasion, enterotoxin and cytolysin genes and also in genes associated with survival within macrophages (Garaizar et al. 2002)

The escalating prevalence in Europe of human monophasic *Salmonella* cases also revealed that both serovars seem to have the same pathogenic behaviour, especially in terms of their ability to infect and cause disease in both animals and humans (EFSA 2010b).

1.7. Virulence factors of *Salmonella* Typhimurium 1,4,[5],12:i:-

Interactions between pathogenic microbes and their hosts are complex and dynamic. *Salmonella*'s ability to infect the host and cause disease is attributed to the acquisition and expression of virulence genes that allow the microorganism to replicate and disseminate within a host, by subverting or eluding its defences and enhancing its potential to cause disease (Cross 2008). In the particular case of *Salmonella* 1,4,[5],12:i:- and considering its molecular mechanism of phase variation, the absence of the second flagellar phase may be considered an important virulence attribute (Switt et al. 2009). This mechanism may allow the evasion of the immune system by silencing the expression of flagellar antigens that are usually recognized by the host (Ikeda et al. 2001).

Virulence attributes in *Salmonella* are complex and vary between serovars and even in strains within a serovar (EFSA 2010b). Unfortunately, studies regarding virulence factors in monophasic *Salmonella* Typhimurium are lacking due to its recent emergence and the presence of multiple distinct clones, so consistent data on virulence mechanisms are scarce (Soyer et al. 2009, EFSA 2010b).

Nevertheless, several reports demonstrated that the virulence gene repertoire of monophasic *Salmonella* Typhimurium and its variability are similar to the biphasic variant (Garaizar et al. 2002, EFSA 2010b, Hauser et al. 2010). A previous report from 2010, demonstrated using microarray analysis that there were no differences between both serovars regarding 102 representative pathogenicity genes, with the exception for minor variations in single strains (Hauser et al. 2010). An other report (Capuano, Mancusi, Capparelli, Esposito & Proroga 2013) confirmed that both *Salmonella* serovars Typhimurium and 1,4,[5],12:i:- were closely related in terms of virulence and antibiotic resistance profiles. A recent study (Yang et al. 2015), showed that *Salmonella* Typhimurium and *Salmonella* 1,4,[5],12:i:- isolates, both with the same Sequence Type (ST) 34, exhibited the same virulence gene profile.

Pathogens deploy virulence effector proteins into host cells, where they interact physically with host proteins to modulate the defence mechanisms (Mukhtar MS et al. 2011). In order to promote disease, *Salmonella* Typhimurium employs a secretory cascade of virulence effectors that interact with host cells (Yoon, Ansong, Adkins & Heffron 2011a). Effector proteins manage multiple functional activities including membrane trafficking, subversion of the host cell cytoskeleton, induction of pro-inflammatory response and regulation of infected cells motility (Worley, Nieman, Geddes & Heffron 2006, McGhie, Brawn, Hume, Humphreys & Koronakis 2009), with other mechanisms still largely undefined (Haraga, Ohlson & Miller 2008). *Salmonella* tightly controls the expression and secretion of virulence determinants to disrupt host cell activities at appropriate times and locations during infection (Yoon et al. 2011a). Failure to properly regulate the expression of these effectors results in attenuation of *Salmonella* virulence (Mouslim, Delgado & Groisman 2004).

1.7.1. Salmonella *Pathogenicity Islands*

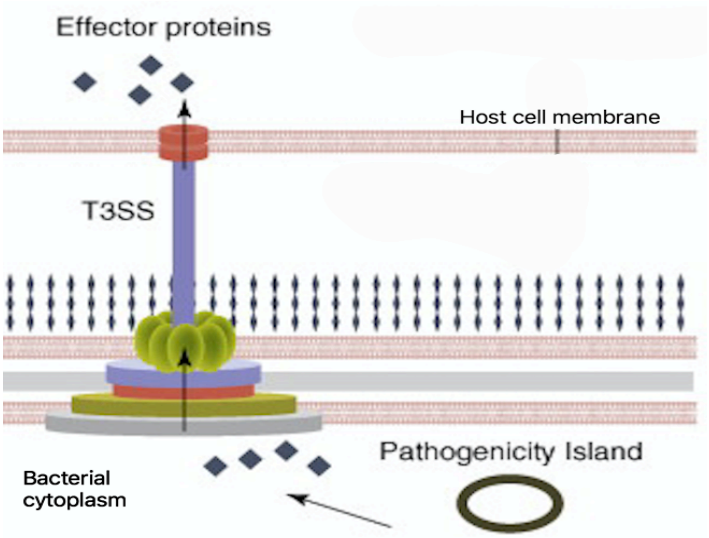
As already mentioned, *Salmonella* virulence depends upon multiple factors, being the result of the coordination of many virulence genes expression in time and space in order to cause disease (Groisman & Ochman 1997). It has been estimated that approximately 4% of the *Salmonella* Typhimurium genome is necessary for lethal infection in mice, which translates into the expression of over 200 virulence genes (Bowe et al. 1998). These genes are not randomly distributed in the *Salmonella* genome, but instead they are clustered in distinct chromosome regions termed *Salmonella* Pathogenicity Islands (SPI) (Marcus, Brumell, Pfeifer & Finlay 2000). However, besides SPI, virulence determinants are also present on a

wide variety of genetic elements, including in other regions of the bacterial chromosome, plasmids and prophages (Foley, Johnson, Ricke, Nayak & Danzeisen 2013).

SPI consist of large regions of genomic DNA of approximately 10–200kb, organized in 12 pathogenicity islands, present in pathogenic strains but missing in non-pathogenic bacteria (Saroj, Shashidhar, Karani & Bandekar 2008). Many pathogenicity islands encode specialized devices for the delivery of virulence proteins into host cells such as type III secretion system (T3SS) encoded by SPI-1 (Fig. 3) (Eswarappa et al. 2008).

Although some SPI are conserved throughout the genus, others SPI are specific of certain serovars. For example, SPI-8 is restricted to *Salmonella* serovars Paratyphi A and Typhi, while SPI-10 is present in *Salmonella* serovars Dublin, Enteritidis, Gallinarum, Paratyphi and Typhi (Saroj et al. 2008). The acquisition of these gene clusters was probably achieved by horizontal gene transfer, since they present a guanine-cytosine content that significantly differs from the remaining chromosome (Marcus et al. 2000). As these islands contain a set of functionally related genes necessary for the expression of a specific virulence phenotype, the acquisition of SPI might have led to a sudden increase in *Salmonella* pathogenicity during evolution (Groisman & Ochman 1997, Eswarappa et al. 2008).

Figure 3 - *Salmonella* Pathogenicity Island I associated type III secretion system. Adapted from Brennan et al. 2009.

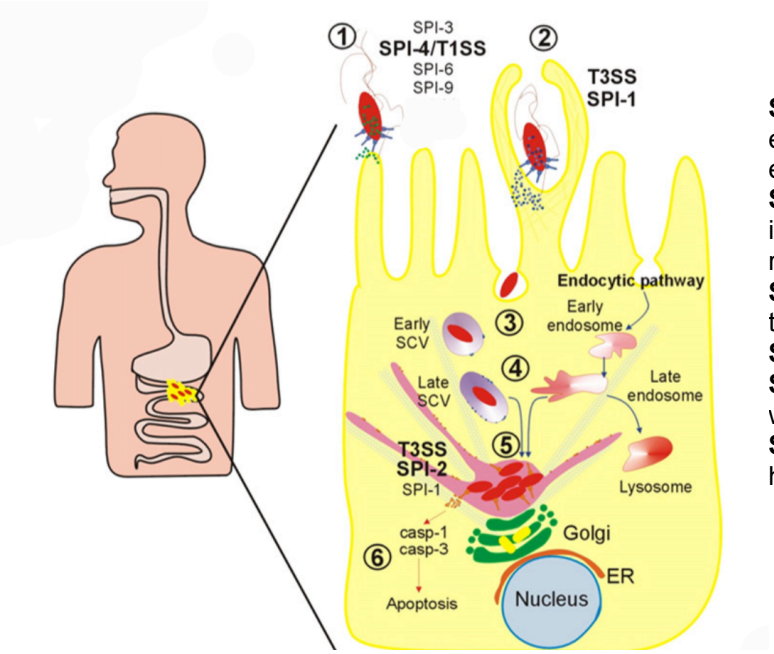


During *Salmonella* infection, T3SS genes encoded on a pathogenicity island (grey ring) are expressed. These include structural genes that make up the secretion complex, in addition to chaperones and effector proteins (grey diamond) that are injected across into a host cell.

Virulence genes responsible for distinct events of *Salmonella* pathogenesis may be distributed in different SPI (Marcus et al. 2000). The different SPIs have specific roles during the establishment of *Salmonella* Typhimurium infection in epithelial cells, including in the following main events of *Salmonella* pathogenesis: adhesion, invasion, *Salmonella*-containing vacuole (SCV) maturation and replication (Fig. 4) (López, Pescaretti, Morero &

Delgado 2012, LaRock, Chaudhary & Miller 2015). For example, virulence genes involved in the intestinal phase of infection are located in SPI-1 and SPI-2 (Marcus et al. 2000).

Figure 4 - Schematic representation of *Salmonella* infection. Adapted from Lopéz et al. 2012.



Step 1 – *Salmonella* induce the expression of fimbriae and adhesins encoded in SPI.
Step 2 – The uptake of *Salmonella* induces cytoskeleton rearrangements.
Step 3 – *Salmonella* invasion leads to an early SVC.
Step 4 – Maturation to a late SVC.
Step 5 – *Salmonella* replication within SCV.
Step 6 – *Salmonella* induce cell host apoptosis.

1.7.1.1. *Adhesion mechanism*

Salmonella adhesion to the intestinal epithelial surface is a critical step of infection pathogenesis which enables bacteria to invade the host (Burkholder & Bhunia 2009). Although *Salmonella* is able to colonize healthy hosts, studies in animal models have shown that the greatest risk for intestinal colonization and infection occur during periods of physiological stress (Hinton, Buhr & Ingram 2000, Humphrey 2004). Data regarding the colonization and infection of *Salmonella* during physiological stress in humans are scarce, but it is likely to follow a similar behaviour as in other animal models (Burkholder & Bhunia 2009).

As soon as *Salmonella* reaches the intestinal tract, many adhesion related structures including the capsule, flagella, fimbriae and lipopolysaccharide (LPS), are able to interact with host receptors (Wagner & Hensel 2011).

From the twelve SPI present in *Salmonella* Typhimurium, 4 are directly involved with adhesion to the host cell, prior to the beginning of disease, including: SPI-3, SPI-4, SPI-6 and SPI-9.

The SPI-3 is a 17kb locus that contains genes involved in intestinal colonization, due to the expression of an autotransporter protein enabling *Salmonella* Typhimurium to bind to fibronectin, and in intracellular survival, due to expression of a high-affinity magnesium transporter encoded by *mgt*ABC (Eswarappa et al. 2008, Rychlik I et al. 2009)

The SPI-4 is a 27kb locus required for the intestinal phase of disease, harboring the *sii*E gene, responsible for the production of a non-fimbrial adhesin, and the *siiABCDEF* operon, which encodes for the Type I secretion system (T1SS) and is suspected to be required for intramacrophage survival (Morgan et al. 2004). T1SS, as well as T3SS, are constituted by several membrane-bound proteins forming a structure designated “needle complex”, also referred as the injectisome, which is responsible for the deliver of virulence effectors into host cells (Brennan & Barford 2009, Misselwitz et al. 2011).

SPI-4 genes, as well as SPI-1 genes are regulated by the two component system (TCS) BarA/SirA, coordinating their expression in response to specific host signals, including low oxygen, high osmolarity and a slightly alkaline pH value (Gerlach, Jäckel, Geymeier & Hensel 2007a). Additionaly, *sii*E expression is negatively regulated by TCS, which is involved in the regulation of the expression of many virulence factors such as flagella, biofilm and invasion effectors (Delgado, Mouslim & Groisman 2006).

The SPI-6 is a 59kb locus present in both serovars Typhi and Typhimurium, which contains genes that encode for a type six secretion system (T6SS) and the *saf*ABCD fimbrial operon (Lambert & Smith 2008).

The SPI-9 is a locus of approximately 16kb that carries four genes, three of which encode for a T1SS and one for a large protein, BapA, which is involved in biofilm formation and host colonization (Gerlach et al. 2007b, Wagner & Hensel 2011).

1.7.1.2. *Invasion mechanism*

After *Salmonella* adhesion, host cell invasion occur (Crum-Cianflone 2008). This process happens due to the expression and regulation of SPI-1 genes, which promote the rearrangement of the host cell cytoskeleton at the interaction site, inducing the enterocyte membrane “ruffling" and the internalization by receptor-mediated endocytosis (Drecktrah et al. 2006). In the interior of the epithelial cell, *Salmonella* Typhimurium is enclosed within an intracellular phagosomal compartment called *Salmonella*-containing vacuole (SCV) (Steele-Mortimer 2008)

The SPI-1, one of the most studied SPI, have an important role in host invasion (Marcus et al. 2000). It is a 43kb chromosomal locus acquired by horizontal gene transfer during *Salmonella* evolution. It harbours at least 31 genes involved in the invasion of host cells and induction of macrophage apoptosis (McGhie et al. 2009).

Major genes present in SPI-1 are responsible for the synthesis of invasion effectors, like SipA, SipB, SipC, AvrA and SptP, a regulator protein HilA, the T3SS like InvA, Spa and Prg proteins and chaperones which are essential for the delivery of virulent effectors (Marcus et al. 2000, Eswarappa et al. 2008, McGhie et al. 2009).

The *inv*A gene codes for an inner membrane protein involved in the formation of a channel through by which polypeptides are exported. SipC and SipA proteins are involved in the

cytoskeletal rearrangement, being able to enhance the ability of *Salmonella* to cross the epithelial barrier and trigger the transduction signal that leads to the migration of PMN through the intestinal epithelia (Lee et al. 2000, Hayward & Koronakis 2002). SipB protein function has not been yet fully understood, but it seems to act both as an effector and as a translocator for other SPI-1 effector proteins, like SptP and AvrA (Hapfelmeier et al. 2004, McGhie et al. 2009, Andrews-Polymenis, Bäumler, McCormick & Fang 2010)

The expression of SPI-1 genes is under the complex regulation of HilA. This regulator is induced by the BarA/SirA TCS (Jones 2005). HilA also activates the T3SS/SPI-1 and SPI-1 effectors and bind to *prg*, *inv*/*spa* and *sip* operon promoters (Marcus et al. 2000, Jones 2005). Consequently, the expression of T3SS/SPI-1 effectors triggers the activation of mitogen-activated protein kinase (MAPK) pathways, leading to the production of pro-inflammatory cytokines, like IL-8, stimulating the recruitment of PMNs and inducing an acute intestinal inflammation (Figueira & Holden 2012).

Other genes important for bacterial invasion include *sop*B and *sop*E, which although encoding for effector proteins translocated by the T3SS/SPI-1 are not located in SPI-1, but in SPI-5 (Marcus et al. 2000). Briefly, SPI-5 is a small locus with 7.6kb in size and has been identified in many serovars, including *Salmonella* Typhimurium (Wood et al. 1998). SPI5 appears to be involved in enteropathogenesis (Wood et al. 1998, Marcus et al. 2000). It includes the *sop*B gene that plays a variety of roles at different stages of infection (Giacomodonato et al. 2014). This gene encodes an inositol phosphate phosphatase, which is required for invasion by localizing the host membrane in the early stages of infection, also promoting the membrane elasticity and vacuole formation (Cain, Hayward & Koronakis 2008). Later, it aids the formation and maintenance of SVC (Zhou, Chen, Hernandez, Shears & Galán 2001) by modifying the phosphoinositide lipids composition and preventing the phagolysosome formation (Giacomodonato et al. 2014).

The gene *sop*E activates the Cdc42 receptor at the host cell, also promoting cytoskeletal rearrangements and the production of pro-inflammatory cytokines (McGhie et al. 2009).

After *Salmonella* Typhimurium internalization, a process mediated by SptP allows the host cytoskeleton to return to a resting state, which down-regulates pro-inflammatory responses (Kaniga, Uralil, Bliska & Galán 1996). In the SCV, the low Mg2+ concentration inhibits *hil*A expression through the PhoP/PhoQ system, a global regulator of *Salmonella* virulence, turning off T3SS/SPI-1 activity (McGhie et al. 2009). Additionally, the transcription of *inv*F and *inv*G genes in SPI-1 is also repressed (Mouslim et al. 2004). The inhibition of these genes suggest that while SPI-1 genes are inhibited, SPI-2 genes expression is activated by PhoP, which is required for the following steps of infection (Jones 2005).

1.7.1.3. Salmonella-*containing vacuole (SCV)*

After the *Salmonella* invasion and internalization, the vacuoles containing the bacteria pass through a maturation process (Gorvel & Méresse 2001).

SCV formation can be separated into 3 steps: early stage, until 30 min post infection (p.i.), intermediate stage, from 30 min to 5h p.i. and late stage, from 5h p.i. (Steele-Mortimer 2008). In the beginning of SCV biogenesis, SCV membrane is enriched with early endosome membrane proteins, as early endosome antigen 1 and transferrin receptor. Then, SCV matures to an intermediate stage characterized by the accumulation of lysosomal membrane glycoproteins (Gorvel & Méresse 2001), which are replaced with late endosomal/lysosomal markers including lysosome-associated membrane proteins (LAMPs) (Steele-Mortimer, Méresse, Gorvel, Toh & Finlay 1999, Smith, Cirulis, Casanova, Scidmore & Brumell 2005). This membrane modification is accompanied by a decrease in the SCV luminal pH to less than 4.5 and a reallocation to a justanuclear position (Drecktrah et al. 2006, Steele-Mortimer 2008).

Regarding the intervention of SPI in SCV formation, some SPI-1 genes, which are induced prior to *Salmonella* engulfing, are down-regulated intracellularly (Smith et al. 2005). T3SS/SPI-1 also contributes to the intracellular pathogenesis, in particular, at early time-points of post invasion, due to the lag-time required for SPI2-associated protein synthesis (Knodler & Steele-Mortimer 2003). For example, SopB T3SS/SPI-1 is required for SCV formation and for delay SCV-lysosomal fusion, while SpiC T3SS/SPI-2 is required for fusion inhibition of the late endosomes/lysosomes with SCV, which shows the importance of both SPIs for an effective colonization of the host (Bakowski, Braun & Brumell 2008).

SPI-2 is also crucial at this stage. It is a 40kb locus that contains more than 40 genes, which are able to encode components of the T3SS apparatus, including *ssr* encoding a regulator, *ssa* for T3SS-2 apparatus, *ssc* encoding the chaperones and *sse* encoding the effector proteins (Figueira & Holden 2012). SPI-2 T3SS-secreted virulence effectors are required for intracellular replication, persistence and also inhibition of the inflammatory response during systemic infections (Yoon, Ansong, Adkins & Heffron 2011b).

At the end of the SCV maturation process, the vacuole location changes and reaches a perinuclear region near the Golgi complex. SCV localization allows the arrest of nutrients from endocytic and exocytic transport vesicles through a mechanism involving SifA, SseG and SseF SPI-2 effectors (Kuhle, Abrahams & Hensel 2006).

SPI-3 effectors are also involved in this maturation process, as SPI-3 contains the *mgt*CB operon encoding for the intramacrophage survival protein MgtC and for the high affinity Mg2+ transporter protein MgtB, which is under the regulation of PhoP/PhoQ TCS (Marcus et al. 2000).

1.7.1.4. *Bacterial replication*

Two important conditions must be achieved in order to initiate bacterial replication: the SCV must be located at a perinuclear region and the nutrients arrested must be sufficient to start this process (Gorvel & Méresse 2001). The main feature of this step is the presence of Sif (*Salmonella*-induced filaments), which are specific tubulovesicular extensions rich in LAMPs. (Birmingham, Jiang, Ohlson, Miller & Brumell 2005). In fact, it has been suggested that Sif structures are a result of a fusion process between a SCV with late endosomes/lysosomes. Sif structures project centrifugally from the SCV, extending throughout the cell (Knodler & Steele-Mortimer 2003).

In this stage of infection, at least 4 virulence effectors are expressed by T3SS/SPI-2, namely *sif*A and *sse*J, encoded outside SPI-2, and *sse*F and *sse*G encoded in SPI-2 (McGhie et al. 2009). The *sif*A gene is responsible for inducing the Sif phenotype and for Sif formation (Birmingham et al. 2005). Absence of *sif*A also promotes SCV instability, due to the lack of cooperation with another SPI-2 effector, SseJ (LaRock, Brzovic, Levin, Blanc & Miller 2012). *Salmonella* Typhimurium lacking *sifA* loses the vacuolar membrane and escapes to the cytoplasm, while bacteria lacking both genes *sifA* and *sseJ,* remain inside the vacuolar membrane. This indicates that membrane disruption is SseJ-dependent (Ruiz-Albert et al. 2002, LaRock et al. 2012), and bacteria lacking *sseJ* present attenuated intracellular replication (Ruiz-Albert et al. 2002).

SPI-2 effectors, SseF and SseG, are also required for Sif formation, since their absence leads to the formation of altered Sif structures (Kuhle & Hensel 2002, Birmingham et al. 2005).

As far as we know, Sif function is still unknown; however, the expression of SifA and of other SPI2-effectors are important during infection (LaRock et al. 2012). It has been demonstrated that, in later stages of *Salmonella* infection, from 14h to 24h p.i. Sif formation is important for the eventual movement of SCV towards the cell periphery before dissemination (Szeto, Namolovan, Osborne, Coombes & Brumell 2009). This mechanism allows bacteria transfer between epithelial cells, a process also dependent on SPI-2 effectors (Szeto et al. 2009).

1.7.2. Salmonella-*host interactions: role of virulence factors in cell apoptosis*

Research on host-pathogen interactions is essential to understand bacterial pathogenesis, including the processes leading to tissue colonization and posterior systemic dissemination. In the past years, it has become clear that *Salmonella* is able to activate host cell death programs (Knodler, Finlay & Steele-Mortimer 2005). Induction of cell death is required for pathogenesis, but the pathways by which *Salmonella* is able to induce this process remains unclear, mainly due to the occurrence of different processes in a variety of cell types (Knodler et al. 2005).

Regardless of the mechanism, it was already demonstrated that *Salmonella* Typhimurium is able to cause cell death in macrophages, dendritic cells and epithelial cells, and to promote systemic dissemination after replication, by mechanisms that involve SPI-1 and SPI-2-related pathways (Hueffer & Galán 2004, Knodler et al. 2005, Fink & Cookson 2007). In macrophages, the SPI-1 effector SipB and also flagellin, both induce a direct activation of host caspase-1, resulting in cell death by a cytokines-dependent process, in a mechanism termed as pyroptosis (Miao et al. 2006, Fink & Cookson 2007). Caspase-1 activation in *Salmonella* infected macrophages results in production of IL-1b and IL-18 and in rapid cell lysis, with release of pro-inflammatory intracellular contents. This process is responsible for the rapid onset of macrophages death, within 1h of infection, and is mediated by a SPI-1-related pathway (Knodler et al. 2005). However, late macrophages death, occurring from 12h to 24h p.i., is SPI-2-depentent, being also mediated by caspase-1 activation through SpvB (van der Velden, Velasquez & Starnbach 2003, Knodler et al. 2005). Additionally, studies regarding SPI-1 mutants genes, including *invA*, *invG*, *invJ*, *prgH*, *sipB*, *sipC*, *sipD* and *spaO*, do not display macrophagic cytotoxicity (Fink & Cookson 2007)

On the contrary, in epithelial cells, *Salmonella* induces cell death via apoptosis, a well-described mechanism with a notably non-inflammatory outcome (Fink & Cookson 2007). The activation of caspase-3 and caspase-8 is a classical feature in the apoptotic process, but not of caspase-1 (Paesold, Guiney, Eckmann & Kagnoff 2002). The induction of apoptosis by *Salmonella* epithelial cells only occurs after prolonged infection, within 24h to 28h p.i. and is mediated by a SPI-2-dependent pathway (Knodler et al. 2005).

It is important to note that pyroptosis is not only limited to macrophages, as caspase-1-dependent death of infected dendritic cells by *Salmonella* has also been observed (van der Velden et al. 2003).

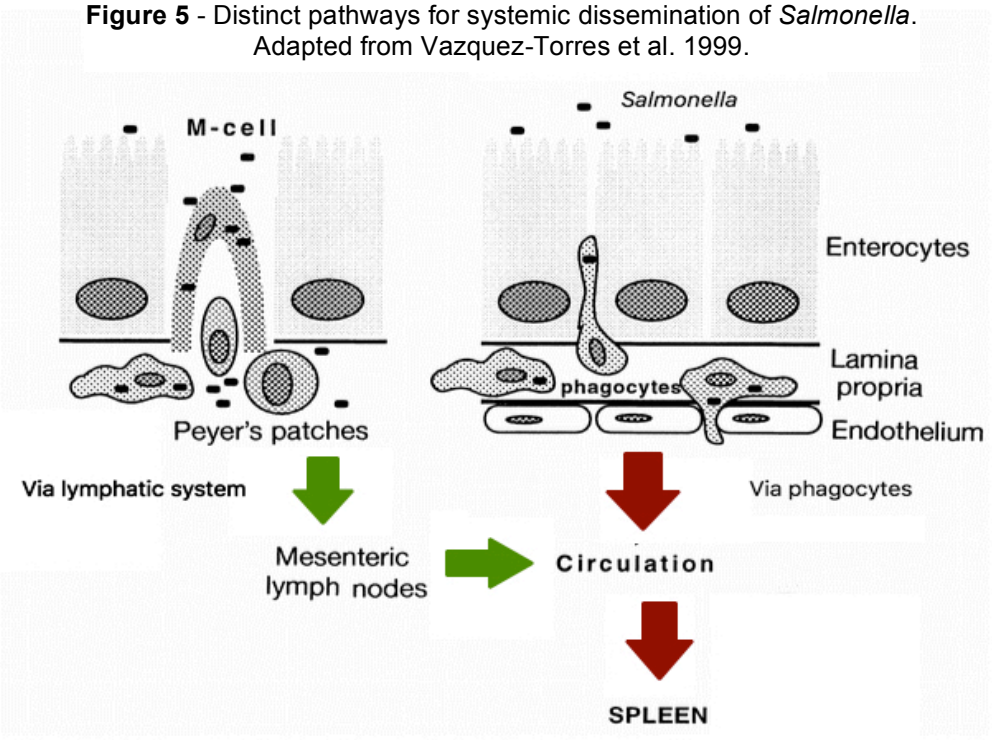
1.7.3. *Systemic dissemination of non-typhoid* Salmonella

Salmonella host-pathogen interactions enable not only the infection of macrophages and subsequent induction of cell death, but also enable bacterial dissemination, allowing *Salmonella* to reach the liver and spleen, via bloodstream and lymphatic system (McGhie et al. 2009). Therefore, intestinal phagocytes can be used as a mechanism for microbial dissemination into deeper tissues. By engulfing bacteria and traversing the epithelial barrier, they accidentally shield *Salmonella* from other components of the immune system (Drecktrah et al. 2006, Worley et al. 2006).

After invasion of M cells that overlie Peyer's patches, *Salmonella* Typhimurium accesses deeper tissues by two distinct pathways. In the intestine, bacteria access systemic tissues within 24h after ingestion, via lymphatic system and the Peyer's patches. In the second one, phagocytes may carry intestinal bacteria directly into the bloodstream, within 15 min after ingestion, and without passing through the Peyer's patches (Fig. 5) (Vazquez-Torres et al.

1999, Worley et al. 2006). In this haematogenous route, *Salmonella* may be carried by $CD18^+$ phagocytes, monocytes or dendritic cells (Vazquez-Torres et al. 1999). In prolonged infections, *Salmonella* can be transported between organs via the bloodstream. Liver infection also leads to bile excretion containing bacteria that reaches the gall bladder (Watson & Holden 2010).

Figure 5 - Distinct pathways for systemic dissemination of *Salmonella*. Adapted from Vazquez-Torres et al. 1999.



Salmonella Typhimurium may promote systemic dissemination by two distinct pathways: via lymphatic system (green arrows) or via phagocytes (red arrows).

It is important to refer that the movement of bacteria-infected cells requires the stimulation of cell motility and the inhibition of inflammatory pathways that could prevent this movement (Stupack, Cho & Klemke 2000). SseI, a virulence effector secreted by SPI-2 across the vacuolar membrane after invasion, is responsible for accelerating the movement of infected $CD18^+$ cells from the lumen of the intestine to the liver and spleen, which are the preferred locations for *Salmonella* replication (Worley et al. 2006). However, others reports demonstrated that SseI may have a different function by blocking migration of macrophages and dendritic cells. This discrepancy between these two roles is due to a single nucleotide polymorphism, which alters the pathway for cell migration (McLaughlin et al. 2009, Thornbrough & Worley 2012).

Bacteria systemic dissemination also relies upon the recently discovered gut-vascular barrier (GVB), present in the human gut, which controls the type of antigens that are translocated across the endothelial cells of blood vessels. The GVB presents morphological and functional features similar to the blood-brain barrier, in particular the characteristic pial vessels in the subarachnoid space, which are prone to disruption during infection, and can be damaged by *Salmonella* (Spadoni et al. 2015).

1.7.4. Salmonella *Virulence Plasmids*

Virulence factors responsible for pathogenicity in enteric bacteria may also be encoded in plasmids.

A plasmid, by definition, is a small, circular, double-stranded DNA molecule distinct from the chromosomal DNA, carryng genes with the ability to provide several genetic advantages to the bacterial cells, including antimicrobial resistance (del Solar, Giraldo, Ruiz-Echevarría, Espinosa & Díaz-Orejas 1998, Sabbagh, Forest, Lepage, Leclerc & Daigle 2010). Although plasmids can replicate autonomously in a self-controlled way independently from the chromosomal DNA, when a bacterium divides all plasmids are copied in order for each cell daughter to receive a copy of each plasmid. Several *Salmonella* serovars have serovar-specific virulence plasmids (Rychlik, Gregorova & Hradecka 2006, Foley et al. 2013). These plasmids are present at a low number (1 to 2 copies per cell) and range from 50 to 100kb, depending on the serovar (Foley et al. 2013). *Salmonella* Typhimurium, as many other non-typhoidal serovars harbor virulence plasmids, which play a important role in systemic infections (Rotger & Casadesús 1999, Marcus et al. 2000, Rychlik et al. 2006)

Most *Salmonella* Typhimurium strains contain a 90kb self-transmissible virulence plasmid harbouring virulence genes, namely the *Salmonella* plasmid virulence (spv) operon, which is involved in the multiplication of *Salmonella* in the reticuloendothelial system. Other virulence genes include the plasmid encoded fimbriae (*pef*) operon (Sabbagh et al. 2010, Foley et al. 2013).

The *spv* operon is a highly conserved 8kb locus of five genes, designated *spv*RABCD, which is positively regulated by the *spv*R product. This region may have been horizontally acquired, since it is located adjacent to an insertional element and has a guanine-cytosine content (46%) lower than the overall chromosome (Marcus et al. 2000)

Genes *spvR*, *spvB*, and *spvC* are required for virulence (Guiney & Fierer 2011). SpvB negatively regulates the formation of SIF structures and is also required for the late macrophage death, observed 12 to 24h after infection (van der Velden et al. 2003, Knodler et al. 2005). The gene *spv*C modulates the host immune response through the inactivation of MAPK, by reducing inflammatory cytokines production during the early stages of infection (Haneda et al. 2012). Immunocompromised patients committed with systemic infections frequently carry the *spv* locus (Guiney & Fierer 2011).

Regarding the *pef* locus, it contains five genes, *pef*BACDI, designated after the homology of their products with those of other fimbrial operons (Rotger & Casadesús 1999). PefA, the major fimbrial subunit of Pef, is expressed in *Salmonella* Typhimurium in static broth cultures at low pH conditions (Humphries et al. 2003). In this serovar, Pef mediates the adhesion to the small intestine in mice, and they are necessary but not sufficient to induce fluid secretion in the murine model. However, in several human cells lines, Pef does not mediate the adhesion cell process (Rotger & Casadesús 1999).

Adhesion by Pef is different from the one induced by the chromosome encoded long polar fimbriae (*lpf*) gene, which promotes the adhesion of *Salmonella* to Peyer's patches (Bäumler, Tsolis & Heffron 1996). Moreover, it is important to refer that the ability to survive in the Peyer's patches is also related to the *gip*A gene, a virulent determinant present at a lambdoid phage integrated in the genome of *Salmonella* Typhimurium (Stanley, Ellermeier & Slauch 2000, Drahovská et al. 2007).

In addition to *spv* or *pef* genes, other virulence related genes may be found, including resistance to complement killing (*rck*), SdiA-regulated gene (*srg*A), a putative disulphide bond oxidoreductase and a macrophage-inducible gene (*mig*-5), able to code for putative carbonic anhydrase (Rychlik et al. 2006).

Salmonella Typhimurium can also carry multidrug resistance plasmids of high molecular weight (up to 200kb) and much smaller plasmids (around 20kb) with unknown functions (Rychlik et al. 2006, Foley et al. 2013).

1.7.5. Salmonella *toxins*

Salmonella Typhimurium pathogenicity has also been attributed to the production of endotoxins and exotoxins. Endotoxins like the lipid A of the outer membrane LPS have been found to promote a wide variety of biological responses (Foley et al. 2013). Regarding exotoxins, they can be classified as cytotoxins and enterotoxins, both with the ability to kill mammalian cells (van Asten & van Dijk 2005, Foley et al. 2013).

Twenty years ago, a different type of exotoxin called salmolysin was reported due to the production of a cryptic hemolysin pattern when its gene, *sly*A, was expressed in *Escherichia coli* (Libby et al. 1994, Ludwig et al. 1995). However, upon further study, it was discovered that expression of the hemolysin is directly regulated by *sly*A and, therefore, is not a function of the gene itself. Later, *sly*A was identified as a virulence-associated transcriptional regulator contributing to the regulation of SPI-2 function and the expression of SPI-2-associated genes (Linehan, Rytkönen, Yu, Liu & Holden 2005).

One of the best described *Salmonella* enterotoxins is a heat-labile 29kDa enterotoxin, encoded by the *stn* gene, and also identified in *Salmonella* Typhimurium (van Asten & van Dijk 2005).

1.8. Antimicrobial Resistance in *Salmonella* Typhimurium 1,4,[5],12:i:-

Resistance to antimicrobial compounds is a natural biological phenomenon. In fact, antimicrobial resistance occurs independently of misuse or abuse of antimicrobials, as a result of selective pressure upon the bacterial ecology due to its administration (Levy & Marshall 2004, Blair, Webber, Baylay, Ogbolu & Piddock 2015). Over the last decades, it has been recognized that the introduction of new antimicrobial agents into clinical practice is

followed by the detection of resistant isolates (Chambers & Deleo 2009). In the past, throughout the 1950s and 1960s, it was considered that the development of new classes of antimicrobials, followed by modifications of these molecules, would allowed clinicians to effectively control bacterial infections (Levy & Marshall 2004). Presently, there are more than 15 classes of antibiotics, whose targets are involved in physiological and metabolic functions of the bacterial cell, and none has escaped resistance development (Levy & Marshall 2004, Blair et al. 2015).

It is important to evaluate the antimicrobial susceptibility profile of significant bacterial isolates, to detect possible drug resistance in pathogens and to assure susceptibility to drugs of choice for particular infections. According to the Clinical and Laboratory Standards Institute (CLSI), an isolate is resistant if when submitted to an antimicrobial susceptibility test, it presents a result that falls in the resistant category. This result implies that isolates are not inhibited by the usually achievable concentrations of the antimicrobial agent administered at normal dosage schedules (Clinical and Laboratory Standards Institute 2012).

Bacterial resistance may be an intrinsic feature associated with the bacterial species; for example, daptomycin is active against Gram-positive bacteria but is not effective against Gram-negative bacteria. The inefficacy towards Gram-negative bacteria is due to the lower amount of anionic phospholipids in the cytoplasmic membrane, which reduce the efficiency of the calcium mediated insertion of daptomycin into the cytoplasmic membrane, necessary for its antimicrobial activity (Blair et al. 2015).

Bacterial resistance to antimicrobials may also emerge due to point mutations or gene transfer (Chambers & Deleo 2009). These mechanisms may confer resistance to other antimicrobials of the same class and occasionally, to multiple antimicrobial classes (Levy & Marshall 2004). An isolate ability to be resistant to several antimicrobial classes is named multidrug-resistant, and the majority of the literature defines a multidrug-resistant bacteria as being resistant to compounds belonging to three or more antimicrobial classes (Magiorakos et al. 2012).

On the other hand, different resistance determinants can be responsible for the same resistance phenotype (EFSA 2010b, Blair et al. 2015). For example, resistance to streptomycin is commonly detected in *Salmonella* 1,4,[5],12:i:-, but the phenotype responsible for streptomycin resistance is encoded by different genes, depending if the isolate belongs to the Spanish or the European clone. In the Spanish clone, the *aad*A gene encodes streptomycin resistance, while in the European variant *str*A and *str*B genes are involved (Switt et al. 2009, EFSA 2010b, Lucarelli et al. 2010).

However, the presence of a resistant gene in an isolate does not always imply resistance to a particular antimicrobial, but it demonstrates its potential to express resistance once a selective pressure is applied (Guerra et al. 2004).

Unfortunately, drug resistance may be mobile and genes conferring antimicrobial resistance may be transferred between bacteria leading to a public Health problem (Levy & Marshall 2004). Horizontal gene transfer has been responsible for the dissemination of numerous antimicrobial resistance determinants throughout diverse bacterial species. The genetic determinants responsible for bacterial resistance may be present in highly efficient transferable elements, which can move between chromosomal and extra-chromosomal DNA elements. The most important vehicles for transfer of resistance genes in bacteria are mobile genetic elements, such as bacteriophages, plasmids, naked DNA or transposons (Levy & Marshall 2004). They may move between bacteria from the same or from different species, and also between bacteria of different taxonomic and ecological groups (Guerra et al. 2004, Blair et al. 2015).

The uncontrolled gene transfer between bacteria is not the only component contributing to resistance dissemination. The widespread use and misuse of antimicrobial compounds also contributed to an increased prevalence of resistance to new antimicrobials (Levy & Marshall 2004). However, due to different antimicrobial administration practices, the antimicrobial resistance patterns may vary between different regions and over time, requiring a continuous surveillance in human and animal populations (EFSA 2010b).

Recently in 2013, the Center for Diseases Control and Prevention considered multidrug-resistant non-typhoidal *Salmonella* as a serious hazard posing a serious economic burden and a public health issue in many countries (EFSA 2010b, CDC 2013). Among these antimicrobial resistant *Salmonella* isolates, the monophasic variants of *Salmonella* Typhimurium represent a major threat to public health (EFSA 2010b). The expansion of this serovar is particularly worrying, not only by its frequent association with invasive human gastroenteritis but also with multidrug-resistant profiles (Parsons, Crayford, Humphrey & Wigley 2013). In fact, the ASSuT tetra-resistant pattern is the most frequent multidrug-resistant profile detected in *Salmonella* 1,4,[5],12:i:-, being isolated from 30% of the human infection cases and also from farm animals (Switt et al. 2009, Lucarelli et al. 2010).

Monophasic *Salmonella* 1,4,[5],12:i:- may vary from pan-susceptible, commonly found in isolates from North and South America (Brazil) to multidrug-resistant isolates, mostly observed in Europe (EFSA 2010b, García, Guerra, Bances, Mendoza & Rodicio 2011). In this continent, the prevalence of *Salmonella* 1,4,[5],12:i:- R-type ASSuT has showed an escalating increase (Hopkins et al. 2010), whereas multidrug-resistant *Salmonella* Typhimurium has registered an decrease in its prevalence (Hopkins et al. 2012, Mandilara et al. 2013).

The first report of multidrug-resistant *Salmonella* 1,4,[5],12:i:- occurred in Spain in 1997, and is related to the Spanish clone. All these isolates were multidrug-resistant, showing resistance to ampicillin, choramphenicol, gentamicin, streptomycin, sulfamethoxazole, tetracyclines and trimethoprim (Echeita et al. 1999, Switt et al. 2009). Most of the Spanish

isolates are classified as phage type U302, though DT208 and DT193 have also been reported, all mainly linked to pigs and pork products (García et al. 2011). Genes responsible for this multidrug-resistant phenotype are mainly encoded by *bla*TEM-1, encoding a broad spectrum β-lactamase that provides resistance to penicillin and amino-penicillins like ampicillin; *aac(3)*-IV and *aad*A2, encoding modified aminoglycoside enzymes that can inactivate gentamicin and streptomycin by modifying different residues in the active sites of these drugs; *cml*A, encoding an efflux pump that promotes resistance to chloramphenicol; *sul*1, *sul*2 and *sul*3, encoding an dihydropteroate synthase that is resistant to sulfonamides, *dfr*A12, encoding an dihydrofolate reductase that is resistant to trimethoprim; and *tet*A, encoding an efflux pump that mediates resistance to tetracycline (Antunes, Machado & Peixe 2007, Switt et al. 2009, García et al. 2011).

The resistance genes of the Spanish clone are located on large non-conjugative plasmids approximately 140kb or 120kb, carrying or lacking the *spv* locus, respectively (Guerra, Soto, Argüelles & Mendoza 2001). Resistance plasmids frequently harbor up to three class 1 integrons: one with a variable region of 1900bp, carrying *dfr*A12 and *aad*A2 genes, a second one with the *estX-psp-aadA2-cmlA1-aadA1* gene cassettes, with *est*X and *psp* encoding a putative esterase and phosphoserine transferase, respectively, and the third lacking of resistance genes in its variable region (Guerra et al. 2004, EFSA 2010b, García et al. 2011).

Regarding the source of the antimicrobial resistance gene clusters found in the Spanish clone, the one found in the Spanish clone of *Salmonella* 1,4,[5],12,i:– is closely related to the antimicrobial resistance gene repertoire of *Salmonella* Cholerasuis (Chiu et al. 2005). Both have several resistance genes located on a plasmid, including *bla*TEM-1, *aad*A2, *cml*A, and *sul*1, but *Salmonella* Cholerasuis has other resistance genes that have not yet been reported in monophasic isolates. Therefore, it is unlikely that the specific resistance plasmid from *Salmonella* Cholerasuis was transferred to the ancestor Spanish 1,4,5,12,i:– isolates. Nevertheless, the antimicrobial resistance gene clusters of both serovars may be related and share a common ancestor (Switt et al. 2009).

The typical R-type ASSuT pattern only appeared a few years later in Italy, by the year 2000, being associated with the European clone. This clone has already been identified in many European countries, including Denmark, the United Kingdom, the Netherlands, Luxembourg, Spain, France, Italy, Poland and the Czech Republic (EFSA 2010b, Lucarelli et al. 2010). Pigs have been identified as the main reservoir of this clone, but it has also been recovered from cattle and poultry (Antunes, Mourão, Pestana & Peixe 2011). These R-type ASSuT *Salmonella* 1,4,[5],12:i:− isolates are mainly assigned to DT 193 and 120, with the antimicrobial resistant determinants present in a chromosomal resistance island that includes the *bla*TEM, *str*A-*str*B, *sul*2 and *tet*(B) genes, which differs from the Spanish clone (EFSA 2010b, Hopkins et al. 2010, Lucarelli et al. 2010). However, some isolates may harbor other resistances genes, for example *dfrA*, gene located at class 1 integron (EFSA 2010b).

Additionally, the European clone of *Salmonella* 1,4,[5],12:i:− may also present a different multidrug-resistant profile confering resistance to extended-spectrum β-lactamases (ESBL), carrying plasmids harbouring the *bla*CTX-M-1 gene (Rodríguez et al. 2012).

1.9. Biofilms in *Salmonella* Typhimurium 1,4,[5],12:i:-

1.9.1. *Biofilm formation: definition, components and mechanism*

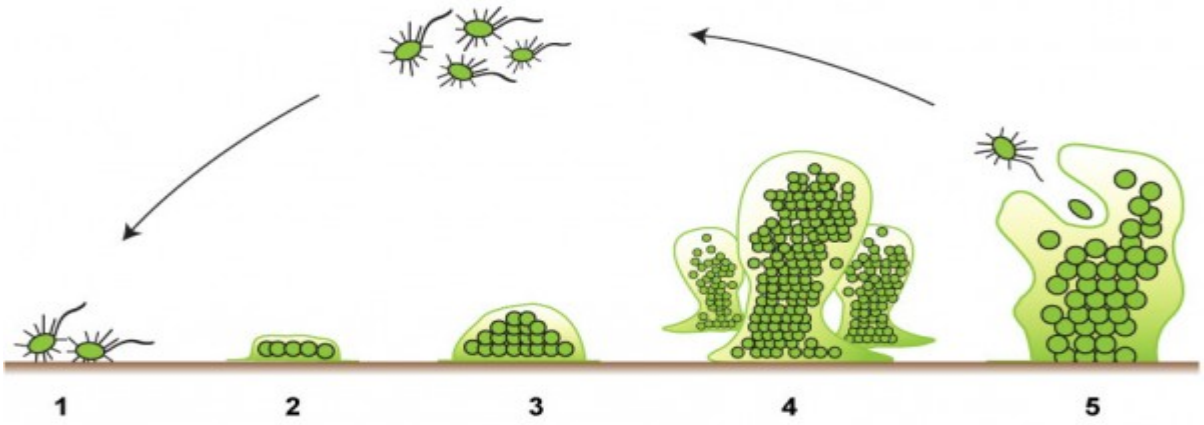
The success of most bacterial infections relies on the formation of bacterial communities, recognized as the predominant mode of bacterial growth (Donlan 2002, Götz 2002, Steenackers, Hermans, Vanderleyden & De Keersmaecke 2012, Kwiecinski, Kahlmeter & Jin 2015). These bacterial communities, known as biofilms, can be described as a community of microorganisms irreversibly adherent to biotic or abiotic surfaces and enclosed in a self-produced matrix (Flemming & Wingender 2010). This matrix is mainly composed by extracellular polymeric substances (EPS), mostly exopolysaccharides (40-95%) and proteins (1-60%), but also includes nucleic acids (1-10%) and lipids (1-40%) (Flemming & Wingender 2010).

The process of biofilm formation is believed to begin when bacteria detect certain environmental parameters (extracellular signals) that trigger the transition from planktonic growth to a biofilm phenotype (Donlan 2002). Currently, four potential incentives for bacterial biofilm formation are described: protection from a harmful environment, sequestration to a nutrient rich area, attainment of cooperative benefits and acquisition of new genetic traits (Davey & O'toole 2000, Molin & Tolker-Nielsen 2003).

The transition to a biofilm phenotype is a sequential process (Fig. 6) (Donlan & Costerton 2002), beginning with the initial attachment of planktonic bacteria to the tissue or abiotic surface. The ability of bacterial cells to adhere differs according to the type of surface. In abiotic surfaces, it is mainly due to physicochemical interactions (Oliveira et al. 2006), whereas in biotic surfaces, it is mainly dependent of specific interactions between bacterial adhesins and the receptors or molecules present on the substrate surface (Vuong & Otto 2002). Afterwards, occurs the production and accumulation of multilayer cell clusters due to intercellular adhesion, which relies on the production of extracellular matrix. Finally, a mature biofilm enclosed in a self-produced matrix is formed (O'Toole, Kaplan & Kolter 2000).

Once the biofilm structure has developed, mature biofilms can undergo a detachment process due to hydrodynamic, mechanical and biochemical signaling, leading to the release and dispersal of planktonic bacteria in order to colonize new surfaces (O'Toole et al. 2000, Donlan & Costerton 2002, Klausen, Gjermansen, Kreft & Tolker-Nielsen 2006).

Figure 6 - Biofilm formation process. Adapted from www.emerypharmaservices.com/blog/eps-offers-quantitative-biofilm-models-for-todays-research-needs, accessed in 12 January 2016.



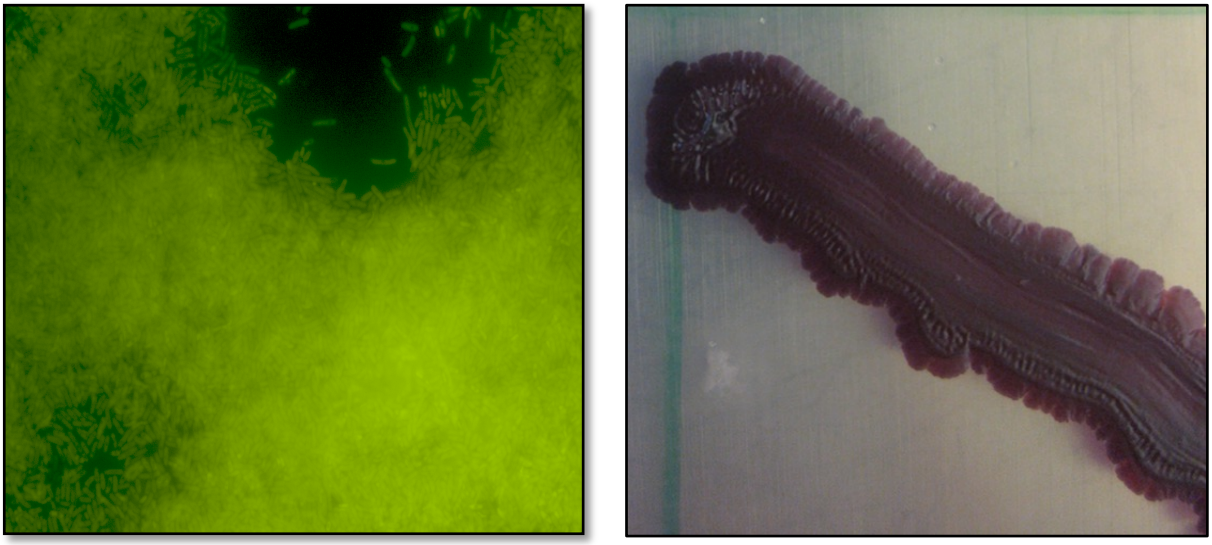
Steps of biofilm formation: 1 – Initial attachment, 2 – Irreversible attachment, 3 – Accumulation of multilayer bacterial clusters, 4 – Mature Biofilm, 5 – Bacterial cell dispersion.

1.9.2. *Structural components and genetic determinants of biofilm formation*

The regulatory network of *Salmonella* biofilm formation is complex and not fully understood, as laboratory results may vary according to the method and environmental conditions used to evaluate biofilm formation (Steenackers et al. 2012). There are several techniques to study biofilm components and its production, including growth characterization in agar and broth medium, optical density determination by microtiter assays and direct observation by fluorescent *in situ* hybridization (FISH) (Vestby, Moretro, Langsrud, Heir & Nesse 2009) (Fig. 7/Left).

The RDAR (red, dry and rough) morphotype detected on agar plates is the best-studied form of multicellular behaviour in *Salmonella* (Steenackers et al. 2012) (Fig. 7/Right). The extracellular matrix of this morphotype is composed by a proteinaceous and an exopolysaccharide fraction (Malcova, Hradecka, Karpiskova & Rychlik 2008, Steenackers et al. 2012). The proteinaceous fraction consists of curli fimbriae, alternatively referred as Tafi (thin aggregative fimbriae), which have been associated with several processes of biofilm formation and also host invasion, colonization, persistence, cell-to-cell interaction and increased motility (Steenackers et al. 2012). The curli fimbriae are formed as a result of the presence of the transcriptional regulator CsgD (curli subunit gene), which was previously referred as agfD in *Salmonella* Typhimurium. CsgD activates the *csg*BAC operon, leading to an increased production of curli fimbrial CsgA and CsgB subunits (Römling, Rohde, Olsén, Normark & Reinköster 2000, Zakikhany, Harrington, Nimtz, Hinton & Römling 2010). The expression of curli fimbriae may respond to environmental signals, being optimally expressed at temperatures below 30°C, in the presence of ethanol and variations in osmolarity and pH (Gerstel & Römling 2001).

Figure 7 - Biofilm phenotype detection using different methodologies (Original).



Left: Biofilm positive phenotype at 24h by FISH using teflon slides. Photo was taken at 1.000 magnification under oil immersion. Right: RDAR morphotype on agar plate.

Cellulose is a glucose polymer and the main component of the exopolysaccharides fraction. It functions as a support for long-range cell to cell interactions, being responsible for the sticky texture, which facilitates bacterial attachment to surfaces (Solano et al. 2002). Previous studies have demonstrated the important role of cellulose during *Salmonella* Typhimurium biofilm formation, including in epithelial cells and glass coverslips (Prouty & Gunn 2003, Ledeboer, Frye, McClelland & Jones 2006). However, reports have suggested that cellulose is not crucial for *Salmonella* biofilm formation on gallstones (Prouty & Gunn 2003, Steenackers et al. 2012).

Additionally, CsgD also indirectly increases cellulose production by activating *adr*A, which expression increases cyclic di-guanosine monophosphate levels, enhancing the expression of a cellulose synthetase and increasing cellulose production (Römling et al. 2000, Solano et al. 2002). However, the production of cellulose may not be necessary for virulence, as determined by *in vivo* studies (Pontes, Lee, Choi & Groisman 2015). In *Salmonella* Typhimurium, cellulose production outside a host may allow bacteria to survive in hostile environments (Römling 2002).

Curli fimbriae and cellulose combined are the main matrix components of *Salmonella* mature biofilms (Gerstel & Römling 2003), in which curli fimbriae provide the thin rigid link between bacterial cells, whereas cellulose is involved in stable attachment through the formation of elastic polysaccharide bonds (Römling 2002).

1.9.3. *Quorum sensing in* Salmonella *biofilms*

Bacteria should not be considered as individual microorganisms. Bacterial cells are able to communicate with each other and respond collectively to environmental stresses by

releasing and sensing small diffusible signal molecules, in a mechanism commonly known as quorum sensing (QS) (Miller & Bassler 2001, Swofford, Van Dessel & Forbes 2015).
QS can be considered as a sort of "social behaviour" and plays a vital role in synchronizing gene expression within the bacterial community (Smith, Fratamico & Yan 2011). QS involves a density-dependent recognition of signaling molecules, termed autoinducers (AI), resulting in modulation of gene expression (Kendall & Sperandio 2014).
In several bacterial genera, including *Salmonella,* it has been reported that biofilm formation is partially regulated by QS (Smith et al. 2011, Steenackers et al. 2012), also involved in the expression of several other features, including genetic competence, virulence, motility and the production of antimicrobial substances (Miller & Bassler 2001).
Three main types of QS systems have been reported in *Salmonella*: acyl-homoserine lactones (AHL), autoinducer (AI)-2 and AI-3 signalling (Smith et al. 2011). In the first system, *Salmonella* encodes a LuxR homologue, named SdiA (Ahmer 2004), which responds to the AHL produced by other bacterial species. In Gram-negative bacteria, this QS system is the most well-studied and usually consists of two proteins: LuxI, responsible for the synthesis of the AHL, and LuxR, recognized and activated by this autoinducer (Kendall & Sperandio 2014). However, *Salmonella*, does not express a signal-generating enzyme similar to LuxI, thus it cannot produce its own AHL (Ahmer 2004). *Salmonella* SdiA can detect AHL produced by a variety of bacterial species, leading to the hypothesis that SdiA can be used for interspecies communication within a mixed-species community (Michael, Smith, Swift, Heffron & Ahmer 2001, Smith & Ahmer 2003). SdiA regulates a few genes in *Salmonella*, including a gene involved in resistance to human complement, *rck* (Ahmer, van Reeuwijk, Timmers, Valentine & Heffron 1998).
The second *Salmonella* quorum sensing system utilizes the LuxS enzyme for the synthesis of AI-2, whereas the third one detects the quorum signal AI-3, as well as the eukaryotic hormones epinephrine and norepinephrine (Kendall & Sperandio 2014).

1.9.4. *Factors affecting biofilm development*

The formation and structure of a biofilm are influenced by three main components: bacterial cells, attachment surface and surrounding environment (Van Houdt & Michiels 2010).
Bacterial cells, in particular their surface, might influence bacterial adhesion due to its physicochemical properties, which may change according to several factors such as microbial growth phase or growth conditions (Giaouris, Chapot-Chartier & Briandet 2009). In general, the surfaces of most bacterial cells are adverse to attachment due to the net negative membrane charge, which creates an electrostatic repulsive force (Donlan 2002). In fact, a positively charged surface is more prone to bacterial adhesion, and a negatively charged surface is more resistant to bacterial adhesion (Song, Koo & Ren 2015). However, bacterial surfaces harbor many cell wall structures like fimbriae, flagella and LPS, which

contribute to the cell surface hydrophobicity. This hydrophobicity helps bacteria to overcome the repulsive forces occurring between the cell and the substratum, allowing the subsequent irreversible attach (Donlan 2002, Donlan & Costerton 2002).

The attachment surface competences, including charge, hydrophobicity, roughness, topography and stiffness, may also affect *Salmonella*-surface interactions and, consequently, biofilm formation (Song et al. 2015). For example, an increased surface roughness promotes bacterial attachment due not only to an increase of the contact area between the surface and bacterial cells (Anselme et al. 2010), but also to the protection from shear forces (Teughels, Van Assche, Sliepen & Quirynen 2006). Thus, smoothening surfaces can reduce biofilm formation (Lonescu et al. 2012).

Environmental factors such as pH, temperature, osmolarity, oxigen levels, nutrient composition and dynamic conditions, also play important roles in the process of biofilm formation (Stepanović, Cirkovic, Mijac & Svabic-Vlahovic 2003, Giaouris, Chorianopoulos & Nychas 2005). Even the presence of mixed bacterial communities also adds additional complexity to attachment and biofilm formation procedure (Rendueles & Ghigo 2015). The integration of these factors ultimately determines the behaviour pattern of a given bacterium with respect to biofilm development (Goller & Romeo 2008).

1.9.5. *Occurrence of* Salmonella *biofilms*

Numerous reports have demonstrated the ability of *Salmonella* isolates to form biofilms on abiotic surfaces, including plastic (Stepanović, Cirković, Ranin & Svabić-Vlahović 2004, Vestby et al. 2009), rubber (Arnold & Yates 2009), glass (Solano et al. 2002), cement (Joseph, Otta, Karunasagar & Karunasagar 2001), stainless steel (Moretro et al. 2009), granite, marble and silestones (Rodrigues, Teixeira, Oliveira & Azeredo 2011), materials commonly encountered in farms, slaughter houses, food processing industry, kitchens and toilets (Steenackers et al. 2012). Biofilm formation in these surfaces increases *Salmonella* resistance to several environmental stresses, which contributes to the survival in non-host settings and to the transmission to new hosts (Vestby et al. 2009).

Regarding biotic surfaces, in particular vegetables, *Salmonella* is able to colonize various parts of a variety of species, including seeds (Mahon et al. 1997), sprouts (O'Mahony et al. 1990), leaves (Oni, Sharma & Buchanan 2015), roots (Klerks, van Gent-Pelzer, Franz, Zijlstra & van Bruggen 2007) and even fruits (Guo, Chen, Brackett & Beuchat 2002), making vegetables important vectors for *Salmonella* transmission between hosts.

Salmonella is also able to adhere and form biofilms on epithelial cells and gallstones (Prouty & Gunn 2003, Steenackers et al. 2012). In fact, biofilm formation on gallstones may be associated with the chronic infection state in *Salmonella* infected individuals, which become asymptomatic chronic gallbladder carriers, shedding *Salmonella*. Then, the shedding process can contaminate food or water supplies, being also a source of recurring infections. This

chronic carrier state is hard to treat with antimicrobial therapy and the surgical gallbladder removal is often the only effective approach to cure these chronic patients (Steenackers et al. 2012).

1.9.6. *Implications of biofilm formation*

1.9.6.1. *In clinical settings*

Biofilm-forming bacteria usually cause chronic infections, despite the administration of an adequate antimicrobial therapy and the host's innate and adaptive defense mechanisms. Chronic infections are characterized by persistent and progressing disease, mainly due to the inflammatory response surrounding the biofilm (Høiby, Bjarnsholt, Givskov, Molin & Ciofu 2010). This persistent response is the only common feature between different biofilm infections, while other signs or symptoms are dependent on the organ or foreign body damage produced by the biofilm formation. The inflammatory cells prevailing in the persisting biofilm infection are polymorphonuclear neutrophil or mononuclear cells, depending on the type of immune response: T helper type 2 (antibodies production) or T helper type 1 (cell-mediated) (Bjarnsholt et al. 2009, Høiby 2014).

Implications of biofilm development are not only limited to chronic infections (Wilson 2001). The nature of biofilm matrix and the physiological attributes of biofilm microorganisms confer an inherent resistance to antimicrobial agents, whether these antimicrobial agents are antibiotics, disinfectants or germicides.

Concerning to the protective mechanisms responsible for biofilm resistance, three factors have to be taken in consideration: poor penetration of reactive agents through the biofilm matrix; implementation of adaptive responses; presence of non-growing cells in the inner layers of the biofilm. The existence of subpopulations of resistant phenotypes in the biofilm have been designated as "persisters" cells (Donlan & Costerton 2002, Høiby et al. 2010). Thus, established biofilms can tolerate antimicrobial agents at concentrations of 10 to 1.000 times higher than the ones required to kill genetically equivalent planktonic bacteria, and are also extremely resistant to phagocytosis, making very difficult to eradicate biofilms from living hosts (Mishra et al. 2015, Anjum & Krakat 2016). Additionally, the biofilm mode of growth also increases the ability to acquire or disseminate antimicrobial resistance determinants by horizontal gene transfer (Madsen, Burmølle, Hansen & Sørensen 2012).

Only recently, a guideline about diagnosis and treatment of biofilm infections was published in order to help clinicians and clinical microbiologists (Høiby et al. 2015).

1.9.6.2. *Food and industrial environment*

In food and food processing environments, bacterial attachment is influenced by the adsorption of macromolecules on the substrate, also known as conditioning film, which

changes the physicochemical properties of the surface and affects the bacterial adhesion and biofilm formation (Bernbom et al. 2009). *Salmonella* is also able to form biofilms on food and food-contact surfaces under adequate conditions, leading to serious hygienic problems and economic losses due to food spoilage (Roberts 1988, Kumar & Anand 1998, Van Houdt & Michiels 2010). In food processing environments, common sources involved in biofilm persistence are floors, waste water pipes, bends in pipes, rubber seals, conveyor belts, stainless steel surfaces and even teflon seals (Kumar & Anand 1998). In fact, adhesion of *Salmonella* to food surfaces was the first published report on a foodborne bacterial biofilm (Duguid, Anderson & Campbell 1966).

The presence of *Salmonella* biofilms in the food industry may cause post-processing contamination, leading to a lower shelf life of the food product and also to a safety problem (Zottola & Sasahara 1994).

Those multicellular structures may also promote several adverse technological effects. For example, biofilm formation in drinking water distribution systems may lead to a decrease in water velocity and carrying capacity, clogging of pipes and an increase in energy utilization, all factors promoting a decrease in efficiency of factory operations (Kumar & Anand 1998). This biofilm formation is mainly possible due to the presence of minimal amounts of nutrients in the water supply, and even in high levels of residual chlorine, their development may not be preventable (Kumar & Anand 1998, Chu, Lu, Lee & Tsai 2003).

Salmonella biofilms are particularly relevant in the context of bacterial long-term persistence on surfaces, playing a role as a reservoir for recurrent bacterial contamination in food processing facilities, which may lead to multiple foodborne outbreaks (Corcoran et al. 2014). Additionally, biofilms may also play a role due to their increased resistance to mechanical actions. These communities are more difficult to mechanically remove from food-contact surfaces and are more resistant to disinfectants compared to planktonic forms (Carpentier & Cerf 1993).

Persistence of *Salmonella* after disinfection may also be related to an inadequate cleaning and sanitation processes, or in well-controlled environments, due to acquired disinfectant resistance (Corcoran et al. 2014, Chen, Zhao & Doyle 2015).

1.10. *Salmonella* surveillance in Portugal

Salmonellosis is a notifiable disease since 1953 in Portugal. The mandatory notification was published in the “Decreto-Lei 39/209”, an update from a former regulation from 1889, only applied to animals, and still in force. The National Health Institute Doutor Ricardo Jorge (INSA) performs the laboratory diagnosis of human salmonellosis, being the National Reference Laboratory from the Ministry of Health. Several public or private laboratories may also perform reference diagnosis if approved by INSA. The European Regulation 882/2004 from April 29 manages the attribution of this competence, and regulates all the laboratories

within the EU, including the methodologies applied in *Salmonella* detection (European Comission 2004).

In Portugal, human cases of salmonellosis must be reported to INSA, responsible for the laboratory-based surveillance. Portuguese medical doctors are obliged to report by post and within 48h any confirmed or suspected case of salmonellosis to the Regional Health Administration (ARS), where the case resides (Diário do Governo-Presidência da República 1949). The ARS sends an anonymous paper copy of each individual notification form to the Directorate-General of Health (Gonçalves, Gouveia & Prasad 2014), which compiles all the data regarding human salmonellosis cases and sends the information, annually, to the European Centre for Disease Prevention and Control (ECDC) (Hugas & Beloeil 2014).

Furthermore, INSA reports to the Portuguese National Authority for Animal Health (DGAV), which compiles all the data regarding the detection of *Salmonella* in human, animal or food and reports to EFSA. The National Institute for Agricultural and Veterinary Research (INIAV) is the national reference laboratory from the Ministry of Agriculture, performing the laboratory diagnosis of animal salmonellosis. The General Directorate of food and veterinary services (DGAV) is the Portuguese authority responsible to put in practice all the European Regulations regarding the surveillance and control of *Salmonella* in food production animals, as well as, in the food chain.

1.11. Thesis objectives and scope

The scarce reports and the lack of knowledge regarding this serovar, particularly in Portugal, guided the scope of this thesis that aimed to address the epidemiology and characterization of the new pandemic serovar *Salmonella* 1,4,[5],12:i:-, obtained in Portugal from 2006 to 2011 from different sources, including human, animal and environmental samples. Therefore, the main objectives of this research project were:

- To perform a demographic characterization of monophasic salmonellosis cases and use geoprocessing techniques combined with spatial analysis to identify spatial clusters for *Salmonella* 1,4,[5],12:i:- in mainland Portugal **(Chapter II)**.
- To perform a characterization of the Portuguese monophasic *Salmonella* isolates obtained from different sources, mostly humans, in terms of country distribution, molecular typing, antimicrobial resistance and virulence factors **(Chapter III)**.
- To investigate the biofilm-forming potential of *Salmonella* 1,4,[5],12:i:- clinical, environmental, and animal isolates, to characterize the time course of biofilm production, and to evaluate the presence of genes involved in biofilm formation, using phenotypic and genotypic techniques **(Chapter IV)**.
- To evaluate the influence of conditions mimicking the intestinal human tract environment on *in vitro* biofilm formation by *Salmonella* 1,4,[5],12:i:-, in order to provide a better insight into the influence of the gastrointestinal environment upon biofilm formation by this serovar **(Chapter V)**.

This thesis provides information regarding the dissemination and epidemiology of this serovar in Portugal, contributing to the application of regulatory measures regarding public health and also to the future establishment of preventive strategies. Close surveillance of *Salmonella* 1,4,[5],12:i:- and its virulence factors, especially regarding antimicrobial resistance profiles and biofilm-forming ability, may help to prevent outbreaks and dissemination to non-problematic regions, as well to establish the most appropriate antimicrobial therapeutic practice for *Salmonella* 1,4,[5],12:i:- infections.

CHAPTER 2

Demographic characterization and spatial cluster analysis of human* Salmonella *1,4,[5],12:i:- infections in Portugal: a 10 year study

2.1. Demographic characterization and spatial cluster analysis of human *Salmonella* 1,4,[5],12:i:- infections in Portugal: a 10 year study

Rui Seixas* • Telmo Nunes • Jorge Machado• Luís Tavares • Fernando Bernardo • Manuela Oliveira

Paper submitted to Journal of Infection and Public Health

* The author participated in the conception and design of the study, conducted the experiments, contributed to the data analysis process and drafted the manuscript.

2.2. Abstract

Salmonella 1,4,[5],12:i:- is presently considered one of the major serovars responsible for human salmonellosis worldwide. Due to its recent emergence, studies assessing the demographic characterization and spatial epidemiology of salmonellosis 1,4,[5],12:i:- at local- or country-level are lacking. In a study conducted over a 10 year period from 2000 to the first quarter of 2011 at the Portuguese National Laboratory in Portugal mainland, a total of 215 human *Salmonella* 1,4,[5],12:i:- isolates obtained from human infections by a passive surveillance system were serotyped. Data regarding source, year and month of sampling, gender, age, district and municipality of the patients were registered. Descriptive statistical analysis and a spatial scan statistic combined with a geographic information system were employed to characterize the epidemiology and identify spatial clusters. Results showed that most districts have reports of infection cases by *Salmonella* 1,4,[5],12:i:-. An increased incidence was observed in the period from 2004 to 2011 with a higher number of cases at the Portuguese coastland, including in districts like Porto (n=60, 27.9%), Lisboa (n=29, 13.5%) and Aveiro (n=28, 13.0%). Most infections occurred during May and October, and fewest in February, being young individuals the most committed. Spatial analysis revealed 4 clusters of higher than expected infection rates. Three were located in the north of Portugal, including two at the coastland (Cluster 1 [RR=3.58, $P \leq 0.001$] and 4 [RR=10.42 $P \leq 0.230$]), and one at the countryside (Cluster 3 [RR=17.76, $P \leq 0.001$]). A larger cluster was detected involving the center and south of Portugal (Cluster 2 [RR=4.85, $P \leq 0.001$]). Although passive surveillance may originate an underestimation of disease burden, this is the first report describing the incidence and the distribution of areas with higher risk of infection in Portugal. *Salmonella* 1,4,[5],12:i:- displayed a significant geographic clustering and these areas should be further evaluated to identify risk factors in order to establish prevention programs.

Keywords:
Cluster analysis; Geographical information system (GIS); Portugal, Public health, *Salmonella* 1,4,[5],12:i:-,

2.3. Introduction

The prevalence of *Salmonella* serovars are constantly changing in many European countries [1] and, in 2010, the European Food Safety Authority (EFSA) Panel on Biological Hazards published a scientific opinion alerting for the increasing number of outbreaks in the European Union member states promoted by "*Salmonella* Typhimurium-like" strains [2].

Nowadays, *Salmonella* 1,4,[5],12:i:- is presently considered one of the major serovars responsible for human salmonellosis worldwide, already isolated from several animal species and food products [3].

Surveillance of *Salmonella*, and in particular of *Salmonella* 1,4,[5],12:i:- strains, may help to monitor disease development, reduce morbidity and mortality and improve health, avoiding unnecessary regulatory measures [4]. Population-based studies are uncommon, especially regarding spatial analysis in order to identify regional clusters and even their association with other factors [5]. As far as we know, there is no spatial-analysis based study evaluating areas of higher risk for *Salmonella* 1,4,[5],12:i:- at a country-level.

Geographic Information Systems (GIS) associated with spatial statistics are important tool for public health maintenance, as they may allow to identify risk areas requiring fast intervention, minimizing costs and rationalizing prevention's procedures [5]. This study aimed to perform the demographic characterization of monophasic salmonellosis 1,4,[5],12:i:- cases and to use spatial analysis combined geoprocessing techniques in order to identify spatial clusters for *Salmonella* 1,4,[5],12:i:- infection, evaluating high-risk areas in Portugal. This study may also provide useful information to understand the spread and epidemiology of this serovar.

2.4. Methods

2.4.1. Data source: study population and area

In Portugal, salmonellosis is a notifiable disease and is defined as the isolation of *Salmonella* spp. (excluding *Salmonella* Typhi or Paratyphi) from an appropriate clinical sample, namely stool, urine and blood, collected from patients with or without clinically compatible signs and symptoms. Medical doctors are obliged to report by post any confirmed or suspected case of salmonellosis, including non-typhoidal salmonellosis, to the local health authority of the municipality where the case lives [6].

In this study, 215 *Salmonella* 1,4,[5],12:i:- isolates were obtained from cases reported from 2001 to to the first quarter of 2011 in mainland Portugal were included. *Salmonella* were isolated at the National Health Institute Doutor Ricardo Jorge from different sources, including feces, blood, peritonial fluid and urine, and serotyped as *Salmonella* 1,4,[5],12:i:- according to the Kauffmann–White scheme [7]. Data including source, year and month of sample, gender, age, district and municipality of the patients were registered.

Isolates were obtained from patients at Portugal mainland, representing an area of 89.015km², corresponding 96.6% of the Portuguese national territory, with 10.047.083

inhabitants [8]. For administrative purpose, this territory is divided into 18 Districts, as follows: Aveiro, Beja, Braga, Bragança, Castelo Branco, Coimbra, Évora, Faro, Guarda, Leiria, Lisboa, Portalegre, Porto, Santarém, Setúbal, Viana do Castelo, Vila Real and Viseu. The Eurostat-based Nomenclature of Territorial Units for Statistics (NUTS) system subdivides Portugal mainland in 308 municipalities [8].

2.4.2. Statistical Analysis

2.4.2.1. Descriptive statistics

Descriptive statistical analyses were performed using SPSS 21.0 software (IBM Corporation, New York, USA). For statistical purposes, age was grouped in three different classes according to National Statistics Institute [8], namely: young (less than 15 years), adult (16-64 years) and elderly (higher than 65). Age mean, median, mode, range and standard deviation were also determined.

2.4.2.1. Spatial analysis

Salmonella 1,4,[5],12:i:- reports were geocoded at the municipality level. The centroids of each municipality were determined using the open-source Quantum Geographic Information System (QGIS) software. Spatial clustering of *Salmonella* 1,4,[5],12:i:- cases was analyzed using spatial scan statistics [9]. Statistical procedures were carried out in SaTScan software using a purely spatial Poisson model. The following data were considered for analysis: the number of positive cases in each municipality, the resident population within each municipality according to the 2011 Portuguese census, and the Cartesian coordinates of the centroids of each municipality included in the survey. The model was first run using the default maximum spatial cluster size of 50% of the total study population to ensure statistical power. The maximum-size parameter was then set at 10% to check for the presence of extreme small risk areas, possibly masked by the 50% scanning window. The number of Monte Carlo replications to estimate the statistical significance of the most likely cluster was set at 9999 iterations. A P value <0.05 was considered statistically significant.

2.4.3. Results

The majority of *Salmonella* 1,4,[5],12:i:- isolates were obtained from feces (n=185, 86%), followed by unknown sources (n=16, 7.6%), blood (n=8, 3.7%), blood and feces (n=3, 1.4%), peritoneal fluid (n=1, 0.5%), blood and urine (n=1, 0.5%) and urine (n=1, 0.5%).

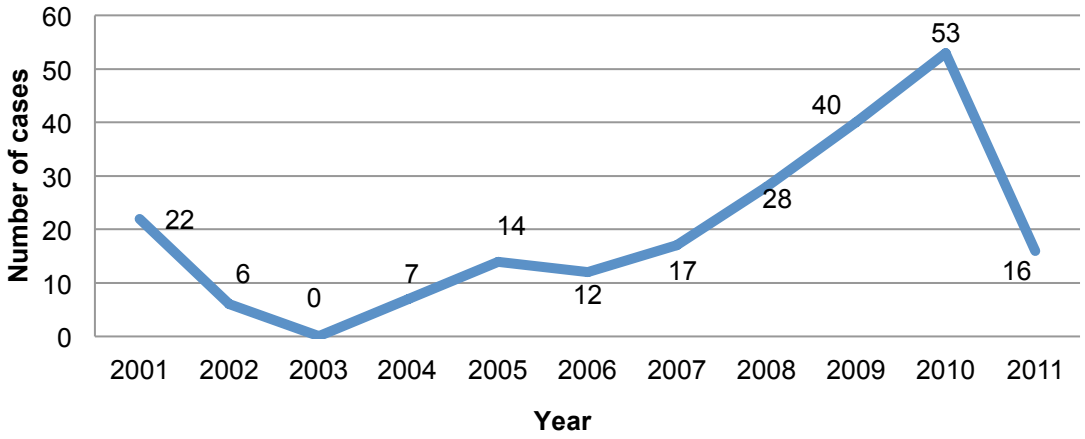
Most cases were reported in Porto (n=60, 27.9%), followed by Lisboa (n=29, 13.5%), Aveiro (n=28, 13.0%), Braga (n=27, 12.6%) and Setúbal (n=24, 11.2%). On the opposite, single cases were reported in Faro at 2010 and Viseu at 2007, as well as, two-single cases in Bragança at 2008 and 2009 and in Leiria at 2002 and 2009 (Table 1).

Table 1 - Distribution of *Salmonella* 1,4,[5],12:i:- clinical cases per district and gender in Portugal from 2000 and to the first quarter of 2011.

District	Gender				
		Not registered	Female	Male	Total
Aveiro	Count	0	21	7	28
	% of Total	0.0%	9.8%	3.3%	13.0%
Beja	Count	0	3	4	7
	% of Total	0.0%	1.4%	1.9%	3.3%
Braga	Count	0	15	12	27
	% of Total	0.0%	7.0%	5.6%	12.6%
Bragança	Count	0	2	0	2
	% of Total	0.0%	0.9%	0.0%	0.9%
Castelo Branco	Count	0	2	6	8
	% of Total	0.0%	0.9%	2.8%	3.7%
Évora	Count	0	2	12	14
	% of Total	0.0%	0.9%	5.6%	6.5%
Faro	Count	0	0	1	1
	% of Total	0.0%	0.0%	0.5%	0.5%
Leiria	Count	1	1	0	2
	% of Total	0.5%	0.5%	0.0%	0.9%
Lisboa	Count	0	16	13	29
	% of Total	0.0%	7.4%	6.0%	13.5%
Porto	Count	2	24	34	60
	% of Total	0.9%	11.2%	15.8%	27.9%
Santarém	Count	0	2	3	5
	% of Total	0.0%	0.9%	1.4%	2.3%
Setúbal	Count	0	8	16	24
	% of Total	0.0%	3.7%	7.4%	11.2%
Vila Real	Count	0	3	4	7
	% of Total	0.0%	1.4%	1.9%	3.3%
Viseu	Count	0	0	1	1
	% of Total	0.0%	0.0%	0.5%	0.5%
Total	Count	3	99	113	215
	% of Total	1.4%	46.0%	52.6%	100.0%

Regarding the distribution of *Salmonella* 1,4,[5],12:i:- infections, 2010 was the year with the higher number of reports (n=53, 24.6%), with an increasing trend in the number of cases from 2004 to 2010 (Figure 1).

Figure 1 - Trend in Portugal of the number of cases per year of *Salmonella* 1,4,[5],12:i:- from 2001 to the first quarter of 2011.

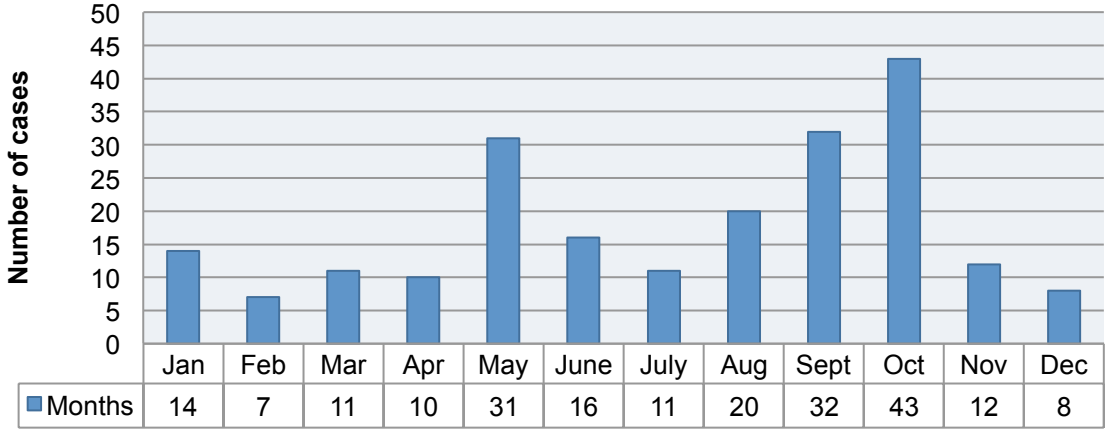


Regarding gender and age, 113 isolates were obtained from men and 99 from women and the male/female ratio was 1.34. In three patients, 1 from Leiria and 2 from Porto, gender was not registered.

Age from 199 individuals was registered. Mean age was 17 years with a median and mode of 3 and 1 year, respectively. Standard Deviation was 25.9 years with a minimum age of 0 years, a newborn, and maximum age of 90 years. Young individuals were the most affected with 69.3% (n=149) of *Salmonella* 1,4,[5],12:i:- cases, followed by adults with 14.0% (n=30) and elderly with 9.3% (n=20).

Seasonal variation is shown in Figure 2. Most of the infections occurred during May and October, with the lowest number of cases being diagnosed in February.

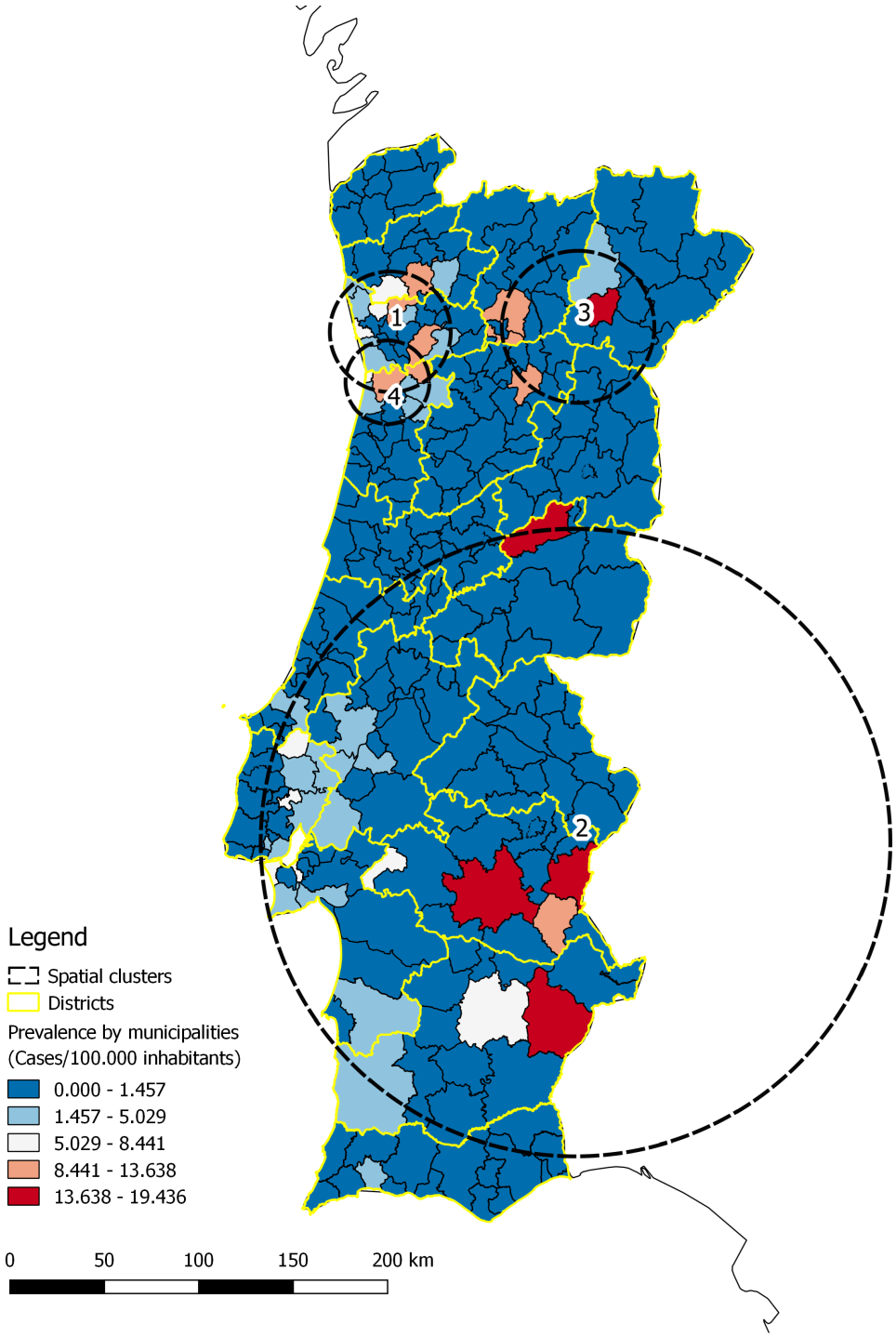
Figure 2 - Number of clinical cases per month of *Salmonella* 1,4,[5],12:i:- from 2001 to the first quarter of 2011.



Spatial analysis revealed 4 clusters of higher than expected infection rates, including two located in the north coastland area involving the districts of Porto and Aveiro (Cluster 1 and 4, respectively), one at the countryside involving the districts Bragança Viana Real, Viseu and Guarda (Cluster 3), and a larger cluster located in the center and south of Portugal which included the districts Castelo Branco, Leiria, Santarém, Lisboa, Portalegre, Évora,

Setúbal and Beja (Cluster 2) (Figure 3). Only clusters one, two and three were statistically significant, attributing a risk of infection of 4, 5 and 18 times higher, respectively, for a individual located in the delimited perimeter compared to other individual outside these areas. The number of cases, observed/expected ratio, relative risk and *p* value for each cluster can be found in Table 2.

Figure 3 - Prevalence and spatial clusters of *Salmonella* 1,4,[5],12:i:- cases in Portugal, 2000 to the first quarter of 2011.



Prevalence of *Salmonella* 1,4,[5],12:i:- per 100.000 inhabitants, by municipalities according to the Eurostat-based Nomenclature of Territorial Units for Statistics system. Spatial clusters results based on a purely spatial Poisson model using the SatScanTM software.

Table 2 - Spatial cluster of high *Salmonella* 1,4,[5],12:i:- infection rates in mainland Portugal from 2000 to the first quarter of 2011.

Cluster	Number of cases	Number of municipalities	Annual cases/ 100000 Habitants	Expected cases	Overlap	Observed /Expected	Relative risk (RR)	Log likelihood ratio	*P* value
1	109	22	4.8	48.02	4	2.27	3.58	41.19	≤0.001
2	84	109	4.9	46.70	No	1.80	4.85	27.49	≤0.001
3	10	18	49.8	0.99	No	10.14	17.76	16.44	≤0.001
4	4	5	37.5	0.55	1	7.28	10.42	5.07	0.230

2.5. Discussion

The first worldwide report on the isolation of *Salmonella* 1,4,[5],12:i:- was from Portugal in 1986 in poultry [10]. Since this first report, a rapid increase in *Salmonella* 1,4,[5],12:i:- isolation was observed on an international scale, becoming the third most common serovar related with humans infections in 2013 in the EU [3].

Data presented in this study represents a passive monitoring based on clinical samples submitted to the national reference laboratory. Passive monitoring often underestimates the number of cases, since not every individual with salmonellosis goes to a physician due to its self-limiting course [11, 12]. In addition, young individuals usually present more severe infections and therefore are more tested than adults [11, 12]. Thus, a limitation to this approach is the probable selection bias [12]

In the present study, as many other reports [13, 14], *Salmonella* was commonly isolated from stool samples. Others sources are scarcely documented and to our knowledge, this report include the first isolation of *Salmonella* 1,4,[5],12:i:- from peritoneal fluid, highlighting an unusual source of isolation, particularly important in clinical settings [15].

The age distribution of individuals with *Salmonella* 1,4,[5],12:i:- infections in the present study is in agreement with others reports [12, 13, 16], where most infections caused by this serovar are observed in young individuals. While this study detected *Salmonella* 1,4,[5],12:i:- infections more frequently in men, others reports also identified both men or women frequently affected [13, 16].

Although in the period of this study an increasing annual incidence was observed, the number of reported isolates is low, which may be attributed to a decreasing in the amount of salmonellosis cases reported in the EU during this period, including Portugal [2].

In this country, most districts have reports of *Salmonella* 1,4,[5],12:i:-, which suggests a wide distribution from the north to south of the country. A higher prevalence in the Portuguese coastland was observed, which the higher human population density in these areas may

explain and because patients are more likely to go to a doctor if gastrointestinal symptoms are presented. It is worth noting that some districts are classified without reports, which do not necessarily represent the absence of the disease, especially when there is evidence of underreporting due to its self-limiting course.

Rates of human *Salmonella* 1,4,[5],12:i:- infection appear to be higher in summer and early autumn, with most cases occurring between May and October, being this last month the one with the higher number of cases. In several studies, *Salmonella* infections among humans generally peak in summer months [17, 18]. The reasons of these seasonal differences are not entirely known. They may reflect a combination of factors, including seasonal human behaviours [17], the parallel *Salmonella* shedding trends by animal reservoirs [19] and environmental variations influencing the pathogen virulence or persistence [17].

Cluster analysis of *Salmonella* 1,4,[5],12:i:- infections showed the existence of areas with a high number of cases, especially in districts located at the northwest, northeast, center and southeast of Portugal. This observation suggests an occurrence of non-random cases, confirmed by the representation of three high rate clusters, which may reveal an increased exposure to human *Salmonella* 1,4,[5],12:i:- infection in these areas.

Interestingly, cluster 1 located at northwest of Portugal presents the highest number of human *Salmonella* 1,4,[5],12:i:- infections. This cluster contains two districts, Porto and Aveiro, with only 22 municipalities involved. Individuals in these areas present a risk about 4 times higher to acquire this infection that inhabitants of other municipalities. Although it may exist several reasons to explain this spatial cluster, one possible explanation is because pig farms are prevalent in these locations, especially in Aveiro [20]. Since pigs can be reservoirs for this serovar, this may justify the spatial cluster and the high frequency of cases [21, 22]. This reason may also be valid for cluster 2, as some of the districts included like Beja, Leiria and Santarém, are also the important locations for pig production in Portugal [20].

2.6. Conclusions

As far as we know, this is the first report describing the incidence and the presence of areas with a higher risk for human *Salmonella* 1,4,[5],12:i:- infections in Portugal. Although passive surveillance may represent an underestimation of disease burden, they provide valuable information on incidence and trends that could aid public health authorities in developing prevention and control programs. There is a need to better understand the demographic, geographic, and seasonal factors associated with the increase of *Salmonella* 1,4,[5],12:i:- infections and to provide evidence-based information for policy makers to prioritize future efforts in addressing the increasing number of infections.

Funding

This study was conducted with the financial support of the “Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária da Universidade de Lisboa” - Project UID/CVT/00276/2013 (funded by Foundation for Science and Technology - FCT). Rui Seixas holds a PhD fellowship (SFRH/BD/75836/2011) from FCT, Portugal

Competing interests

The authors declare they have no competing interests.

Ethics approval

Not applicable.

Acknowledgements

The authors would like to acknowledge the data provided by the Portuguese national reference laboratory, the National Health Institute Doutor Ricardo Jorge (INSA).

References

1.Hugas M, Beloeil P. Controlling *Salmonella* along the food chain in the European Union - progress over the last ten years. Euro Surveill 2014; 19(19):pii=20804.

2.EFSA Panel on Biological Hazards. Scientific Opinion on monitoring and assessment of the public health risk of "*Salmonella* Typhimurium-like" strains. EFSA Journal 2010; 8(10):1826-74.

3.EFSA and ECDC. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013. EFSA Journal 2015; 13(1):3991.

4.Mølbak K, Simonsen J, Jørgensen CS, Krogfelt KA, Falkenhorst G, Ethelberg S *et al*. Seroincidence of human infections with nontyphoid *Salmonella* compared with data from public health surveillance and food animals in 13 European countries. Clin Infect Dis 2014; 59(11):1599-606.

5.Varga C, Pearl DL, McEwen SA, Sargeant JM, Pollari F, Guerin MT. Evaluating area-level spatial clustering of *Salmonella* Enteritidis infections and their socioeconomic determinants in the greater Toronto area, Ontario, Canada (2007 - 2009): a retrospective population-based ecological study. BMC Public Health 2013; 13(1078).

6.Gonçalves G, Gouveia E, Prasad L. The response of a local health authority to reported cases of salmonellosis in a Portuguese municipality, 2007 to 2011. BMC Res Notes 2014; 7(161).

7.Grimont PA, Weill FX.Antigenic formulae of the *Salmonella* serovars.9th ed.WHO Collaborating Centre for Reference and Research on *Salmonella*, Institut Pasteur, Paris, France; 2007.

8.Instituto Nacional de Estatística. Census 2011 - XV recenciamento geral da população, http://censos.ine.pt/ngt_server/attachfileu.jsp?look_parentBoui=148313382&att_display=n&att_download=y; 2011 [accessed 10.09.2016].

9.Kulldorff M. A spatial scan statistic. Commun Stat Theory Methods 1997; 26(6):1481-96.

10.Machado J, Bernardo F. Prevalence of *Salmonella* in chicken carcasses in Portugal. J Appl Bacteriol 1990; 69(4):477-80.

11.Asmar BI, Abdel-Haq N. Nontyphoidal *Salmonella* infection in children: relation to bacteremia, age, and infecting serotype. Infect Dis (Lond) 2016; 48(2):147-51.

12.Almeida C, Moreira D, Machado A, Terra I, Vieira L, Cunha J. *Salmonella* gastroenteritis in children. Acta Med Port 2012; 25(4):219-23.

13.Ke B, Sun J, He D, Li X, Liang Z, Ke C. Serovar distribution, antimicrobial resistance profiles, and PFGE typing of *Salmonella enterica* strains isolated from 2007–2012 in Guangdong,China. BMC Infect Dis 2014; 14(338).

14.Capuano F, Mancusi A, Capparelli R, Esposito S, Proroga YT. Characterization of drug resistance and virulotypes of *Salmonella* strains isolated from food and humans. Foodborne Pathog Dis 2013; 10(11):963-8.

15.Joseph T, Sobhan P, Bahuleyan S, John A, Sathar SA, Sreesh S *et al*. Non-typhoidal *Salmonella*: an unusual cause of spontaneous bacterial peritonitis in decompensated cirrhosis. Gastroenterol Rep (Oxf) 2014; 2(3):242-44.

16.Gossner CM, van Cauteren D, Le Hello S, Weill FX, Terrien E, Tessier S *et al*. Nationwide outbreak of *Salmonella enterica* serotype 4,[5],12:i:- infection associated with consumption of dried pork sausage, France, November to December 2011. Euro Surveill 2012; 17(5):pii=20071.

17.Ravel A, Smolina E, Sargeant JM, Cook A, Marshall B, Fleury MD *et al*. Seasonality in human salmonellosis: assessment of human activities and chicken contamination as driving factors. Foodborne Pathog Dis 2010; 7(7):785-94.

18.Collard JM, Bertrand S, Dierick K, Godard C, Wildemauwe C, Vermeersch K *et al*. Drastic decrease of *Salmonella* Enteritidis isolated from humans in Belgium in 2005, shift in phage types and influence on foodborne outbreaks. Epidemiol Infect 2008; 136(6):771-81.

19.Wales A, Breslin M, Carter B, Sayers R, Davies R. A longitudinal study of environmental *Salmonella* contamination in caged and free-range layer flocks. Avian Pathol 2007; 36(3):187-97.

20.Almeida J: Produção intensiva de suínos - Projecto de exploração (master dissertation). Portugal: Universidade de Lisboa; 2008.

21.Hopkins KL, Kirchner M, Guerra B, Granier SA, Lucarelli C, Porrero MC *et al*. Multiresistant *Salmonella enterica* serovar 4,[5],12:i:- in Europe: a new pandemic strain? Euro Surveill 2010; 15(22).

22.Mandilara G, Lambiri M, Polemis M, Passiotou M, Vatopoulos A. Phenotypic and molecular characterisation of multiresistant monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) in Greece, 2006 to 2011. Euro Surveill 2013; 18(22).

CHAPTER 3

Phenotypic and molecular characterisation of* Salmonella *1,4,[5],12:i:- R-type ASSuT isolates from humans, animals and environment in Portugal, 2006-2011

3.1. Phenotypic and molecular characterisation of *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates from humans, animals and environment in Portugal, 2006-2011

Rui Seixas*, Tânia Santos, Jorge Machado, Luís Tavares, Fernando Bernardo, Teresa Semedo-Lemsaddek, Manuela Oliveira

Paper published in *Foodborne Pathogens and Diseases* 2016 Nov;13(11):633-641

* The author contributed in the conception and design of the study, conducted the experiments, performed the data analysis process and drafted the manuscript.

3.2. Abstract

The increase in prevalence of *Salmonella* 1,4,[5],12:i:- related infections over the last few years has been considered a public health issue in many European countries, especially as this serovar may be associated with tetraresistance to ampicillin, streptomycin, sulfonamides, and tetracyclines (R-type ASSuT). *Salmonella* 1,4,[5],12:i:- isolates (n = 187) obtained by the Portuguese National Laboratory from different sources, including human clinical cases (n=170), veterinary (n=10), environmental (n=6), and food samples (n=1), were collected from 15 districts between 2006 and 2011. All isolates were serotyped using the slide agglutination method and results were confirmed by multiplex PCR for the monophasic variant. From the confirmed *Salmonella* 1,4,[5],12:i:-, R-type ASSuT isolates were selected by disc diffusion and minimal inhibitory concentration (MIC) determination for further characterization by pulsed-field gel electrophoresis restriction with XbaI, virulence genes determination by PCR, additional antimicrobial resistance profiling by disc diffusion, and epidemiological distribution evaluation. Out of the 187 serotyped isolates, 133 were confirmed as *Salmonella* 1,4,[5],12:i:- with a R-type ASSuT occurrence of 61.7%. Distribution among Portuguese districts showed a higher percentage of reported cases in coastal areas, in particular, in Porto (24.8%), Setúbal (13.5%), and Aveiro (12.8%), probably due to the higher population density. Clonality analysis revealed a high diversity of pulsotypes with the majority of human salmonellosis cases being attributed to sporadic events. All isolates harbored 14 out of the 18 virulence genes evaluated and 87.8% of the isolates showed all the resistance genes frequently associated with the European clone, *bla*TEM+*sul*2+*stra*A-*stra*B+*tet*B+. This study shows that *Salmonella* 1,4,[5],12:i:- resistant isolates are widely distributed in Portugal. This may be related to a selective advantage offered by R-type ASSuT profile, the presence of multiple virulent features, including the ability to form biofilms, which along with a high diversity of pulsotypes may be responsible for the dissemination through the country.

Keywords: Portugal, R-type ASSuT, *Salmonella* 1,4,[5],12:i:-, virulence factors

3.3. Introduction

Salmonella 1,4,[5],12:i:- is the monophasic variant of *Salmonella* Typhimurium, being presently considered one of the major serovars responsible for human salmonellosis worldwide (Switt et al., 2009). A marked increase in prevalence of *Salmonella* 1,4,[5],12:i:- showing resistance to ampicillin, streptomycin, sulfonamides, and tetracyclines (R-type ASSuT) has been identified in several European Union member states over the last decade (Switt et al., 2009). This resistance profile is the most frequently detected, related to 30% of the human cases and livestock (Lucarelli et al., 2010).

Genes responsible for this resistance phenotype are present in a chromosomal resistance island that frequently includes the *bla*TEM, *str*A-*str*B, *sul*2, and *tet*B genes, with some isolates having additional resistances (Lucarelli et al., 2010). Also, the presence of multiple virulent determinants, along with biofilm formation, enables *Salmonella* to infect several host species and persist in the environment (Steenackers et al., 2012).

The aim of the present study was to characterize, in terms of distribution, molecular typing, antimicrobial resistance, and virulence factors, *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates obtained in Portugal from different sources, mainly of human origin and also from animal and environment. This study provides useful information for understanding the dissemination and epidemiology of this serovar in Portugal and within Europe, contributing to the future establishment of preventive and therapeutic strategies, as well as suitable regulatory measures regarding public health safety.

3.4. Materials and Methods

3.4.1. Bacterial isolates

A total of 187 *Salmonella* 1,4,[5],12:i:- isolates were included in the study. The samples were collected in Portugal between 2006 and the first quarter of 2011 from different sources, including human clinical cases (n = 170), veterinary (n = 10), environmental (n = 6), and food samples (n = 1). Human salmonellosis is a mandatory notifiable disease in Portugal, respecting a passive surveillance system, and the isolates represent all reported cases of *Salmonella* 1,4,[5],12:i:- in Portugal during this period of time. All *Salmonella* were isolated at the Portuguese National Laboratory, the National Health Institute Doutor Ricardo Jorge, and serotyped using the slide agglutination method according to the Kauffmann–White scheme (Grimont and Weill, 2007). Identification of serotyped isolates was confirmed by multiplex PCR (mPCR), and confirmed *Salmonella* 1,4,[5],12:i:- isolates were selected for detection of R-type ASSuT. *Salmonella* 1,4,[5],12:i:- presenting the tetraresistance pattern were further submitted to phenotypic and molecular characterization. Regarding the human samples, data, including year of sample collection, gender, age, and district location, were recorded.

3.4.2. DNA extraction and mPCR identification of *Salmonella* 1,4,[5],12:i:-

Salmonella isolates were plated onto Columbia agar with 5% sheep blood (BioMérieux) and incubated at 37°C for 24h. DNA was extracted from a single colony grown overnight using the guanidine thiocyanate method (Pitcher et al., 1989). Identification of *Salmonella* 1,4,[5],12:i:- isolates was confirmed using mPCR (Tennant et al., 2010) as recommended by the European Food Safety Authority (EFSA, 2010). A total volume of 25 µL containing 2.5 U of Supreme NZYTaq 2x Green Master Mix (NZYTech, Portugal), 0.4 µM of primers sense-59 and antisense-83, which amplify the phase 2 (*flj*B) flagellar gene, 0.8 µM of FFLIB and RFLIA, amplifying the *fli*B-*fli*A intergenic region (Stabvida, Portugal), and 3µL of DNA template were submitted to the following conditions: initial denaturation at 95°C for 2min, 25 cycles of denaturation at 95°C for 30 s, annealing at 64°C for 30 s, extension at 72°C for 1.5 min, and a final delay at 72°C for 5 min. Amplified products were resolved in a 1.5% agarose gel (Bioline, UK) stained with GelRed (Biotium) and visualized by transillumination under UV (Pharmacia Biotech, Thermal Imaging System FTI-500). NZYDNA ladder VI (NZYTech, Portugal) was used as molecular weight marker. *Salmonella enterica* subsp. *enterica* CECT 7162 and CECT 722 were used as monophasic and biphasic *Salmonella* controls, respectively, for PCR amplification.

3.4.3. Detection of R-type ASSuT pattern

3.4.3.1. Antimicrobial susceptibility testing

Salmonella 1,4,[5], 12:i:- isolates, the identification of which was confirmed by mPCR, were evaluated to detect the R-type ASSuT pattern. Antimicrobial susceptibility testing (AST) was performed by disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI, 2012) guidelines using ampicillin (AMP, 30 µg), streptomycin (S, 25 µg), sulfamethoxazole (SXT, 10 µg), and tetracycline (TE, 30 µg) and confirmed by determination of minimal inhibitory concentration (MIC) using E-test (BioMérieux) as recommended by the WHO Global Salm-Surv (2003). Test performance was monitored using *Escherichia coli* ATCC 25922 and executed in duplicate.

3.4.4. Characterisation of *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates

3.4.4.1. Evaluation of additional resistances

AST was performed by disc diffusion method according to CLSI guidelines (CLSI 2012) using amoxicillin/clavulanic acid (AMC, 30 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), ciprofloxacin (CIP, 5 µg), chloramphenicol (C, 30 µg), gentamicin (CN, 10 µg), nalidixic acid (NA, 30 µg), Test performance was monitored using *E. coli* ATCC 25922 and executed in duplicate.

3.4.4.2. Detection of antimicrobial resistance determinants

Genes coding for resistance to amoxicillin (*bla*TEM), streptomycin (*str*A-*str*B), sulfamethoxazole (*sul*2) and tetracycline (*tet*B) were evaluated by PCR using primers and conditions previously described (Lucarelli et al. 2012). *Salmonella* Typhimurium monophasic variant 105/07/03 gently provided by Dr. Lucarelli's laboratory was used as a positive control for all PCR amplifications.

3.4.4.3. Genotyping of *Salmonella*

Genomic fingerprinting of *Salmonella* was achieved by PFGE using a CHEF-DRIII apparatus (Bio-Rad Laboratories, San Diego, USA) following DNA digestion with XbaI (Invitrogen, USA) according to the PulseNet protocol (Ribot et al. 2006). BioNumerics software, version 7.5 (Applied Maths, Kortrijk, Belgium) was used to register macrorestriction patterns, clustering analysis was performed using DICE similarity coefficient and the unweighted-pair group method with arithmetic mean (UPGMA) with optimisation and position tolerance set at 0.8% and 0.8%, respectively. *Salmonella* Braenderup strain H9812 was used as molecular weight marker.

3.4.4.4. Detection of virulence associated genes

Genes coding for virulence factors such as virulence plasmid (*spv*C), invasion (*inv*A, *inv*H, *sop*B), enterotoxin (*stn*), genes associated with survival within macrophages (*pho*P, *pho*Q, *sly*A) and with the formation of fimbriae (*agf*A, *sef*A, *saf*C, *pef*A) were evaluated by PCR using primers previously described (Huehn et al. 2009). The Quorum-sensing gene (*sdi*A) was also evaluated by PCR using the primers described (Halatsi et al. 2006). The presence of genes associated with adhesion to Peyer's patches (*gip*A, *lpf*D) was also assessed (Borriello et al. 2012). Genes associated with biofilm formation (*adr*A, *csg*D, *gcp*A) were included in the study as previous published (Seixas et al. 2014). Reproducibility was evaluated by selecting 10% replicates using Research Randomizer (Urbaniak and Plous 2013). *Salmonella enterica* subsp. *enterica* CECT 443 and CECT 722 were used as positive controls for PCR amplifications.

3.4.5. Data Analysis

Descriptive statistics (reported as proportions) were described. Incidence rates by district and by year were estimated dividing the number of reported cases by the district population or by the population country respectively, according Census of 2011, conducted by the National Statistical Institute (Instituto Nacional de Estatística 2011) and multiplied by 100.000.

3.5. Results

Out of the 187 serotyped isolates, 133 (71.1%) were confirmed by mPCR as monophasic. Confirmed *Salmonella* 1,4,[5],12:i:- isolates (n=133) were obtained from human clinical cases (n=125), environmental (n=5) and veterinary (n=3) samples (Table 1). The male/female ratio of the human clinical samples was 1.33 (69 male and 52 female). It wasn't possible to register the patient's gender for four samples and for nine it was not possible to register their age. Distribution included 14 of the 15 districts reported. Three samples were from unknown locations.

Table 1 – Isolation source and year of collection of *Salmonella* 1,4,[5],12:i:- isolates from Portugal, 2006 to the first quarter of 2011

Isolation		2006		2007		2008		2009		2010		2011	
		S	P	S	P	S	P	S	P	S	P	S	P
Environmental (water) and food		1	0	-	-	-	-	1	1	5	4	-	-
Veterinary (Pig, Lymph node)		-	-	-	-	-	-	-	-	10	3	-	-
Human clinical	Feces	8	4	14	8	28	19	36	28	40	33	23	15
	Fluids	1	0	1	1	-	-	1	0	7	6	1	1
	Unknown	-	-	-		-	-	2	2	8	8	-	-
Total		10	4	15	9	28	19	40	31	70	54	24	16

Data include isolates the total number of samples identified by serotyping **(S)** and confirmed isolates by mPCR **(P)**. Fluids include blood, urine and peritoneal fluid.

Salmonella 1,4,[5],12:i:- isolates (n=133) distribution through Portugal, evaluated by district, showed that Porto had the higher number of cases (24.8%), followed by Setúbal (13.5%) and Aveiro (12.8%) (Figure 1). The incidence rates per 100.000 inhabitants were 0.09 in 2006, 0.14 in 2007, 0.27 in 2008, 0.38 in 2009, 0.66 in 2010 and 0.23 in the first quarter of 2011.

Salmonella 1,4,[5],12:i:- isolates (n=133) revealed an ASSuT profile occurrence of 61.6% (n=82). The percentage of districts presenting R-type ASSuT isolates was 78.6%.

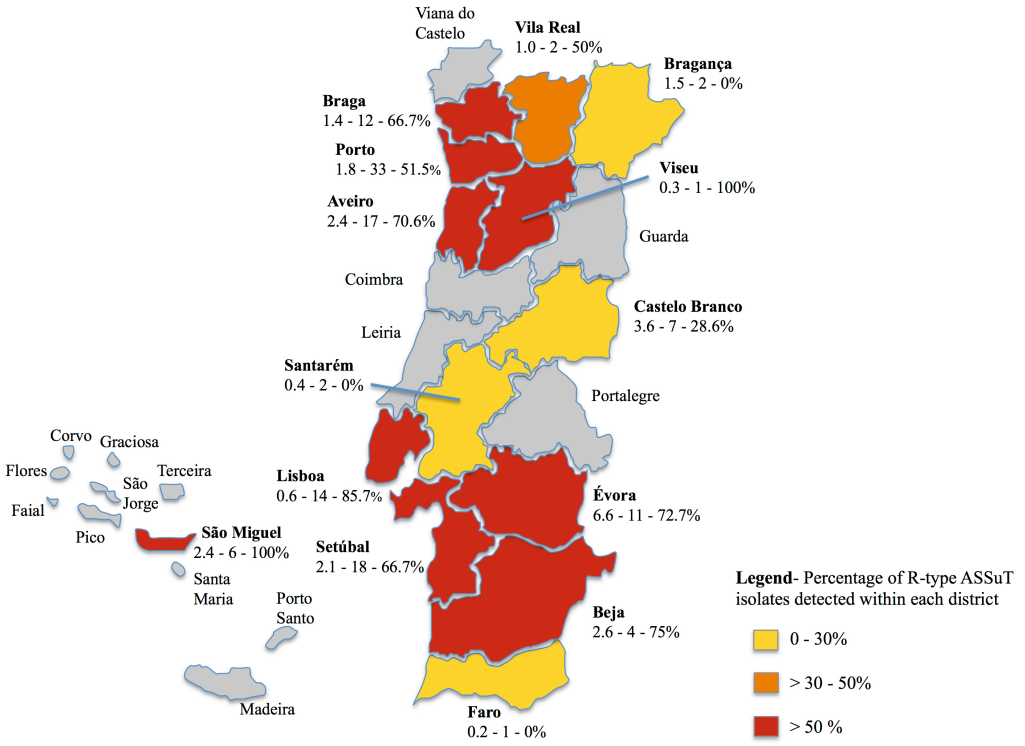
Forty out of the 82 isolates confirmed as R-type ASSuT presented additional resistances to at least one antimicrobial agent (48.8%) but none were resistant to all seven antimicrobials tested. The highest frequencies of additional resistances were observed for amoxicillin (28%, n=23), followed by chloramphenicol (15.9%, n=13), gentamicin (9.8%, n=8), nalidixic acid (8.5%, n=7), ceftazidime (4.9%, n=4), cefotaxime (4.9%, n=4) and ciprofloxacin (1.2%, n=1)

Regarding the resistance profiles of the 82 *Salmonella* 1,4,[5],12:i:- isolates, 18 multiresistant profiles were observed. ASSuT (n=42), AAMCSSuT (n=16) and ACSSuT profiles (n=4) were the three most prevalent profiles. All of the 82 ASSuT isolates, showed the highest MIC for ampicillin (>256 µg/mL) and sulfamethoxazole (>1024 µg/mL). The MIC_{50} value for streptomycin was 512 µg/mL and the MIC_{90} value was 1024 µg/mL. Tetracycline MIC_{50} and MIC_{90} values were 64 µg/mL and 96 µg/mL, respectively.

Almost all the isolates presented antimicrobial resistance genes, including *bla*TEM (93.9%), *sul*2 (95.1%), *stra*A-*stra*B (95.1%) and *tet*B (93.9%). The most common antimicrobial

resistance gene profile was the presence of four determinants, *bla*TEM+*sul*2+*stra*A-*stra*B+*tet*B+ in 72 isolates (87.8%). Other resistance genes profiles detected were *bla*TEM+*sul*2+*stra*A-*stra*B+ in three isolates and *tet*B+ in a further 3 isolates (3.7%), followed by single isolates with one of the following profiles *bla*TEM+*sul*2+*tet*B+, *bla*TEM+*stra*A-*stra*B+, *sul*2+*stra*A-*stra*B+*tet*B+ and *sul*2+*stra*A-*stra*B+ (1.2%).

Figure 1 - Schematic illustration of Portugal representing the distribution of *Salmonella* 1,4,[5],12:i:- R-type ASSuT by district from 2006 to the first quarter of 2011



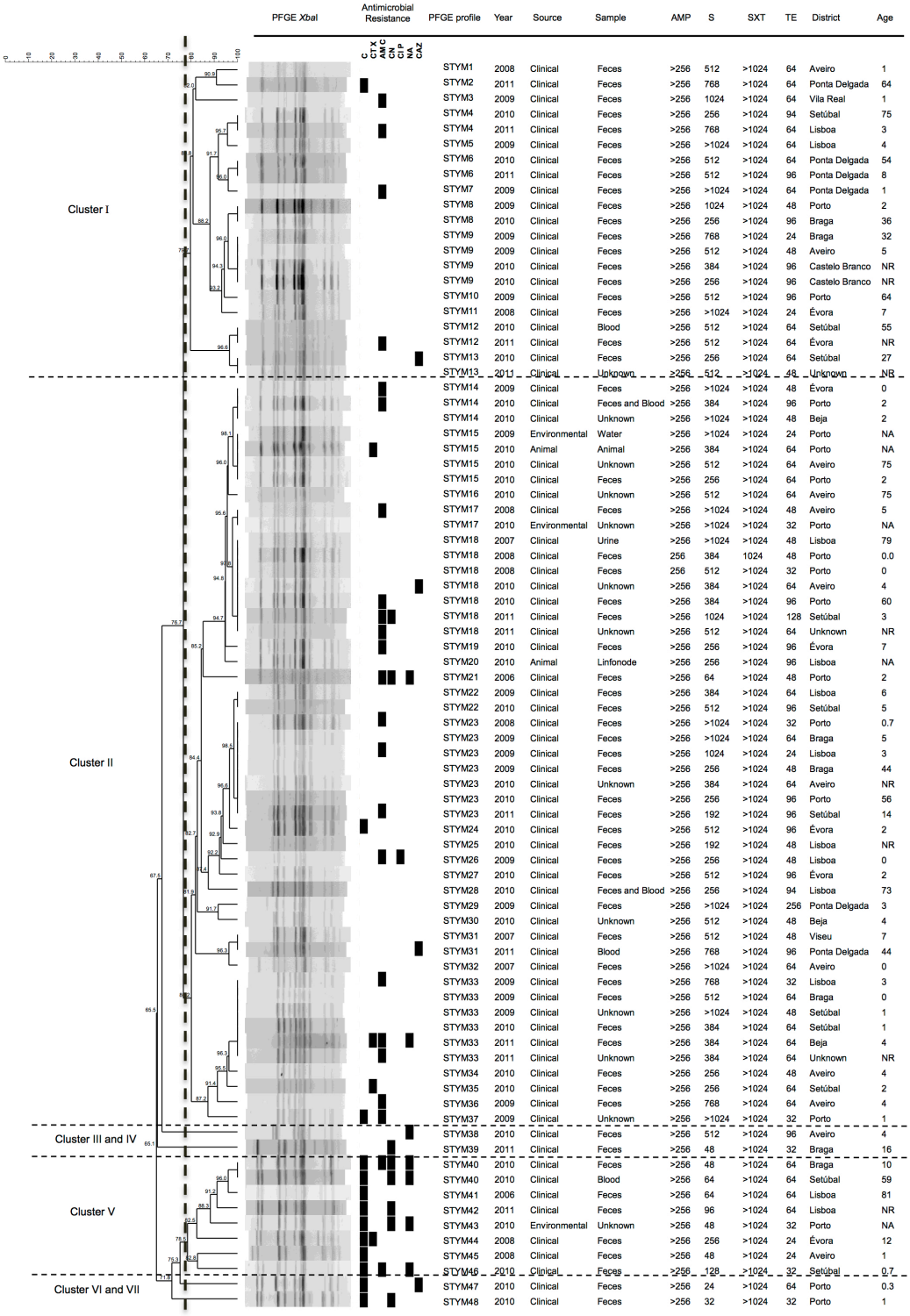
Each district include the following information: Number of *Salmonella* 1,4,[5],12:i:- confirmed isolates per 100.000 inhabitants of the corresponding district/Total number of reported isolates/Percentage of R-type ASSuT isolates detected within each district. For example, Lisboa presents 0.6 confirmed isolates per 100.000 inhabitants – a total of 14 reported isolates – 85.7% of R-type ASSuT isolates detected

PFGE typing identified 48 different PFGE profiles among the 82 isolates examined (Figure 2). Three predominant clones (STYM18; n=7, STYM23; n=7, STYM33; n=6) were identified and correspond to 24.4% of *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates. Cluster analysis allowed grouping of the isolates into 7 clusters at approximately 80% similarity, with 4 clusters including only one isolate.

Virulence gene detection revealed that *pho*P, *pho*Q *inv*A, *inv*H, *sop*B, *stn*, *sly*A *agf*A, *saf*C, *sdi*A, *gip*A, *ipf*D, *adr*A, *csg*D genes were present in 100% (82/82) of the isolates. The remaining virulence genes had different frequencies. However, independent of the sample's year, district and source of isolation, *gcp*A and *spv*C genes were present in 95.1% (78/82) and 67.1% (55/82), respectively. *sef*A and *pef*A genes were not present in any isolate (0/82). Overall, four different virulence profiles were identified (*gcp*A+*spv*C-, *gcp*A-*spv*C+,

*gcp*A+*spv*C+, *gcp*A-*spv*C-), with all isolates showing at least 14 virulence-associated genes out of the 18 genes tested (Table 2).

Figure 2 - Dendrogram based on *Xba*I-PFGE patterns of the Portuguese *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates.



PFGE profile	Year	Source	Sample	AMP	S	SXT	TE	District	Age
STYM1	2008	Clinical	Feces	>256	512	>1024	64	Aveiro	1
STYM2	2011	Clinical	Feces	>256	768	>1024	64	Ponta Delgada	64
STYM3	2009	Clinical	Feces	>256	1024	>1024	64	Vila Real	1
STYM4	2010	Clinical	Feces	>256	256	>1024	94	Setúbal	75
STYM4	2011	Clinical	Feces	>256	768	>1024	64	Lisboa	3
STYM5	2009	Clinical	Feces	>256	>1024	>1024	64	Lisboa	4
STYM6	2010	Clinical	Feces	>256	512	>1024	64	Ponta Delgada	54
STYM6	2011	Clinical	Feces	>256	512	>1024	96	Ponta Delgada	8
STYM7	2009	Clinical	Feces	>256	>1024	>1024	64	Ponta Delgada	1
STYM8	2009	Clinical	Feces	>256	1024	>1024	48	Porto	2
STYM8	2010	Clinical	Feces	>256	256	>1024	96	Braga	36
STYM9	2009	Clinical	Feces	>256	768	>1024	24	Braga	32
STYM9	2009	Clinical	Feces	>256	512	>1024	48	Aveiro	5
STYM9	2010	Clinical	Feces	>256	384	>1024	96	Castelo Branco	NR
STYM9	2010	Clinical	Feces	>256	256	>1024	96	Castelo Branco	NR
STYM10	2009	Clinical	Feces	>256	512	>1024	96	Porto	64
STYM11	2008	Clinical	Feces	>256	>1024	>1024	24	Évora	7
STYM12	2010	Clinical	Blood	>256	512	>1024	64	Setúbal	55
STYM12	2011	Clinical	Feces	>256	512	>1024	64	Évora	NR
STYM13	2010	Clinical	Feces	>256	256	>1024	64	Setúbal	27
STYM13	2011	Clinical	Unknown	>256	512	>1024	96	Unknown	NR
STYM14	2009	Clinical	Feces	>256	>1024	>1024	48	Évora	0
STYM14	2010	Clinical	Feces and Blood	>256	384	>1024	96	Porto	2
STYM14	2010	Clinical	Unknown	>256	>1024	>1024	48	Beja	2
STYM15	2009	Environmental	Water	>256	>1024	>1024	24	Porto	NA
STYM15	2010	Animal	Animal	>256	384	>1024	64	Porto	NA
STYM15	2010	Clinical	Unknown	>256	512	>1024	64	Aveiro	75
STYM15	2010	Clinical	Feces	>256	256	>1024	64	Porto	2
STYM16	2010	Clinical	Unknown	>256	512	>1024	64	Aveiro	75
STYM17	2008	Clinical	Feces	>256	>1024	>1024	48	Aveiro	5
STYM17	2010	Environmental	Unknown	>256	>1024	>1024	32	Porto	NA
STYM18	2007	Clinical	Urine	>256	>1024	>1024	48	Lisboa	79
STYM18	2008	Clinical	Feces	256	384	1024	48	Porto	0.0
STYM18	2008	Clinical	Feces	256	512	>1024	32	Porto	0
STYM18	2010	Clinical	Unknown	>256	384	>1024	64	Aveiro	4
STYM18	2010	Clinical	Feces	>256	384	>1024	96	Porto	60
STYM18	2011	Clinical	Feces	>256	1024	>1024	128	Setúbal	3
STYM18	2011	Clinical	Unknown	>256	512	>1024	64	Unknown	NR
STYM19	2010	Clinical	Feces	>256	256	>1024	96	Évora	7
STYM20	2010	Animal	Linfonode	>256	256	>1024	96	Lisboa	NA
STYM21	2006	Clinical	Feces	>256	64	>1024	48	Porto	2
STYM22	2009	Clinical	Feces	>256	384	>1024	64	Lisboa	6
STYM22	2010	Clinical	Feces	>256	512	>1024	96	Setúbal	5
STYM23	2008	Clinical	Feces	>256	>1024	>1024	32	Porto	0.7
STYM23	2009	Clinical	Feces	>256	>1024	>1024	64	Braga	5
STYM23	2009	Clinical	Feces	>256	1024	>1024	24	Lisboa	3
STYM23	2009	Clinical	Feces	>256	256	>1024	48	Braga	44
STYM23	2010	Clinical	Unknown	>256	384	>1024	64	Aveiro	NR
STYM23	2010	Clinical	Feces	>256	256	>1024	96	Porto	56
STYM23	2011	Clinical	Feces	>256	192	>1024	96	Setúbal	14
STYM24	2010	Clinical	Feces	>256	512	>1024	96	Évora	2
STYM25	2010	Clinical	Feces	>256	192	>1024	48	Lisboa	NR
STYM26	2009	Clinical	Feces	>256	256	>1024	48	Lisboa	0
STYM27	2010	Clinical	Feces	>256	512	>1024	96	Évora	2
STYM28	2010	Clinical	Feces and Blood	>256	256	>1024	94	Lisboa	73
STYM29	2009	Clinical	Feces	>256	>1024	>1024	256	Ponta Delgada	3
STYM30	2010	Clinical	Unknown	>256	512	>1024	48	Beja	4
STYM31	2007	Clinical	Feces	>256	512	>1024	48	Viseu	7
STYM31	2011	Clinical	Blood	>256	768	>1024	96	Ponta Delgada	44
STYM32	2007	Clinical	Feces	>256	>1024	>1024	64	Aveiro	0
STYM33	2009	Clinical	Feces	>256	768	>1024	32	Lisboa	3
STYM33	2009	Clinical	Feces	>256	512	>1024	64	Braga	0
STYM33	2009	Clinical	Unknown	>256	>1024	>1024	48	Setúbal	1
STYM33	2010	Clinical	Feces	>256	384	>1024	64	Setúbal	1
STYM33	2011	Clinical	Feces	>256	384	>1024	64	Beja	4
STYM33	2011	Clinical	Unknown	>256	384	>1024	64	Unknown	NR
STYM34	2010	Clinical	Feces	>256	256	>1024	48	Aveiro	4
STYM35	2010	Clinical	Feces	>256	256	>1024	64	Setúbal	2
STYM36	2009	Clinical	Feces	>256	768	>1024	64	Aveiro	4
STYM37	2009	Clinical	Unknown	>256	>1024	>1024	32	Porto	1
STYM38	2010	Clinical	Feces	>256	512	>1024	96	Aveiro	4
STYM39	2011	Clinical	Feces	>256	48	>1024	32	Braga	16
STYM40	2010	Clinical	Feces	>256	48	>1024	64	Braga	10
STYM40	2010	Clinical	Blood	>256	64	>1024	64	Setúbal	59
STYM41	2006	Clinical	Feces	>256	64	>1024	64	Lisboa	81
STYM42	2011	Clinical	Feces	>256	96	>1024	64	Lisboa	NR
STYM43	2010	Environmental	Unknown	>256	48	>1024	32	Porto	NA
STYM44	2008	Clinical	Feces	>256	256	>1024	24	Évora	12
STYM45	2008	Clinical	Feces	>256	48	>1024	24	Aveiro	1
STYM46	2010	Clinical	Feces	>256	128	>1024	32	Setúbal	0.7
STYM47	2010	Clinical	Feces	>256	24	>1024	64	Porto	0.3
STYM48	2010	Clinical	Feces	>256	32	>1024	32	Porto	1

AMC - amoxicillin/clavulanic acid; AMP – ampicillin; C – chloramphenicol; CAZ – ceftazidime; CIP – ciprofloxacin; CN - gentamicin; CTX – cefotaxime; NA - nalidixic acid; S – streptomycin; SXT – sulfamethoxazole; TE – tetracyclin. NR – Not Registered, NA – Not Applicable.

Table 2 – Biofilm formation, antimicrobial resistance and virulence determinants present in *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates from Portugal, 2006 to the first quarter of 2011

N.º	Year	Source	Additional Resistances	*spv*C	*gcp*D	*bla*TEM	*sul*2	*str*A	*tet*B
272	2008	Clinical		+	+	+	+	+	+
197	2011	Clinical	C	+	+	+	+	+	+
349	2009	Clinical	AMC	+	+	+	+	+	+
127	2010	Clinical		+	+	+	+	+	-
230	2011	Clinical	AMC	+	+	-	-	-	+
299	2009	Clinical		+	+	+	+	+	+
480	2010	Clinical		-	+	+	+	+	+
199	2011	Clinical		-	-	+	-	+	-
546	2009	Clinical	AMC	+	+	+	+	+	+
416	2009	Clinical		+	+	+	+	+	+
231	2010	Clinical		-	+	+	+	+	+
241	2009	Clinical		+	+	+	+	+	+
502	2009	Clinical		+	+	+	+	+	+
301	2010	Clinical		-	+	+	+	+	+
302	2010	Clinical		-	+	+	+	+	+
128	2009	Clinical		+	+	+	+	+	+
698	2008	Clinical		+	+	+	+	+	+
371	2010	Clinical		+	+	+	+	+	+
225	2011	Clinical	AMC	-	+	+	+	+	+
368	2010	Clinical	CAZ	+	+	+	+	+	+
338	2011	Clinical		-	+	+	+	+	+
404	2009	Clinical	AMC	+	+	+	+	+	+
243	2010	Clinical	AMC	-	+	+	+	+	+
22	2010	Clinical		+	-	+	+	+	+
200	2009	Enviro.		-	+	+	+	+	+
340	2010	Animal	CTX	+	+	+	+	+	+
217	2010	Clinical		+	+	+	+	+	+
309	2010	Clinical		+	+	-	+	+	+
331	2010	Clinical		+	+	+	+	+	+
553	2008	Clinical	AMC	+	+	+	+	+	+
111	2010	Enviro.		-	+	+	+	+	+
84	2007	Clinical		+	+	+	+	+	+
145	2008	Clinical		+	+	+	+	+	+
521	2008	Clinical		+	+	+	+	+	+
215	2010	Clinical	CAZ	-	+	+	+	+	+
254	2010	Clinical	AMC	-	+	+	+	+	+
215	2011	Clinical	AMC; CN	-	+	+	+	+	+
332	2011	Clinical	AMC	+	+	+	+	+	+

Table 2 – Biofilm formation, antimicrobial resistance and virulence determinants present in *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates from Portugal, 2006 to the first quarter of 2011 (Continued)

N.º	Year	Source	Additional Resistances	*spv*C	*gcp*D	*bla*TEM	*sul*2	*str*A	*tet*B
271	2010	Clinical	AMC	-	+	+	+	+	+
282	2010	Animal		-	+	+	+	+	+
300	2006	Clinical	AMC; CN; NA	+	+	+	+	+	+
251	2009	Clinical		+	+	+	+	+	+
351	2010	Clinical		-	+	+	+	+	+
665	2008	Clinical	AMC	+	+	+	+	+	+
217	2009	Clinical		+	+	+	+	+	+
335	2009	Clinical	AMC	+	+	+	+	+	+
384	2009	Clinical		+	+	+	+	+	+
173	2010	Clinical		+	+	+	+	+	+
413	2010	Clinical		+	+	+	+	+	+
192	2011	Clinical	AMC	-	+	+	+	+	+
341	2010	Clinical	C	+	+	+	+	+	+
431	2010	Clinical		+	+	+	+	+	+
325	2009	Clinical	AMC; CIP	+	+	+	+	+	+
319	2010	Clinical		-	+	+	+	+	+
186	2010	Clinical		-	+	+	+	+	+
547	2009	Clinical		+	+	+	+	+	+
68	2010	Clinical		+	+	+	+	+	+
115	2007	Clinical		+	+	+	+	+	+
103	2011	Clinical	CAZ	-	+	+	+	+	+
146	2007	Clinical		+	+	+	+	+	+
326	2009	Clinical	AMC	+	+	+	+	+	+
587	2009	Clinical		+	+	+	+	+	+
644	2009	Clinical		+	+	+	+	+	+
347	2010	Clinical		+	+	+	+	+	+
159	2011	Clinical	AMC; CTX; NA	+	+	+	+	+	+
328	2011	Clinical	AMC	+	+	+	+	+	+
94	2010	Clinical		-	+	+	+	+	+
379	2010	Clinical	CTX	-	+	+	+	+	-
503	2009	Clinical	AMC	+	+	+	+	+	+
601	2009	Clinical	C; AMC	+	+	+	+	+	+
81	2010	Clinical	NA	+	+	+	+	+	+
156	2011	Clinical	CN	-	+	+	+	+	+
233	2010	Clinical	AMC; C; CN; NA	+	+	+	+	+	+
358	2010	Clinical	C; CN; NA	-	+	+	+	+	+
292	2006	Clinical	C	+	+	+	+	+	-
122	2011	Clinical	C; CN	+	-	-	-	-	+
119	2010	Enviro.	C; CN; NA	+	-	+	+	-	+
472	2008	Clinical	C; CTX	+	+	+	+	+	+

Table 2 – Biofilm formation, antimicrobial resistance and virulence determinants present in *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates from Portugal, 2006 to the first quarter of 2011 (Continued)

N.º	Year	Source	Additional Resistances	*spv*C	*gcp*D	*bla*TEM	*sul*2	*str*A	*tet*B
263	2008	Clinical	C	+	+	+	+	+	+
122	2010	Clinical	C; AMC; NA	+	-	-	-	-	+
321	2010	Clinical	C; CAZ	-	+	-	-	-	+
307	2010	Clinical	C; CN	+	+	+	+	+	+

Enviro. – Environmental; AMC - amoxicillin/clavulanic acid; AMP – ampicillin; C – chloramphenicol; CAZ – ceftazidime; CIP – ciprofloxacin; CN - gentamicin; CTX – cefotaxime; NA - nalidixic acid; S – streptomycin; SXT – sulfamethoxazole; TE – tetracyclin.

3.6. Discussion

A rapid increase in *Salmonella* 1,4,[5],12:i:- was observed on an international scale, becoming the third most common serovar in humans in 2013 in the EU (EFSA and ECDC 2015). Accurate identification of serovar 1,4,[5],12:i:- is important, since misclassification could result in unnecessary regulatory actions. In fact, serotyping alone can lead to misidentifications and should be combined with mPCR, as recommend by EFSA for identification of *Salmonella* Typhimurium and 1,4,[5],12:i:-, two serovars included in European regulations aiming the reduction of *Salmonella* prevalence in poultry (EFSA 2010).

According to our results, 71.1% of isolates serotyped as 1,4,[5],12:i:- were confirmed as monophasic variants by PCR assays. Several studies reported similar or higher percentages: 71.4% (Tavechio et al. 2004), 82% (Mandilara et al. 2013), 90.3% (Barco et al. 2011), 91.7% (Wasyl and Hoszowski 2012), 94.2% (Lailler et al. 2013) and 95.1% (Hopkins et al. 2010). Almost all *Salmonella* 1,4,[5],12:i:- isolates (28.3%) that weren't confirmed as monophasic by mPCR, represent isolates which the second flagellar phase was not detected by serotyping but was detected by PCR. This can be explained by deletions in fljB gene that don't compromise the primer binding sites but impair the phase inversion, leading to misidentifications in serotyping (Barco et al. 2011). One remaining isolate (0.6%), didn't produce the 1 kb amplicon, which could be attributed to the complete loss of the IS200 fragment (Barco et al. 2011).

The rapid dissemination of this serovar is allied with an escalating incidence of antimicrobial resistant in 1,4,[5],12:i:- strains (Antunes et al. 2011). The ASSuT tetraresistance pattern, already identified in many European countries (Lucarelli et al. 2010) was detected in 61.6% of the isolates included in this study, which was identical to another study where the occurence of ASSuT isolates was 60% (Hopkins et al. 2012). In this study, similar to others reports (Mulvey et al. 2013; Wasyl and Hoszowski 2012), ASSuT was the most frequent R-type detected and resistance to amoxicillin and chloramphenicol were also additional resistances frequently identified. For many years, ampicillin or trimethoprim-sulfamethoxazole were recommended as antimicrobial agents for the treatment of severe

Salmonella human infections, which has contributed for the increased resistance levels and reduced efficacy of these agents (Winokur et al. 2000). In this study, resistance to ampicillin and trimethoprim-sulfamethoxazole was also observed, and tetracycline was the most active of the four antimicrobial compounds tested.

Distribution for Portuguese districts showed a higher percentage of *Salmonella* 1,4,[5],12:i:- isolates in coastal areas, namely Porto, Setúbal and Aveiro. Most districts have reports of *Salmonella* 1,4,[5],12:i:-, including the Portuguese islands Azores, which suggests a wide distribution from the north to south of the country with a higher prevalence in the west, which may be explained by the higher human population density in these areas. Although in the period of this study an increasing annual incidence was observed, the number of reported isolates is low, which may be attributed to a decreasing in the amount of salmonellosis cases reported in the EU in this period, including Portugal (EFSA 2010). However, it is of concern that more than half of the reported cases in most districts are promoted by isolates presenting the ASSuT pattern. This is critical issue for public health safety since ASSuT isolates are most frequently collected from young people (72.1%), which are more susceptible, and may lead to severe infections (EFSA 2010; Ke et al. 2014).

In our study, quinolone and 3rd generation cephalosporin resistance were low. Nevertheless, resistance to ciprofloxacin, cefotaxime and ceftazidime were mostly detected in isolates from young people, as already observed for the R-type ASSuT. Although most of *Salmonella* gastrointestinal infections are self-limiting and may not require antimicrobial therapy, treatment of complicated infections may be difficult due to high levels of resistance to frequently used antimicrobial compounds. Therefore, resistance to fluoroquinolones and 3rd generation cephalosporins can pose a major challenge to clinicians for the effective control of these infections, particularly relevant in young people (Ke et al. 2014).

Taking together with previous published results regarding biofilm formation (Seixas et al. 2014), it is particularly important to point out that almost all isolates resistant to fluoroquinolones and 3rd generation cephalosporins were also moderate biofilm producers, harboring the gene *gcp*A gene responsible to form biofilm under low nutrient conditions.

The molecular analysis of antibiotic resistance in R-type ASSuT isolates demonstrated that almost all these isolates, regardless of their origin, harbored the same resistance genes, *bla*TEM, *str*A-*str*B, *sul*2, and *tet*B, commonly found in several studies (Antunes et al. 2011; Hopkins et al. 2010; Lucarelli et al. 2010). These genes are present in a chromosomal resistance island and are typically associated to the European clone (Lucarelli et al. 2010), suggesting that the Portuguese clones may be related to those present in other European countries (Hopkins et al. 2010). Recently, a study demonstrated the high prevalence of metal tolerance, especially to silver and cooper, associated with antimicrobial resistant determinants in *Salmonella* 1,4,[5],12:i:- from Portugal, which may facilitate the dissemination of these isolates (Mourão et al. 2015).

The dissemination in Portugal may be also due to a relatively high diversity of clones during the study period, also detected in others reports, even among isolates from a single country (Mandilara et al. 2013; Soyer et al. 2009). PFGE identified 48 profiles with no relation to the epidemiological data, which indicates that human cases were most likely sporadic and that the associated strains were not responsible for outbreaks. The unrelated epidemiological background of all cases supports this hypothesis. Interestingly, cluster analysis of PFGE profiles showed that all isolates belonging to cluster V were resistant to chloramphenicol, also including isolates with the highest rates of antimicrobial resistance with most isolates displaying additional resistances to at least two or three antimicrobials. Bionumerics cluster analysis showed that ASSuT and ACSSuT-resistant isolates were mainly included in two different clusters with similarity level of 70% each, which may suggest that the ASSuT-resistant isolates belong to a same clonal lineage different from that of the ACSSuT isolates.

The high genetic diversity does not relate to the low variability of the virulence gene repertoire of the *Salmonella* 1,4,[5],12:i:- isolates, since only four different virulotypes were observed, also demonstrated in other report (Capuano et al. 2013). This can be explained by the fact that most isolates were collected from humans and therefore, in order to promote invasive salmonellosis, it is required a common core of virulence genes (Suez et al. 2013). Additionally the gene spvC, frequently identified in isolates that cause systemic infections in immunocompromised patients (Guiney and Fierer 2011) was detected in some isolates expressing resistance to fluoroquinolones and 3rd generation cephalosporins, which is particularly relevant in the clinical context.

3.7. Conclusions

Salmonella 1,4,[5],12:i:- is an emerging pathogen worldwide. In Portugal, although the number of reports were low, there was an increasing incidence of *Salmonella* 1,4,[5],12:i:- cases during this 5-year study. It was observed a wide distribution through the country, with reports of the R-type ASSuT in the majority of districts.

This rapid spread of *Salmonella* 1,4,[5],12:i:- R-type ASSuT in Portugal might be related with the diversity of pulsotypes, the advantage offered by R-type ASSuT and the presence of a core of virulence genes, also along with biofilm formation.

Close surveillance of *Salmonella* 1,4,[5],12:i:- and its resistance patterns, especially important for young people, as revealed in this report, may help to prevent outbreaks and dissemination to non-problematic districts, to track potential transmission pathways and to rationalize salmonellosis antimicrobial therapeutics.

Disclosure Statement

The authors declare no competing interests.

Acknowledgments

This study was conducted with the financial support of the “Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária da Universidade de Lisboa” - Project UID/CVT/00276/2013 (funded by Foundation for Science and Technology - FCT). Rui Seixas and Raquel Santos hold a PhD fellowship (SFRH/BD/75836/2011 and SFRH/BD/100571/2014, respectively) from FCT, Portugal.

References

Antunes P, Mourão J, Pestana N, Peixe L. Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms. J Antimicrob Chemother 2011; 66:2028-32.

Barco L, Lettini AA, Ramon E, Longo A, Saccardin C, Pozza MC, Ricci A. A rapid and sensitive method to identify and differentiate *Salmonella enterica* serotype Typhimurium and *Salmonella enterica* serotype 4,[5],12:i:- by combining traditional serotyping and multiplex polymerase chain reaction. Foodborne Pathog Dis 2011; 8:741-3.

Borriello G, Lucibelli M, Pesciaroli M, Carullo M, Graziani C, Ammendola S, Battistoni A, Ercolini D, Pasquali P, Galiero G. Diversity of *Salmonella* spp. serovars isolated from the intestines of water buffalo calves with gastroenteritis. BMC Vet Res 2012; 8:1-9.

Capuano F, Mancusi A, Capparelli R, Esposito S, Proroga YT. Characterization of drug resistance and virulotypes of *Salmonella* strains isolated from food and humans. Foodborne Pathog Dis 2013; 10:963-8.

CLSI. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. Wayne, PA 2012.

EFSA. Panel on Biological Hazards: Scientific Opinion on monitoring and assessment of the public health risk of "*Salmonella* Typhimurium-like" strains. EFSA Journal 2010; 8:1826-74.

EFSA and ECDC. The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013. EFSA Journal 2015; 13:3991.

Grimont PAD, Weill FX. Antigenic formulae of the *Salmonella* serovars, 9th edition. Paris, France.: WHO Collaborating Center for Reference and Research on *Salmonella*, Institute Pasteur, 2007.

Guiney DG, Fierer J. The Role of the spv Genes in *Salmonella* Pathogenesis. Front Microbiol 2011; 14:129.

Halatsi K, Oikonomou I, Lambiri M, Mandilara G, Vatopoulos A, Kyriacou A. PCR detection of *Salmonella* spp. using primers targeting the quorum sensing gene sdiA. FEMS Microbiol Lett 2006 259:201-7.

Hopkins K, de Pinna E, Wain J. Prevalence of *Salmonella enterica* serovar 4,[5],12:i:- in England and Wales, 2010. Euro Surveill 2012; 17:pii=20275.

Hopkins KL, Kirchner M, Guerra B, Granier S, Lucarelli C, Porrero MC, Jakubczak A, Threlfall EJ, Mevius DJ. Multiresistant *Salmonella enterica* serovar 4,[5],12:i:- in Europe: a new pandemic strain? Euro Surveill 2010; 15:pii=19580.

Huehn S, Bunge C, Junker E, Helmuth R, Malorny B. Poultry-associated *Salmonella enterica* subsp. *enterica* serovar 4,12:d:- reveals high clonality and a distinct pathogenicity gene repertoire. Appl Environ Microbiol 2009; 75:1011-20.

Instituto Nacional de Estatística. Census 2011 - XV recenciamento geral da população. 2011. Acessed on 8th June, 2016. Available at http://censos.ine.pt.

Ke B, Sun J, He D, Li X, Liang Z, Ke C. Serovar distribution, antimicrobial resistance profiles, and PFGE typing of *Salmonella enterica* strains isolated from 2007–2012 in Guangdong,China. BMC Infect Dis 2014; 14.

Lailler R, Grout J, Marault M, Oudart C, Moury F, Brisabois A. Molecular confirmation method for monophasic and non-motile variant strains of *Salmonella* serovar Typhimurium. Euro Reference 2013; 11.

Lucarelli C, Dionisi AM, Filetici E, Owczarek S, Luzzi I, Villa L. Nucleotide sequence of the chromosomal region conferring multidrug resistance (R-type ASSuT) in *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium strains. J Antimicrob Chemother 2012; 67:111-4.

Lucarelli C, Dionisi AM, Torpdahl M, Villa L, Graziani C, Hopkins K, Threlfall J, Caprioli A, Luzzi I. Evidence for a second genomic island conferring multidrug resistance in a clonal group of strains of *Salmonella enterica* serovar Typhimurium and its monophasic variant circulating in Italy, Denmark, and the United Kingdom. J Clin Microbiol 2010; 48:2103-9.

Mandilara G, Lambiri M, Polemis M, Passiotou M, Vatopoulos A. Phenotypic and molecular characterisation of multiresistant monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) in Greece, 2006 to 2011. Euro Surveill 2013; 18:pii=20496.

Mourão J, Novais C, Machado J, Peixe L, Antunes P. Metal tolerance in emerging clinically relevant multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- clones circulating in Europe. Int J Antimicrob Agents 2015; 45:610-6.

Mulvey M, Finley R, Allen V, Ang L, Bekal S, El Bailey S, Haldane D, Hoang L, Horsman G, Louie M, Robberts L, Wylie J, McCracken M, Langner S, Ahmed R, Tabor H, Gilmour M. Emergence of multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- involving human cases in Canada: results from the Canadian Integrated Program on Antimicrobial Resistance Surveillance (CIPARS), 2003-10. J Antimicrob Chemother 2013; 68:1982-6.

Pitcher DG, Saunders N, Owen R. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 1989; 8:151-6.

Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis 2006; 3:59-67.

Seixas R, Machado J, Bernardo F, Vilela C, Oliveira M. Biofilm formation by *Salmonella enterica* serovar 1,4,[5],12:i:- Portuguese isolates: a phenotypic, genotypic, and socio-geographic analysis. Curr Microbiol 2014; 68:670-7.

Soyer Y, Moreno SA, Davis MA, Maurer J, McDonough PL, Schoonmaker-Bopp DJ, Dumas NB, Root T, Warnick LD, Gröhn YT, Wiedmann M. *Salmonella enterica* serotype 4,5,12:i:-, an emerging *Salmonella* serotype that represents multiple distinct clones. Journal of Clinical Microbiology 2009; 47:3546-56.

Steenackers H, Hermans K, Vanderleyden J, De Keersmaecke SCJ. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. Food Res Int 2012; 45:502-31.

Suez J, Porwollik S, Dagan A, Marzel A, Schorr YI, Desai PT, Agmon V, McClelland M, Rahav G, Gal-Mor O. Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. PLoS One 2013; 8:e58449.

Switt A, Soyer Y, Warnick LD, Wiedmann M. Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i:-. Foodborne Pathog Dis 2009; 6:407-15.

Tavechio AT, Ghilardi ACR, Fernandes SA. Multiplex PCR identification of the atypical and monophasic *Salmonella enterica* subsp. *enterica* serotype 1,4,[5],12:i:- in São Paulo State, Brazil: frequency and antibiotic resistance patterns. Rev Inst Med trop S Paulo 2004; 46:115-7.

Tennant SM, Diallo S, Levy H, Livio S, Sow SO, Tapia M, Fields PI, Mikoleit M, Tamboura B, Kotloff KL, Nataro JP, Galen JE, Levine MM. Identification by PCR of non-typhoidal *Salmonella enterica* serovars associated with invasive infections among febrile patients in Mali. PLoS Negl Trop Dis 2010; 4:e621.

Urbaniak GC, Plous S. Research Randomizer (Version 4.0) [Computer software]. 2013. Acessed on 12th January, 2015. Available at http://www.randomizer.org/.

Wasyl D, Hoszowski A. Occurrence and characterization of monophasic *Salmonella enterica* serovar Typhimurium (1,4,[5],12:i:-) of non-human origin in Poland. Foodborne Pathog Dis 2012; 9:1037-43.

WHO Global Salm-Surv. A global *Salmonella* surveillance and laboratory support project of the WHO - Laboratory protocols. Agar diffusion using E-test. Hendriksen RS, (ed.): WHO, 2003, pp 1-11.

Winokur P, Brueggemann A, DeSalvo D, Hoffmann L, Apley M, Uhlenhopp E, Pfaller M, Doern G. Animal and human multidrug-resistant, cephalosporin-resistant *Salmonella* isolates expressing a plasmid-mediated CMY-2 AmpC beta-lactamase. Antimicrob Agents Chemother 2000; 44:2777-83.

CHAPTER 4

Biofilm formation by* Salmonella *enterica serovar 1,4,[5],12:i:- Portuguese isolates: a phenotypic, genotypic and socio-geographic analysis

4.1. Biofilm formation by *Salmonella enterica* serovar 1,4,[5],12:i:- Portuguese isolates: a phenotypic, genotypic and socio-geographic analysis

Rui Seixas* • Jorge Machado • Fernando Bernardo • Cristina Vilela • Manuela Oliveira

Paper published in *Current Microbiology* 2014 May;68(5):670-7.

* The author contributed in the conception and design of the study, conducted the experiments, performed the data analysis process and drafted the manuscript.

4.2. Abstract

Biofilm-forming ability is well established as an important virulence factor. However, there are no studies available regarding biofilm formation of *Salmonella* Typhimurium 1,4,[5],12:i:-, the new pandemic serovar in Europe. To address this problem, biofilm expression by *Salmonella* 1,4,[5],12:i:- was evaluated using 133 isolates from clinical, environmental and animal origins, collected in Portugal from 2006 to 2011. Biofilm detection was performed by phenotypic and genotypic methods, such growth characterization in agar and broth medium, optical density determination by microtiter assays and direct observation by fluorescent in situ hybridization. Biofilm-related genes *adr*A, *csg*D and *gcp*A were detected by PCR. A socio-geographic characterization of strains as biofilm producers was also performed. Results showed that biofilm formation in monophasic *Salmonella* is widely distributed in Portuguese isolates and could be one of the reasons for its dissemination in this country. Biofilm expression varies between locations, showing that isolates from some regions like Lisboa or Ponta Delgada have an increased ability to persist in the environment due to an enhanced biofilm production. Biofilm formation also varies between risk groups, with a higher prevalence in isolates from salmonellosis infections in women. Therefore, the analysis of the socio-geographic distribution of biofilm-forming bacteria should be considered for the establishment of more adequate regulatory measures or therapeutics regimens, especially important due to the continuous increase of infections caused by antimicrobial resistant microorganisms.

Keywords: Biofilm, *Salmonella* 1,4,[5],12:i:-, Portugal, FISH, microtiter assay

4.3. Introduction

In the mid-1990s, the emergence of a pandemic monophasic variant of *Salmonella* Typhimurium, S. *enterica* subsp. *enterica* serovar 1,4,[5],12:i:-, was reported in Europe. This serotype is now considered one of the main serovars responsible for human salmonellosis worldwide [29].

Biofilm is well established as a major virulence factor in many bacterial species, including *Salmonella* spp., being one of the major reasons for the establishment of chronic infections and for environmental persistence [26]. The most important extracellular components contributing to form biofilm in *Salmonella* include curli fimbriae and cellulose, and their differential expression is responsible for the development of different morphotypes in agar plates supplemented with Congo red and Coomassie brilliant blue. Isolates can be classified into five morphotypes: red, dry and rough (RDAR), able to coexpress curli fimbriae and cellulose; brown, dry and rough (BDAR), able to express fimbriae, but not cellulose; and pink, dry and rough (PDAR), which produce cellulose, but not fimbriae. Isolates that do not express any of these two components are designated as smooth and white (SAW) [3]. Smooth, brown and mucoid (SBAM) isolates are also not able to produce cellulose or curli fimbriae, but in this morphotype biofilm formation is completely dependent on the overproduction of capsular polysaccharide [10, 14].

Salmonella Typhimurium isolates are able to form biofilm on several abiotic and biotic surfaces [13, 15], including in the liquid–air interface [24]. Biofilm formation in *Salmonella* can proceed through different pathways and time ranges and detection may differ according to evaluation time. The synthesis of components involved in biofilm formation by this bacterium is regulated by a highly complex regulatory network, which includes various genes. The *csg*D gene is part of the *csg*DEFG operon, the main control unit in biofilm formation by *Salmonella*, which positively regulates *csg*BA and *adr*A expression [3, 11, 26]. While *csg*BA encodes for the curli subunits *csg*A and *csg*B, the *adr*A gene of *Salmonella* Typhimurium controls the levels of cyclic di-GMP, which regulates cellulose production and consequently biofilm formation. In 2011, Bhowmick et al. [3] described the role of another gene, *gcp*A, in biofilm formation by *Salmonella* Weltevreden under low nutrient conditions.

There are many studies available about biofilm formation by *Salmonella* serovars. However, to the best of our knowledge, there are no reports regarding biofilm production by the pandemic monophasic variant of *Salmonella* Typhimurium. Therefore, the present study aimed to investigate the ability of *Salmonella* 1,4,[5],12:i:- clinical, environmental, and veterinary isolates to express biofilm, to characterize the time course of biofilm production, and to evaluate the presence of genes involved in biofilm formation, using phenotypic and genotypic techniques. This study may provide useful information for the establishment of more adequate therapeutic regimens or disinfection procedures.

4.4. Materials and Methods

4.4.1. Bacterial Isolates Identification and Socio-geographic Characterization

In this study, 133 *Salmonella* 1,4,[5],12:i:- isolates, belonging to a collection from the National Reference Laboratory of Gastrointestinal Infections, National Health Institute Doutor Ricardo Jorge, Lisbon, Portugal, were analyzed. Isolates were obtained throughout the country from 2006 to 2011, from clinical (n = 125), environmental (n = 5), and veterinary (n = 3) samples. Four samples were collected in 2006, 9 in 2007, 19 in 2008, 31 in 2009, 54 in 2010, and 16 in 2011. Clinical samples were obtained from 2006 to 2011; one environmental sample was collected in 2009 and the remaining four in 2010; all animal samples were from 2010. From these samples, 52 belong to women and 69 belong to men. For four samples was not possible to register the patient's gender. Sample distribution included 14 districts (Table 1). Three samples are from unknown locations.

Table 1 – Geographic distribution of biofilm production in Portugal evaluated by microtiter biofilm assay

District Location	Mean	Number of isolates	Std. Deviation
Aveiro	0.868	17	0.079
Beja	0.882	4	0.106
Braga	0.878	12	0.063
Bragança	0.810	2	0.038
Castelo Branco	0.770	7	0.115
Évora	0.830	11	0.103
Faro	0.759	1	NA
Lisboa	0.882	14	0.089
Ponta Delgada	0.969	6	0.113
Porto	0.830	33	0.082
Santarém	0.817	2	0.032
Setúbal	0.859	18	0.089
Vila Real	0.904	2	0.153
Viseu	0.798	1	NA
Unknowm	1.001	3	0.079

NA – Not applicable

All *Salmonella* isolates were serotyped and their identification was confirmed by multiplex PCR using the protocol recommended by EFSA [9].

A biofilm producer isolate, *Salmonella* Enteritidis 3934 [24], was used as a positive control in all phenotypic assays. *Salmonella enterica* subsp. *enterica* CECT 443 was used as positive control for PCR amplification of biofilm-related genes.

4.4.2. Biofilm Formation Assays

4.4.2.1. Curli Fimbriae and Cellulose Phenotypic Expression on Agar Plates

Biofilm phenotypic expression of curli fimbriae and cellulose was evaluated on Luria–Bertani (LB) (Scharlau, Portugal) agar without NaCl supplemented with Congo red (40 mg/mL) (Sigma-Aldrich, USA) and Coomassie brilliant blue (20 mg/mL) (Sigma-Aldrich, USA). Cellulose production was also determined on LB supplemented with calcofluor (20 mg/mL) (Sigma-Aldrich, USA). All plates were incubated for 96h at 28°C. After incubation, colony morphology was registered. For cellulose detection, colony fluorescence was evaluated visually under UV light at 366 nm [12].

All assays were performed in duplicate and repeated twice on different occasions.

4.4.2.2. Biofilm Formation in the Air–Liquid Interface

Biofilm formation in the air–liquid interface was assessed by inoculation of an overnight culture in LB (Scharlau, Portugal) without NaCl (1:10), incubated at 28°C for 8 days. Each isolate was visually examined every day for pellicle formation [30].

All assays were performed in duplicate and repeated twice on different occasions.

4.4.2.3. Evaluation of Biofilm Formation by a Microtiter Biofilm Assay

Assays were performed using flat-bottom, polystyrene, microtiter plates (Orange Scientific, Belgium) containing $5x10^5$ CFU/mL in Mueller–Hinton Broth (MHB) (Liofilchem, Italy). Plates were incubated in a humid chamber at 37°C for 24, 48, and 72h. After each time point, microplates were processed according to the protocol described by Pettit et al. [18] using resazurin (Alamar Blue, AB, ThermoScientific, Spain), and their optical density (OD) values were registered. The OD cut-off (ODc) was defined as three standard deviations above the mean OD of the negative control, and isolates were classified as follows: if O.D. ≤ O.D.c, as non biofilm producers; if O.D.c < O.D. ≤ 2 x O.D.c, as weak biofilm producers; if 2 x O.D.c < O.D. ≤ 4 x O.D.c, as moderate biofilm producers; and if O.D. > 4 x O.D.c, as strong biofilm producers [28].

Assays were performed in triplicate, repeated in three different occasions and results were averaged.

4.4.2.4. Biofilm Detection by Fluorescent *In Situ* Hybridization

Biofilm production by bacterial suspensions was evaluated using a fluorescent *in situ* hybridization (FISH) protocol already described with few modifications [17]. As hybridization supports, ten-well teflon slides (Heinz Herenz, Germany) were used. The slides were previously rinsed in ethanol (Merck, Portugal) and incubated in a 2% 3-trimethoxysilylpropilamine solution (Merck, Portugal) in acetone (Panreac, Spain) for 1 min, followed by two washing steps in acetone for 1 min and one in distilled water. Ten microlitres

of overnight bacterial cultures in Tryptic Soy Broth (TSB) (Liofilchem, Italy), previously diluted at 1:40 in TSB, were placed in each well and incubated in a humid chamber for 24, 48, and 72h at 37°C, to allow biofilm formation. After the incubation period and air-drying, bacterial cultures were fixed with a 4% paraformaldehyde (w/v) (Sigma-Aldrich, USA) solution in PBS for 3h at room temperature. After fixation, bacteria were dehydrated with ethanol at 50, 80, and 96%, during 3 min at each concentration. Afterward, 10 µl of hybridization buffer (0.9 M NaCl, 20 mM Tris–HCl, pH 7.2, 0.01% SDS) was added, containing 5 ng/µl of a 23S rRNA oligonucleotide probe, Sal3, specific for *Salmonella* spp. and labeled with fluorescein in the 50'-end (Stabvida, Portugal) [16]. Slides were incubated in a humid chamber (Omnislide Thermal Cycling Block, Thermoelectron Corporation, USA) at 45°C for 3h. Slides were then washed in a buffer solution (0.9 M NaCl, 20 mM Tris–HCl, pH 7.2, 0.1% SDS) at 45°C during 15 min, mounted in Vectashield Mounting Medium (Vector Laboratories, United Kingdom) and immediately visualized by fluorescent microscopy at 1,000x (objective HCX PLAN APD) in a Leica DMR microscope (Leica Microsystems Lda., Portugal), equipped with a mercury lamp of 100 W.

All assays were performed in duplicate and repeated twice on different occasions.

4.4.3. DNA Extraction and PCR Detection of *adr*A, *csg*D, and *gcp*D

Isolates were plated onto Columbia agar supplemented with 5% sheep blood (BioMérieux, France) and incubated at 37°C for 24h. DNA was extracted from a single colony from an overnight culture using the guanidine thiocyanate method [19]. Primer sequences and PCR amplification protocol were performed as described by Bhowmick et al. [3] with minor modifications. In brief, 25 µl of PCR mix containing 2.5 U of Supreme NZYTaq 2x Green Master Mix (NZYTech, Portugal), 0.4 µM of primers (Stabvida, Portugal), and 0.4 µM of DNA template were submitted to the following PCR conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for *csg*D and *adr*A genes and at 57°C for *gcp*A gene for 1 min, extension at 72°C for 1 min, and a final delay at 72°C for 5 min. Amplified products were analyzed in a 2% agarose gel (Bioline, UK) stained with GelRed (Biotium, Portugal) and visualized by transillumination under UV light (Pharmacia Biotech, Thermal Imaging System FTI-500). NZYDNA ladder VI (NZYTech, Portugal) was used as molecular weight marker.

4.4.4. Statistical Analysis

Statistical analyses were performed using the SPSS 20.0 software (IBM Corporation, New York, USA). Time course of biofilm production by FISH and pellicle formation in the air–liquid interface was evaluated by Cochran's Q test. Association between rough (RDAR/BDAR) or smooth (SAW) morphotypes and pellicle formation was assessed by Pearson Chi-square, and the strength of this association was determined by the Phi Coefficient. Time course by

the microtiter biofilm assay was evaluated by repeated measures (ANOVA), and differences between OD values and morphotypes were determined by the Kruskal–Wallis test.

Comparison between results obtained at the time points by the microtiter biofilm plate assay and FISH was performed using the Cochran's Q test. Association between a biofilm-positive phenotype by the microtiter assay or by FISH and the presence of biofilm related genes was determined by the Binomial test. Differences between biofilm production results determined by the microtiter assay and the groups considered in the socio-geographic evaluation, which included year of sample collection, district location and patient´s gender, were studied by the Kruskal-Wallis test.

Results are presented by mean value ± Standard Deviation. A *p*-value≤0.05 was considered statistically significant.

4.5. Results

4.5.1. Curli fimbriae and cellulose phenotypic expression on agar plates

Three morphotypes were detected on LB agar without NaCl supplemented with Congo red and Coomassie brilliant blue. RDAR was the most frequent morphotype, found in 104 isolates (78.2%). SAW was observed in 24 isolates (18.0%), while BDAR was detected in only 5 isolates (3.8%). Cellulose production on LB supplemented with calcofluor was detected in 104 isolates (78.2%). No other morphotypes, such as PDAR and SBAM, were detected.

4.5.2. Biofilm formation in the air-liquid interface

Pellicle formation was observed in 104 isolates (78.2%) within 8 days of incubation. The percentage of isolates expressing pellicle increased with incubation time: 0.7% (n=1) at day 1; 6.0% (n=8) at day 2; 30.8% (n=41) at day 3, 59.4% (n=79) at day 4, 70.7% (n=94) at day 5, 74.4% (n=99) at day 6, 77.4% (n=103) at day 7, and 78.2% (n=104) at day 8. Most of the isolates expressing the SAW morphotype (83.3%) did not form pellicle in the air-liquid interface (Table 2).

Table 2 - Distribution of pellicle forming isolates among the morphotypes

Morphotype	Day								Total
	Absence of formation	1	2	3	4	5	6	7	8	
BDAR	4	0	0	0	0	0	0	1	0	5
RDAR	6	1	7	33	35	15	4	2	1	104
SAW	19	0	0	0	3	0	1	1	0	24
Total	29	1	7	33	38	15	5	4	1	133

4.5.3. Biofilm detection by FISH

The percentage of biofilm-positive isolates evaluated by FISH increased with incubation time: 38.3% (n=51), 82.0% (n=109) and 98.5% (n=131) of the isolates expressed biofilm at 24, 48 and 72h, respectively. FISH detected biofilm-positive isolates from all three morphotypes. All isolates able to produce curli fimbriae and cellulose (RDAR and BDAR) presented a biofilm-positive phenotype by FISH at 72h. Almost all biofilm-positive isolates expressing SAW were also detected by FISH at the same time point (Table 3).

Table 3 - Number of biofilm-positive isolates determined by FISH at 24h, 48h and 72h

Number of FISH biofilm-positive isolates [n (%)]			
Morphotypes	24 hours	48 hours	72 hours
RDAR	42 (40.4%)	88 (84.6%)	104 (100%)
BDAR	2 (40.0%)	4 (80%)	5 (100%)
SAW	6 (25.0%)	17 (70.8%)	22 (91.7%)
Total	50 (37.6%)	109 (82.0%)	131 (98.5%)

4.5.4. Microtiter biofilm plate assay

O.D. values obtained in this assay ranged from 0.542 to 1.339. Results revealed that all isolates tested produced biofilm in MHB at 24h. O.D. mean values obtained increased with incubation time (0.857±0.145 at 24h, 0.978±0.105 at 48h and 1.044±0.112 at 72h). Classification by O.D. mean values, as described by Stepanović et al. [28], also showed an increase in *Salmonella* biofilm-forming ability over time (Table 4). Isolates presenting the RDAR morphotype had O.D. mean values higher at 24h (0.866±0.097) than the BDAR (0.837±0.093) and SAW (0.823±0.079) isolates.

Table 4 - Biofilm production by 133 monophasic *Salmonella* isolates determined by microtiter plate assay at three time points

Biofilm producing isolates [n (%)]			
	24 hours	48 hours	72 hours
Strong Biofilm producer	0	0	18 (13.5%)
Moderate Biofilm producer	72 (54.1%)	88 (66.2%)	92 (69.2%)
Weak Biofilm producer	61 (45.9%)	45 (33.8%)	23 (17.3%)

4.5.5. PCR detection of *adr*A, *csg*D and *gcp*D

Out of the 133 isolates analyzed, all (100%) presented the *adr*A and *csg*D genes, and 129 isolates (97.0%) were positive for *gcp*A.

4.5.6. Statistical analysis

Pellicle formation at the air-liquid interface was evaluated over 8 days and an increase of pellicle-positive isolates between each time point observed was statistically significant (Cochran's Q test, $P<0.001$). The presence of curli fimbriae and cellulose production was associated with the presence of pellicle formation (Pearson Qui-Square, $P<0.001$). Association between this two variables was strong (Phi Coeficient = 0.682, $P<0.001$).

The increase of biofilm-positive isolates revealed by the FISH protocol was statistically significant between each time point (Cochran's Q test, $P<0.001$).

Regarding the microtiter biofilm assay, differences between results from the three time points were statistically significant (repeated measures ANOVA, $P<0.001$). However, differences between the O.D. mean values from the three morphotypes detected by plate assays were not statistically significant (Kruskal-Wallis test, $P = 0.087$).

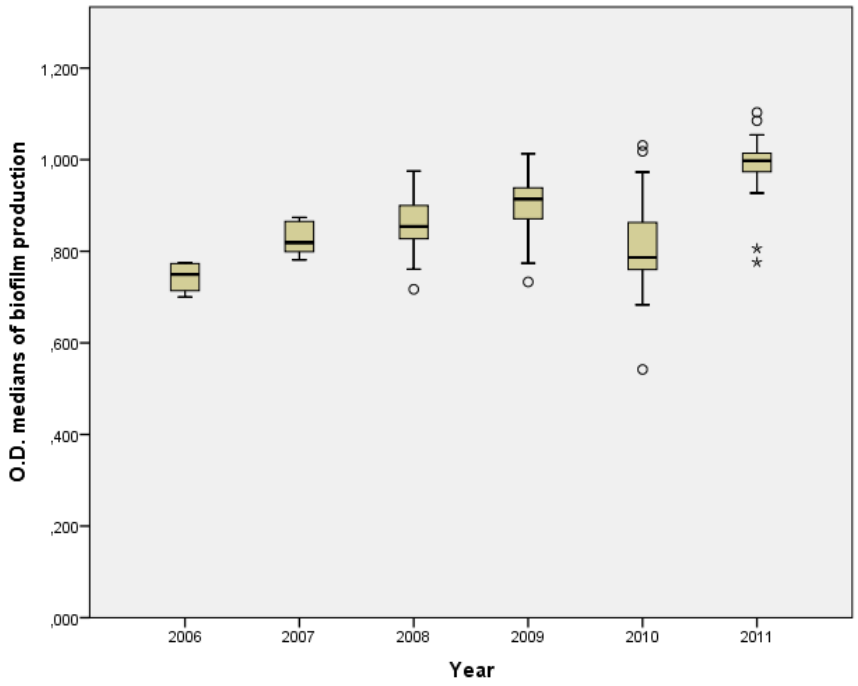
Comparing the results obtained by FISH and by the microtiter biofilm assay with the presence of biofilm related genes, it was observed an association of a FISH biofilm-positive phenotype at 72h with all the genes tested, namely with *adr*A (Binomial test, $P = 0.500$), *csg*D (Binomial test, $P = 1.000$) and *gcp*D (Binomial test, $P = 0.687$). Association of a biofilm-positive phenotype by microtiter biofilm assay and the presence of biofilm related genes were also found for all genes tested.

4.5.7. Socio-geographic characterization of biofilm production

Regarding patients' socio-geographic characterization, biofilm-forming ability at 24h evaluated by the microtiter assay increased along the years of sample collection, with an exception for the year of 2010 (Figure 1). Regarding district distribution of biofilm-positive isolates, it was observed that Ponta Delgada (n=6) and Vila Real (n=2) had higher O.D. mean values for biofilm formation at 24h, 0.969±0.113 and 0.904±0.153, respectively. Differences between sample collection years (Kruskal-Wallis test, $P<0.001$) and locations were statistically significant (Kruskal-Wallis test, $P = 0.033$).

Higher O.D. mean values for biofilm production were found in women (W) isolates in comparison with isolates obtained from men (M) at 24h (W: 0.873±0.084; M: 0.839±0.095), at 48h (W: 0.993±0.095; M: 0.975±0.110) and at 72h (W: 1.039±0.117; M: 1.038±0.118). These differences were statistically significant only at 24h (t-test, $P = 0.048$).

Figure 1 – O.D. medians values of biofilm production according to the year of sample collection.



Error Bars represents standard deviation. Circles and stars represent outliers and extreme outliers (outliers that are farther than tree interquartile ranges from the near edge of the box), respectively.

4.6. Discussion

Biofilm is a major bacterial virulence factor which presence may influence the outcome of an infection [22]. Clinical or environmental isolates expressing biofilm present an increased ability to survive and persist within the host or the environment, decreasing the efficacy of antimicrobial therapy or biocides [5]. Most studies examining biofilm formation in *Salmonella* clinical isolates have predominantly focused upon serovars such as *Salmonella* Typhimurium and *Salmonella* Enteritidis [24,5]. However, to our knowledge, biofilm-forming ability of the pandemic monophasic variant of *Salmonella* Typhimurium has not yet been described. This report aims to evaluate biofilm production by a large collection of Portuguese *Salmonella* 1,4,[5],12:i:- isolates, using several methods applied at different time points.

The most important extracellular components of biofilm matrix in *Salmonella* include curli fimbriae and cellulose. *Salmonella* isolates expressing RDAR morphotype can persist longer in the environment in comparison with other morphotypes such as BDAR or SAW [30]. The RDAR morphotype was the most predominant in the isolates tested, followed by SAW and BDAR. This is in accordance with other studies, which showed a higher prevalence of this morphotype in isolates associated with human salmonellosis [12,20]. Steenackers et al. showed that most isolates expressing SAW morphotype are more invasive, indicating that loss of curli and cellulose production could enhance bacteria's ability to evade host defenses and cause systemic infections [26]. The author described a prevalence of approximately 10% of SAW isolates in a collection of 800 strains of *Salmonella* Typhimurium and *Salmonella*

Enteritidis [26]. In this study, the percentage found was higher (18.0%). Although our collection is smaller (n=133), it is important to mention that is only composed by monophasic *Salmonella* Typhimurium isolates. More than half of RDAR isolates (65.4%) were able to express pellicle formation at day 3 (n=33) and day 4 (n=35); by the contrary, almost all isolates with SAW and BDAR morphotypes were not able to produce this trait or were able to express pellicle only in the last days of the assay (Table 2). This suggests that RDAR isolates have an increased ability to produce pellicle, which may contribute to their persistence in the environment. Similar results were observed in a study regarding *Escherichia coli* isolates from the gastrointestinal tract, which showed that bacteria expressing the RDAR morphotype presented higher biofilm-forming ability, while isolates expressing the SAW morphotype displayed lower biofilm-forming ability [4].

Expression of rough or smooth morphotypes was respectively associated with the presence and absence of pellicle formation in the air-liquid interface, which may indicate that the pellicle formed in LB broth is mainly constituted by curli and cellulose, which is in accordance with other reports [2,21].

Pellicle formation ability is highly disseminated, especially in RDAR isolates. In fact, the percentage of pellicle positive isolates obtained in this study is higher in comparison with results reported by other authors [24,30]. Colonization of air-liquid interfaces by *Salmonella* can be selectively advantageous for aerobic bacteria and may cause serious problems, especially in industrial water systems [23].

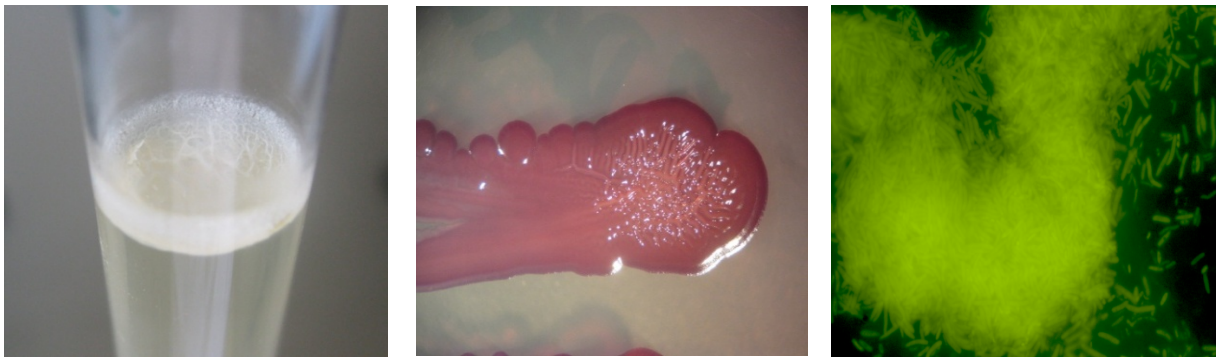
Microtiter assay revealed that these isolates possess a high ability to form biofilm on plastic surfaces, with 100% of biofilm-positive isolates detected by the microtiter assay at 24h. These results are in accordance with previous studies regarding biofilm production by *Salmonella* Typhimurium, which showed that these isolates are able to form biofilm on plastic surfaces [12,28,27]. Biofilm formation classification showed that more than half of the isolates exhibit moderate biofilm producing ability at 24h, being only able to express strong biofilms at 72h. Most of biofilm microtiter studies regarding *Salmonella* serovars, including *Salmonella* Typhimurium, are only performed for 24 or 48h, with isolates revealing strong biofilms after these incubation times [28,27,1,7]. Since biofilm formation in *Salmonella* can be serovar-dependent [15], performing this assay until 72h could demonstrate that monophasic *Salmonella* Typhimurium isolates may express stronger biofilms later than *Salmonella* Typhimurium.

Isolates presenting the RDAR morphotype had O.D. mean values higher at 24h than the BDAR and SAW isolates; however, differences between O.D. mean values from different morphotypes were not statistically significant. Other study also reported no significant differences between these three morphotypes [25].

Besides their capacity to produce biofilm on plastic surfaces, isolates also showed the ability to form biofilm on glass slides, with 38.2% of biofilm positive isolates detected by FISH at

24h (Figure 2). It was observed that it was necessary a 72h incubation for the large majority of the isolates (98.5%) to adhere and form biofilm on glass slides. Differences in biofilm detection results were observed for FISH and the microtiter technique at 24h (38.2% and 100%, respectively) and at 48h (82.0% and 100%, respectively). At 72h both techniques presented similar results (98.5% biofilm positive isolates determined by FISH and 100% by the microtiter plate technique). Although media composition can influence biofilm formation [27], this finding may be explained by the fact that *Salmonella* isolates adhere in higher numbers to hydrophobic materials, such as plastic, in comparison with hydrophilic materials, such as glass [8].

Figure 2 – Multicellular phenotype using different methodologies.



Left - Pellicle formation at day 4. Middle – RDAR morphotype. Right - Biofilm-positive phenotype by FISH protocol. Photo was taken at 1000X magnification under oil immersion.

The FISH protocol was performed for the first time as a high-throughput screening method to study biofilm formation in a large collection of *Salmonella* monophasic isolates, showing that almost all SAW isolates expressed biofilm at 72h (Table 3). At 24 and 48h the percentage of biofilm-positive isolates was lower in SAW when compared with the other two morphotypes. FISH does not produce the same results as the microtiter assay standard technique. However, FISH should be considered an important low cost and high-throughput screening method, especially when evaluating isolates obtained in environments where glass surfaces are relevant for biofilm formation. Microtiter assays are able to detect biofilm production by all SAW isolates at 24h, and it could also be used as a good indicator for the presence of biofilm related genes.

SAW is presented when *csg*D is not expressed, but this doesn´t mean that isolates expressing this morphotype do not form an extracellular matrix that can be detected by other biofilm protocols, such as the microtiter assay. In fact, *Salmonella* can produce other matrix components at 25ºC and 37ºC that have an important role in biofilm formation, such as capsular polysaccharide, and even the production of curli fimbriae can occur upon iron depletion at 37°C [6]. Genes responsible for biofilm formation (*adr*A, *csg*D and *gcp*A) are present in almost all isolates. However, their expression in plate assays was not always observed, which can be explained by the fact that biofilm formation in *Salmonella* is

influenced by environmental conditions, including temperature [23]. The high frequency of *gcp*A may indicate a high ability to form biofilm in low nutrient conditions [3].

Microbial biogeography studies, especially regarding human foodborne pathogens such as *Salmonella*, may contribute to understand the effect of environmental selection upon microbial communities, which is extremely relevant in terms of public health safety. As determined by microtiter assays, biofilm production ability has increased from 2006 (0.743±0,035) to 2011 (0.981±0.085). Since its first report in Europe in the mid-1990s, the presence of this monophasic *Salmonella* has been increasing, being one of the major serovars responsible for human salmonellosis worldwide. The improvement of biofilm-forming ability through the years may be responsible for an increased aptitude to persist in the environment, showing that biofilm could be one of the reasons accountable for the rise of this serovar, not only in Portugal but also worldwide, and also for its higher fitness to persist in the ecosystem.

Considering district distribution, statistically significant differences were found between isolates from different districts, despite the lower number of samples available from some locations (Table 1). Differences in the isolates ability to form biofilm throughout the country may be responsible for differences in the capability to persist in the environment; therefore, regulatory measures should take this fact into consideration and should be adapted to the different locations. Differences regarding biofilm formation at 24h by the two genders were also statistically significant, demonstrating that isolates collected from women have an increased ability to produce biofilm in comparison with isolates obtained from men. Microbial biogeography analysis, especially concerning virulence factors such as biofilm, may allow for the adaptation of antimicrobial therapeutic protocols to different population risk groups.

As far as we know, this is the first study describing biofilm formation by *Salmonella* 1,4,[5],12:i:- isolates, revealing that this virulence trait is widely disseminated in Portugal and can represent an important concern regarding the public health safety.

Acknowledgments

We dedicate this work to Professor Cristina Lobo Vilela, 1958–2013, a major contributor in the research presented in this manuscript. We also like to thank to Cristina Solano from, Instituto de Agrobiotecnología, Idab, Universidad Publica de Navarra-CSIC-Gobierno de Navarra, Pamplona, Spain, for the isolate *Salmonella* Enteritidis 3934. This study was conducted with the financial support of the Interdisciplinary Centre of Research in Animal Health (CIISA) from the Faculty of Veterinary Medicine from the University of Lisbon. Rui Seixas holds a PhD fellowship (SFRH/BD/ 75836/2011) from Foundation for Science and Technology (FCT), Portugal.

References

1.Agarwal RK, Singh S, Bhilegaonkar KN, Singh VP (2011) Optimization of microtitre plate assay for the testing of biofilm formation ability in different *Salmonella* serotypes. Int Food Res J 18 (4):1493-1498

2.Anriany YA, Weiner RM, Johnson JA, De Rezende CE, Joseph SW (2001) *Salmonella enterica* serovar Typhimurium DT104 displays a rugose phenotype. Appl Environ Microbiol 67 (9):4048-4056

3.Bhowmick PP, Devegowda D, Darshanee Ruwandeepika HA, Srikumar S, Karunasagar I, Karunasaga I (2011) *gcp*A (stm1987) is critical for cellulose production and biofilm formation on polystyrene surface by *Salmonella enterica* serovar Weltevreden in both high and low nutrient medium. Microb Pathog 50 (2011):114-122

4.Bokranz W, Wang X, Tschäpe H, Römling U (2005) Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. J Med Microbiol 54 (Pt 12):1171-1182

5.Castelijn GA, van der Veen S, Zwietering MH, Moezelaar R, Abee T (2012) Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* Typhimurium strains of different origin in high and low nutrient medium. Biofouling 28 (1):51-63

6.de Rezende CE, Anriany Y, Carr LE, Joseph SW, Weiner RM (2005) Capsular polysaccharide surrounds smooth and rugose types of *Salmonella enterica* serovar Typhimurium DT104. Appl Environ Microbiol 71 (11):7345-7351

7.Díez-García M, Capita R, Alonso-Calleja C (2012) Influence of serotype on the growth kinetics and the ability to form biofilms of *Salmonella* isolates from poultry. Food Microbiol 31 (2):173-180

8.Donlan RM (2002) Biofilms: microbial life on surfaces. Emerg Infect Dis 8 (9):881-890

9.EFSA Panel on Biological Hazards (2010) Scientific Opinion on monitoring and assessment of the public health risk of "*Salmonella* Typhimurium-like" strains. EFSA Journal 8 (10):1826-1874

10.Jain S, Chen J (2007) Attachment and biofilm formation by various serotypes of *Salmonella* as influenced by cellulose production and thin aggregative fimbriae biosynthesis. J Food Prot 70 (11):2473-2479

11.Jonas K, Tomenius H, Kader A, Normark S, Römling U, Belova LM, Melefors O (2007) Roles of curli, cellulose and BapA in *Salmonella* biofilm morphology studied by atomic force microscopy. BMC Microbiol 7 (70)

12.Karaca B, Akcelik N, Akcelik M (2013) Biofilm-producing abilities of *Salmonella* strains isolated from Turkey. Biologia 68 (1):1-10

13.Ledeboer NA, Jones BD (2005) Exopolysaccharide sugars contribute to biofilm formation by *Salmonella enterica* serovar Typhimurium on HEp-2 cells and chicken intestinal epithelium. J Bacteriol 187 (9):3214−3226.

14.Malcova M, Hradecka H, Karpiskova R, Rychlik I (2008) Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: Identification of a new colony morphology type and the role of SGI1 in biofilm formation. Veterinary Microbiology 129 (2008):360-366

15.Moretro T, Vestby LK, Nesse LL, Storheim SE, Kotlarz K, Langsrud S (2009) Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. J Appl Microbiol 106 (3):1005−1012

16.Oliveira M, Vieira-Pinto M, Martins da Costa P, Vilela CL, Martins C, Bernardo F (2012) Occurrence of *Salmonella* spp. in samples from pigs slaughtered for consumption: A comparison between ISO 6579:2002 and 23S rRNA Fluorescent In Situ Hybridization method. Food Res Int 45 (2):984-988

17.Oliveira M, Bexiga R, Nunes SF, Carneiro C, Cavaco LM, Bernardo F, Vilela CL (2006) Biofilm-forming ability profiling of *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates. Vet Microbiol 118 (1-2):133-140

18.Pettit RK, Weber CA, Kean MJ, Hoffmann H, Pettit GR, Tan R, Franks KS, Horton ML (2005) Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. Antimicrob Agents Chemother 49 (7):2612-2617

19.Pitcher DG, Saunders NA, Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett Appl Microbiol 8:151-156

20.Römling U, Bokranz W, Rabsch W, Zogaj X, Nimtz M, Tschape H (2003) Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. Int J Med Microbiol 293 (4):273−285

21.Römling U, Rohde M, Olsén A, Normark S, Reinköster J (2000) AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* Typhimurium regulates at least two independent pathways. Mol Microbiol 36 (1):10-23

22.Sanchez CJJ, Mende K, Beckius ML, Akers KS, Romano DR, Wenke JC, Murray CK (2013) Biofilm formation by clinical isolates and the implications in chronic infections. BMC Infect Dis 13 (47)

23.Scher K, Romling U, Yaron S (2005) Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol 71 (3):1163-1168.

24.Solano C, García B, Valle J, Berasain C, Ghigo JM, Gamazo C, Lasa I (2002) Genetic analysis of *Salmonella* Enteritidis biofilm formation: critical role of cellulose. Mol Microbiol 43 (3):793-808

25.Solomon EB, Niemira BA, Sapers GM, Annous BA (2005) Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. J Food Prot 68 (5):906-912

26.Steenackers H, Hermans K, Vanderleyden J, De Keersmaecke SCJ (2012) *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. Food Res Int 45 (2012):502-531

27.Stepanović S, Cirkovic I, Mijac V, Svabic-Vlahovic M (2003) Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. Food Microbiol 20 (3):339-343

28.Stepanović S, Cirković I, Ranin L, Svabić-Vlahović M (2004) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 38 (5):428-432

29.Switt A, Soyer Y, Warnick LD, Wiedmann M (2009) Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i:-. Foodborne Pathog Dis 6 (4):407-415. doi:10.1089/fpd.2008.0213

30.Vestby LK, Moretro T, Langsrud S, Heir E, Nesse LL (2009) Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal-and feed factories. BMC Vet Res 5 (20)

CHAPTER 5

Effect of simulated gastrointestinal conditions on biofilm formation by* Salmonella *1,4,[5],12:i:-

5.1. Effect of simulated gastrointestinal conditions on biofilm formation by *Salmonella* 1,4,[5],12:i:-

Rui Seixas • Marta Gabriel • Jorge Machado • Luís Tavares • Fernando Bernardo • Manuela Oliveira

Paper published in ScientificWorldJournal 2014;2014:153956

* The author contributed to the conception and design of the study, conducted the experiments, performed the data analysis process and drafted the manuscript.

5.2. Abstract

Salmonella Typhimurium 1,4,[5],12:i:- is a major serovar responsible for human salmonellosis whose biofilm forming ability, influenced by environmental conditions like those found in the gastrointestinal tract is one of the main contributing factors to its ability to persist in the host and thus one of the main causes of chronic relapsing infections.

Most studies to evaluate biofilm formation are performed in microtiter assays using standard media. However, no reports are available on the ability of this serovar to produce biofilm under *in vitro* simulated gastrointestinal conditions which better correlate to the environment found in the gastrointestinal tract.

To address this, a modified biofilm assay simulating intestinal fluid was conceived to assess the biofilm formation of 133 *Salmonella* Typhimurium 1,4,[5],12:i:- isolates with and without agitation and at three different time points (24h, 48h and 72h). The results were then compared to the existing microtiter method using conventional biofilm growth medium (Mueller Hinton Broth).

Statistical analysis revealed significant differences in the results obtained between the three protocols used. The simulated human intestinal environment impaired biofilm production demonstrating that conditions like pH, agitation or the presence of enzymes can influence biofilm production. Therefore, results from *in vitro* simulation of *in vivo* conditions may contribute to unravel factors relating to biofilm formation and persistence in the context of the human host

Keywords: Biofilm, Gastrointestinal conditions, Microtiter biofilm assay, *Salmonella* Typhimurium 1,4,[5],12:i:-.

5.3. Introduction

The emergence of a pandemic monophasic variant of *Salmonella* Typhimurium, S. *enterica* subsp. *enterica* serovar 1,4,[5],12:i:-, was first reported in Europe in the mid-1990s, and is presently considered to be one of the major serovars responsible for human salmonellosis worldwide [1].

Many studies have demonstrated that *Salmonella* bacteria are capable of forming biofilms on a wide variety of abiotic and biotic surfaces [2, 3]. These highly organized multicellular bacterial structures, responsible for chronic or persistent infections, decrease antimicrobial therapy efficacy and improve resistance to environmental stresses such as desiccation, high temperatures and antiseptics [4, 5].

Since its conception by Christensen and collaborators in 1985, the 96-well microtiter plate test has been the most frequently used assay for high throughput quantitative evaluation of biofilm-forming ability by bacteria [6, 7]. Over the years, modifications have been made to improve its accuracy [8, 9]. It is generally performed under static conditions using different media, such as Mueller Hinton Broth (MHB) or Tryptic Soy Broth (TSB), and enables quantitative biofilm determination through the application of different dyes such as crystal violet, resazurin or dimethyl methylene blue [7, 8].

However these *in vitro* conditions differ greatly from the human intestinal environment, in terms of organic composition (enzymes), pH or dynamics (peristalsis), which is the preferential location for *Salmonella* infection,

Several factors including pH, temperature and media composition [10, 11], affect biofilm formation. We aimed to evaluate the influence of conditions mimicking the intestinal human tract environment on biofilm formation by *Salmonella* Typhimurium 1,4,[5],12:i:- *in vitro*. With these modifications, which better simulate real conditions, we aim to provide a better insight into the influence the gastrointestinal environment has upon the biofilm forming ability of this serovar and ultimately provide more reliable laboratory and clinically relevant results.

5.4. Materials and Methods

5.4.1. Bacterial isolates and identification

In this study, 133 *Salmonella* Typhimurium 1,4,[5],12:i:- isolates, collected in Portugal from 2006 to 2011 from different origins were used. Isolates were obtained from clinical (n=125), environmental (n=5) and animal (n=3) samples. All *Salmonella* isolates were serotyped and identification was confirmed by multiplex PCR as recommended by EFSA (EFSA Panel on Biological Hazards 2010).

5.4.2. Evaluation of biofilm formation by a standard microtiter biofilm assay

Alamar blue (AB) (Thermo Fisher Scientific, Oxford, United Kingdom) biofilm assay was performed according to the protocol described by Pettit et al., (2005) [12], with minor

modifications. Overnight cultures were used to prepare bacterial suspensions with $5x10^5$ CFU/mL in MHB (Liofilchem, Roseto Degli Abruzzi, Italy. Suspensions were placed in flat-bottom, polystyrene, tissue-culture-treated 96-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). Three microtiter-wells were used per isolate. Plates were incubated in a humidity chamber at 37°C without agitation for 24h, 48h and 72h. After each time point, plates were removed from the incubator and 5 µl of AB were added to the wells, gently shaken and incubated for 1h at 37°C, in order to stain the adherent and viable bacteria. Absorbances at 570 nm were determined using a Spectra MAX 340PC microplate reader (Molecular Devices, Sintra, Portugal). All microtiter assays were carried out in triplicate, repeated on three different occasions and the results were averaged.

5.4.3. Biofilm formation under *in vitro* simulated intestinal conditions by a microtiter biofilm assay

5.4.3.1. *In vitro* passage of *Salmonella* Typhimurium 1,4,[5],12:i:- under simulated gastric conditions

Microtiter biofilm assay was also performed using simulated gastrointestinal conditions as described by De Angelis et al., (2006) [13]. Briefly, stationary-phase bacteria grown in 5 mL of TSB were harvested at 6000 g (Hermle Labortechnik, Wehingen, Germany) for 10 min and suspended in 5 mL of simulated gastric fluid which contained NaCl (125 mM/L), KCl (7 mM/L), NaHCO (45 mM/L), and pepsin (3 g/L) (Sigma-Aldrich, St. Louis, USA), pH 3. Bacterial suspensions were submitted to agitation conditions for 180 min with a mini shaker apparatus (VWR, Lisboa, Portugal) at 175 rpm, in order to simulate the passage through the stomach. Aliquots were taken in order to determine the number of colony forming units per mL by measuring Optical Density (O.D.) values, based on standard curves previously determined (data not shown).

5.4.3.2. *In vitro* biofilm formation under simulated intestinal conditions

After gastric digestion, bacteria cells were harvested using the same conditions, washed with 0.9% sterile sodium chloride solution and suspended in simulated intestinal fluid (SIF), containing 0.1% (w/v) pancreatin (AppliChem, Darmstadt, Germany) and 0.15% (w/v) bile bovine (Sigma-Aldrich, St. Louis, USA), pH 8.0 [13].

Then, 100 µl of bacterial suspensions in SIF were incubated in flat-bottom, polystyrene, tissue-culture-treated 96-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). For each isolate, three microtiter wells were used. Plates were incubated in a humidity chamber at 37°C under stationary and agitation conditions with a mini shaker apparatus (VWR, Lisboa, Portugal) at 100 rpm for 24, 48 and 72h and after each time point, plates were removed from the incubator and 5 µl of AB was added to the wells. The plates were then incubated for a further 1h at 37°C. Absorbances at 570 nm were determined using a Spectra

MAX 340PC microplate reader (Molecular Devices, Sintra, Portugal). All microtiter assays were carried out in triplicate, repeated on three different occasions and the results were averaged.

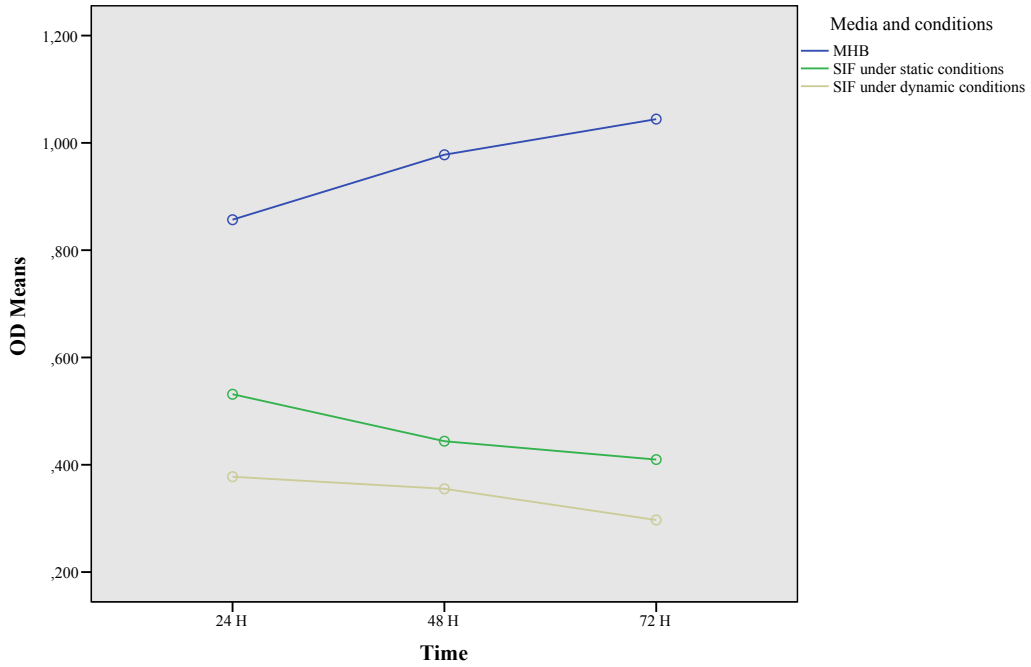
5.4.4. Classification of biofilm-forming ability by microtiter plates

Based on the O.D. and O.D. cut-off (O.D.c) values, isolates were classified into different categories according to their biofilm-forming ability, as previously described by Stepanović et al. 2000 [9]. The O.D. cut-off was defined as three standard deviations above the mean O.D. of the negative control and isolates were classified as follows: if O.D. ≤ O.D.c, isolates were considered to be non biofilm producers; if O.D. ≤ 2 x O.D.c, weak biofilm producers; if 2 x O.D.c < O.D. ≤ 4 x O.D.c, moderate biofilm producers; and if 4 x O.D.c < O.D., strong biofilm producers [14]. AB assays were performed in triplicate, repeated on different occasions and results were averaged. Results are presented as mean value ± Standard Deviation (SD). Statistical analyses were performed using the SPSS 20.0 software (IBM Corporation, New York, USA). Differences between time points and techniques were evaluated by repeated measures ANOVA and one-way ANOVA, respectively. Tukey post hoc tests were used to compare biofilms O.D. mean values. Correlation between CFU/mL after gastric passage and biofilm production at 24h was determined by Pearson coefficient. *P* values ≤ 0.05 were considered statistically significant.

5.5. Results and Discussion

Standard microtiter biofilm assay staining with resazurin (Alamar Blue), a metabolic activity indicator frequently used for quantitative biofilm determination revealed that *Salmonella* Typhimurium 1,4,[5],12:i:- isolates possess a high ability for biofilm formation on plastic surfaces, which is in accordance with previous studies [7, 10, 15]. O.D. mean values in MHB increased over time, it was observed that biofilms with the highest O.D. mean values are produced at 72h (Figure 1). This increase was statistical significant (repeated measures ANOVA, $P \leq 0.001$).

Figure 1: Time course of biofilm production by 133 *Salmonella* Typhimurium 1,4,[5],12:i:- isolates using an Alamar Blue microtiter assay applied in different incubation conditions.



Mean and standard deviation for MHB were at 24h 0,856 ± 0,095, at 48h 0,977 ± 0,105, and at 72h 1,044 ± 0,118. SIF under static conditions were at 24h 0,531 ± 0,217, at 48h 0,443 ± 0,222, and at 72h 0,409 ± 0,146. SIF under agitation conditions were at 24h 0,377 ± 0,136, at 48h 0,355 ± 0,142, and at 72h 0,297.

Following the simulated gastric passage using the modified microtiter biofilm assay, CFU/mL values have a significant positive correlation, although weak, with biofilm production at 24h in SIF under static conditions (Pearson r=0,183, *P* = 0.018) and in SIF under dynamic conditions (Pearson r=0,158, *P* = 0.035). Higher numbers of CFU/mL can lead to a higher biofilm formation, even though the effects of gastric stress conditions on biofilm formation may be strain-specific, as demonstrated by others authors [16].

The largest number of isolates forming weak biofilms was found in SIF under dynamic conditions (83.5% at 24h; 51.1% at 48h and 57.9% at 72h), while the largest number of isolates able to form moderate and strong biofilms was found in MHB at 48h and at 72h (66.2% and 99.2%, respectively) (Table 1). However, 21% of the isolates showed strong biofilm-forming ability at 24h in SIF under static conditions, and this percentage decreased with time (9% at 48h and 3% at 72h). In MHB, more than one third (37.6%) of the *Salmonella* Typhimurium 1,4,[5],12:i:- isolates tested were only able to produce strong biofilms at 72h.

Human gastrointestinal conditions may decrease bacteria's ability to adhere to a substratum, the first step required for biofilm formation and impaired the ability to form strong biofilm [17].

Table 1 - Characterization of biofilm-forming ability of 133 *Salmonella* Typhimurium 1,4,[5],12:i:- isolates using a Alamar Blue microtiter assay applied in different incubation conditions

Biofilm formation ability	Nº. of strains that produced biofilm (n=, %)								
	24h			48h			72h		
	MHB	SIF with static conditions	SIF with dynamic conditions	MHB	SIF with static conditions	SIF with dynamic conditions	MHB	SIF with static conditions	SIF with dynamic conditions
Strong biofilm producer	0.0%	21.8% (n=29)	0.8% (n=1)	0.0% (n=0)	9.0% (n=12)	0.8% (n=1)	37.6% (n=50)	6.8% (n=9)	3.0% (n=4)
Moderate biofilm producer	54.1% (n=72)	42.1% (n=46)	15.8% (n=21)	66.2% (n=88)	39.8% (n=53)	37.6% (n=50)	61.7% (n=82)	52.6% (n=70)	39.1% (n=52)
Weak biofilm producer	45.9% (n=61)	36.1% (n=58)	83.5% (n=111)	33.8% (n=45)	51.1% (n=68)	61.7% (n=82)	0.8% (n=1)	40.6% (n=54)	57.9% (n=77)

MHB, Mueller Hinton Broth; SIF, Simulated Intestinal Fluid.

O.D. mean values of biofilm production in SIF under dynamic conditions decreased significantly with incubation time (repeated measures ANOVA $P \leq 0.001$), and are significantly lower in comparison with static conditions (ANOVA, $P \leq 0.001$), at all the time points studied. This can be explained by the fact that the dynamic conditions applied may have impaired bacterial adhesion and is in accordance with other reports that used dynamic methodologies [11, 17].

Biofilm O.D. mean values obtained in SIF with static conditions, although lower than the ones from MHB, are higher than in SIF with dynamic conditions, these differences are statistically significant (ANOVA, $P \leq 0.001$) showing that conditions, like agitation, have a significant influence on biofilm formation. Dynamics of intestinal peristalsis may strongly influence bacteria's ability to adhere to a surface, and should be included as a parameter during biofilm evaluation, as already stated in previous studies [17].

The decrease in biofilm OD mean values between 48h and 72h at SIF with dynamic condition was significantly higher than in SIF under static conditions (Tukey, $P \leq 0.001$), which can be due to a decrease in the number of viable bacteria. The higher number of dead bacteria cells may be due to a decrease in nutrients together with an accumulation of toxic compounds originating from bacterial metabolism that were disseminated by the agitation conditions during this assay [18].

There were significant differences between results obtained by the three protocols at the three time point evaluated (ANOVA, $P \leq 0.001$), which indicates that intestinal conditions can influence biofilm production by *Salmonella*. White et al. 2008 [19], previously showed that expression of biofilm related genes like curli genes are turned off during *in vivo* infection, but turned on again once the bacteria is shedded into the environment. This may explain why biofilm production is lower in SIF than in MHB, especially if considering the dynamic conditions present in the intestinal tract due to peristalsis.

5.6. Conclusions

The simulated gastrointestinal environment impaired biofilm production by *Salmonella*, demonstrating that conditions simulating those encountered *in vivo* like pH, agitation or the presence of enzymes can influence *in vitro* biofilm formation results, emphasizing the importance of experimental conditions on the results obtained. In conclusion, the provision of dynamic and environmental conditions that better simulate the *in vivo* gastrointestinal stress that *Salmonella* is subjected to, should be included as one of the parameters in the evaluation of biofilm producing strains, enabling a more accurate correlation between *in vitro* biofilm formation and what happens in the gastrointestinal tract. By approximating experimental conditions to those that bacteria encounter in the human host it may be possible to obtain more insight into the real ability and importance of biofilm production when compared with MHB used in standard biofilm assays.

Acknowledgments

The authors would like to thank Dr. Sinclair Owen for the revision of the manuscript. We dedicate this work to Professor Cristina Lobo Vilela, 1958 – 2013, Interdisciplinary Centre of Research in Animal Health (CIISA) / Faculty of Veterinary Medicine from the University of Lisbon. This study was conducted with the financial support of the "Centro de Investigação Interdisciplinar em Sanidade Animal, Faculdade de Medicina Veterinária da Universidade de Lisboa" (CIISA/FMV/ULisboa). Rui Seixas holds a PhD fellowship (SFRH/BD/75836/2011) from FCT, Portugal.

References

[1]. Switt A, Soyer Y, Warnick LD, Wiedmann M: Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i:-. Foodborne Pathog Dis 2009, 6(4):407-415.

[2]. Moretro T, Vestby LK, Nesse LL, Storheim SE, Kotlarz K, Langsrud S: Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. J Appl Microbiol 2009, 106(3):1005−1012.

[3]. Ledeboer NA, Jones BD: Exopolysaccharide sugars contribute to biofilm formation by *Salmonella enterica* serovar Typhimurium on HEp-2 cells and chicken intestinal epithelium. J Bacteriol 2005, 187(9):3214−3226.

[4]. Marin C, Hernandiz A, Lainez M: Biofilm development capacity of *Salmonella* strains isolated in poultry: risk factors and their resistance against disinfectants. Poult Sci 2009, 88:424-431.

[5]. Scher K, Romling U, Yaron S: Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. Appl Environ Microbiol 2005, 71:1163-1168.

[6]. Peeters E, Nelis HJ, Coenye T: Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods 2008, 72(2):157-165.

[7]. Stepanović S, Cirković I, Ranin L, Svabić-Vlahović M: Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 2004, 38(5):428-432.

[8]. Pettit RK, Weber CA, Kean MJ, Hoffmann H, Pettit GR, Tan R, Franks KS, Horton ML: Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. Antimicrob Agents Chemother 2005, 49(7):2612-2617.

[9]. Stepanović S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M: A modified microtiter-plate test for quantification of staphylococcal biofilm formation. J Microbiol Methods 2000, 40(2):175-179.

[10]. Solomon EB, Niemira BA, Sapers GM, Annous BA: Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. J Food Prot 2005, 68(5):906-912.

[11]. Stepanović S, Cirkovic I, Mijac V, Svabic-Vlahovic M: Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. Food Microbiol 2003, 20(3):339-343.

[12]. Pettit RK, Weber CA, Kean MJ, Hoffmann H, Pettit GR, Tan R, Franks KS, Horton ML: Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. Antimicrob Agents Chemother 2005, 49(7):2612-2617.

[13]. De Angelis M, Siragusa S, Berloco M, Caputo L, Settanni L, Alfonsi G, Amerio M, Grandi A, Ragni A, Gobbetti M: Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Res Microbiol 2006, 157(8):792-801.

[14]. Stepanović S, Cirković I, Ranin L, Svabić-Vlahović M: Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. Lett Appl Microbiol 2004, 38(5):428-432.

[15]. Vestby LK, Moretro T, Langsrud S, Heir E, Nesse LL: Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. BMC Vet Res 2009, 5(20).

[16]. Lianou A, Koutsoumanis KP: Strain variability of the biofilm-forming ability of *Salmonella enterica* under various environmental conditions. Int J Food Microbiol 2012, 160(2):171-178.

[17]. Stepanović S, Vukovic D, Jezek P, Pavlovic M, Svabic-Vlahovic M: Influence of dynamic conditions on biofilm formation by staphylococci. Eur J Clin Microbiol Infect Dis 2001, 20(7):502-504.

[18]. Sillankorva S, Neubauer P, Azeredo J: *Pseudomonas fluorescens* biofilms subjected to phage phiIBB-PF7A. BMC Biotechnol 2008, 8(79).

[19]. White AP, Gibson DL, Grassl GA, Kay WW, Finlay BB, Vallance BA, Surette MG: Aggregation via the red, dry, and rough morphotype is not a virulence adaptation in *Salmonella enterica* serovar Typhimurium. Infect Immun 2008, 76 (3):1048−1058.

CHAPTER 6

General Discussion

6.1. Discussion

Salmonellosis is one of the most frequent foodborne diseases. An estimated 93.8 million cases of gastroenteritis caused by *Salmonella* species occur globally each year and among these, nearly 80.3 million cases are foodborne (Majowicz et al. 2010). Recently, a multidrug resistant serovar has emerged, *Salmonella* 1,4,[5],12:i:-, a monophasic variant of *Salmonella* Typhimurium, which is now the third most frequent serovar isolated from humans in many countries (EFSA and ECDC 2015).

In Portugal, *Salmonella* 1,4,[5],12:i:- was isolated for the first time in poultry in 1986 (Machado & Bernardo 1990). It's incidence increased sharply thereafter, and in 2012 was even more frequently detected than *Salmonella* Typhimurium (Silveira, Marques & Machado 2013).

Despite the escalating expansion of this serovar, research on its incidence, characterization and pathogenesis is still scarce. In fact, Pubmed websearch on *Salmonella* 1,4,[5],12:i:- produces under 1.800 results, while *Salmonella* Typhimurium originates 19 times more results. Nowadays, there is still much to discover regarding this monophasic variant, including its epidemiology and distribution, phenotypic and genotypic features, biofilm-forming ability and its relevance in host and environment persistence. These topics were the focus of this thesis, which aims to contribute for unravelling the importance of such isolates in Portugal.

Portugal have locations with a higher risk for human *Salmonella* 1,4,[5],12:i:- infections

Using data provided by INSA, the application of both statistic and geoprocessing methods combined with spatial cluster analysis allowed the definition of high-risk areas in Portugal for human *Salmonella* 1,4,[5],12:i:- infections. In fact, the determination of areas of higher risk of infection for this serovar, or other foodborne pathogens, may be critical for assessing population at risk or for establishing prevention programs.

One of the main findings was the detection of three statistically significant clusters of higher than expected infection rates, two located in the north of Portugal, including one at the coastland (Cluster 1 [RR=3.58, $P \leq 0.001$]) and one at the countryside (Cluster 3 [RR=17.76, $P \leq 0.001$]) and a larger cluster involving the center and south of Portugal (Cluster 2 [RR=4.85, $P \leq 0.001$]).

Custer analysis suggests the occurrence of non-random cases in these regions, which may reveal an increased exposure to human *Salmonella* 1,4,[5],12:i:- infection in these areas. The

risk of infection was determined, being observed that it could be 18 times higher for an individual located inside the clusters compared to individuals outside these areas.

Several reasons may explain the occurrence of these spatial clusters. Although not evaluated in this study, one hypothesis is the association with pig farms, which are prevalent near these locations (Almeida 2008). In fact, there is increasing evidence suggesting pigs act as reservoirs for this serovar (Antunes et al. 2011). Moreover, foodborne outbreaks caused by *Salmonella* 1,4,[5],12:i:- have been reported in Luxembourg in 2006, due to contaminated pork products, and France in 2010 (Mandilara et al. 2013).

Despite the increasing evidence of pigs as a reservoir host, other animals may be also implicated in serovar 1,4,[5],12:i:- dissemination, including cows and wild animals, as pointed out by other reports (Zamperini et al. 2007, Ido et al. 2015). Furthermore, several foodborne outbreaks involved different vehicles have been documented, including a multistate outbreak in the USA in 2011, which was associated to the consumption of contaminated alfalfa sprouts and an outbreak in Denmark in 2012, which was linked to beef (Mandilara et al. 2013). The contamination of irrigation water channels was also suggested to be related in an outbreak in Italy that occurred between 2013 and 2014 (Cito et al. 2016).

Regarding the distribution of this serovar throughout Portugal, most districts have reports of *Salmonella* 1,4,[5],12:i:-; however, data analysed in this study was gathered by a passive monitoring system, which often leads to the underestimation of the number of cases. Comparing with other serovars commonly documented, such as *Salmonella* Enteritidis and Typhimurium, *Salmonella* 1,4,[5],12:i:- isolation reported in Portugal is low, despite the increasing annual incidence observed during the 10-year period considered in this study.

Additionally, a decrease in the total number of *Salmonella* isolates obtained by the Portuguese national laboratory was observed, a trend also observed in many EU countries (EFSA 2010b, Mandilara et al. 2013). This reduction resulted from the decrease of *Salmonella* Enteritidis isolates reported, as the total number of *Salmonella* Typhimurium isolates was relatively consistent over time and *Salmonella* 1,4,[5],12:i:- numbers increased, as already stated. The fact that the reduction of the total number of isolates is mainly attributed to the decrease of *Salmonella* Enteritidis isolation can be related to the successful establishment of *Salmonella* control programs (Mandilara et al. 2013).

In order to increase the efficacy of such programs regarding *Salmonella* 1,4,[5],12:i:-, it is necessary to understand the features of this serovar, in particular the ones promoting infections in humans' populations.

Salmonella 1,4,[5],12:i:- R-type ASSuT isolates are commonly associated with multiple virulent features and a high diversity of pulsotypes

The incidence of infections caused by different *Salmonella* serovars appears to change considerably over time and regarding *Salmonella* 1,4,[5],12:i:-, this issue is even more complex. It is difficult to have a clear characterization of the spread of this emergent serovar because it appears to be underreported due to the lack of a consistent identification method allowing its differentiation from *Salmonella* Typhimurium. At the molecular level, 1,4,[5],12:i:- serovar is very similar to *Salmonella* Typhimurium, being characterised by the lack of *fljB* gene expression, which encodes the second phase flagellar antigen (Switt et al. 2009). The correct identification of *Salmonella* 1,4,[5],12:i:- solely according to traditional serotyping must reveal the detection of somatic antigens of serogroup B and phase-1 flagellar antigen "i", and the repeated phase inversion assays performed must not provide evidence for phase-2 flagellar antigen production.

There are several concerns regarding serotyping. In fact, the number of times that phase inversion assays should be repeated to ensure that the strain is truly monophasic is not standardized; the failure to detect phase-2 antigen due to a low of expression is also a possibility; finally the entire procedure requires several days before allowing to confirm the identification of monophasic variant strains, hampering the timely application of public health measures (EFSA 2010b, Barco et al. 2011).

Identification of *Salmonella* 1,4,[5],12:i:- isolates by molecular methods such as PCR, as recommended by EFSA, is of major importance for guarantying surveillance accuracy, since serotyping alone can lead to misidentifications. Such methods are especially relevant since *Salmonella* Typhimurium and its monophasic variant identification are mandatory by the European regulations aiming at the reduction of the prevalence of *Salmonella* serovars in poultry (Official Journal of the European Union 2003). Our research, as other reports, showed that standard serotyping needs to be combined with multiplex PCR in order to correctly identify and report isolates as *Salmonella* 1,4,[5],12:i:- (Barco et al. 2011)

According to our results, 71.1% of isolates serotyped as 1,4,[5],12:i:- were confirmed as monophasic variants by PCR assays. Several studies from other countries reported similar or higher isolation percentages: 71.4% in Brazil (Tavechio, Ghilardi & Fernandes 2004), 82% in Greece (Mandilara et al. 2013), 90.3% in Italy (Barco et al. 2011), 91.7% in Poland (which also included isolates from the Ukraine and Belarus) (Wasyl & Hoszowski 2012), and 94.2% in France (Lailler et al. 2013).

Regarding virulent features, the most frequent R-type isolated from 30% of the human infection cases and farming animals is the ASSuT tetraresistance pattern, also known as R-Type ASSuT, which shows co-resistance to ampicillin, streptomycin, sulphonamides and

tetracyclines (Lucarelli et al. 2010). Due to the high prevalence of this antimicrobial resistance profile and its scarce reports until 2010, it was relevant to characterize the *Salmonella* 1,4,[5],12:i:- R-type ASSuT Portuguese isolates using phenotypic and genotypic approaches. This characterization was only performed using confirmed monophasic *Salmonella* 1,4,[5],12:i:- isolates by multiplex PCR.

The ASSuT tetraresistance pattern was detected and confirmed by minimal inhibitory concentration (MIC) determination in 61.6% of the Portuguese isolates under study, in a similar percentage observed in another European study (60%) (Hopkins et al. 2012) and in a study performed in Asia (54%) (Yang et al. 2015). This pattern has also been identified in many other countries, including Czech Republic, Denmark, France, Italy, Poland, Spain, United Kingdom and USA (Hopkins et al. 2010, Lucarelli et al. 2010, Hopkins et al. 2012, Mandilara et al. 2013, Madajczak et al. 2014). A report from 2013, including isolates from Canada, described one of the highest occurrence percentage of R-type ASSuT (76.5%) (Mulvey et al. 2013).

In Portugal, the ASSuT pattern is widely disseminated, being present in isolates from 10 of the 14 districts under study. These results point out for the importance isolates regarding public health safety.

The antimicrobial genetic determinants responsible for this resistance phenotype are present in a chromosomal resistance island that frequently includes *bla*TEM, *str*A-*str*B, *sul*2 and *tet*B genes (EFSA 2010b, Ke et al. 2014). Almost 90% of the Portuguese R-type ASSuT isolates tested presented all these genetic determinants, as also documented in several reports (Hopkins et al. 2010, Lucarelli et al. 2010, Antunes et al. 2011), The remaining 10% of the isolates exhibited deletions of one, two or three of these determinants. These genes are typical associated with the European clone, which differs from the Spanish clone by the presence of different antimicrobial resistance determinants and even by their position on the bacterial DNA. Therefore, the Portuguese clones may be related to the European clone, already disseminated in many countries, and to the one which appeared in Italy sixteen years ago (Hopkins et al. 2010).

Recently, it was demonstrated that the horizontal acquisition of this resistance region may be responsible for the monophasic phenotype of *Salmonella* 1,4,[5],12:i:- (García et al. 2016). This study revealed that this region includes genes that replaced the ones responsible for the expression of the second phase flagellar antigen and may represent an example of stabilization of plasmid material, now present in the bacterial chromosome (García et al. 2016).

In addition to the typical R-type ASSut pattern found in *Salmonella* 1,4,[5],12:i:- isolates, additional resistances to quinolones, aminoglycosides or cephalosporins are reported in the European clone (EFSA 2010b). However, the resistance rates of quinolones and fluoroquinolones, in particular nalidixic acid and ciprofloxacin, and also of 3rd generation

cephalosporins, including cefotaxime and ceftazidime, are usually low (Frasson, Bettanello, De Canale, Richter & Palù 2016).

In this study, as well in several others (Mulvey et al. 2013, Frasson et al. 2016), the resistance rates of serovar 1,4,[5],12:i:- human isolates for these antimicrobials does not exceed 10%. Moreover, resistance rates for nalidixic acid are usually higher comparing to ciprofloxacin and 3rd generation cephalosporins (EFSA and ECDC 2014, Ke et al. 2014, Frasson et al. 2016). Similar percentages are also observed in isolates of different origins, including food (Yang et al. 2015) and animal isolates (Tu et al. 2015).

It is important to note that resistance to nalidixic acid, although detected in only seven isolates from these study, is critical, as it may represent a reduced susceptibility to ciprofloxacin (Ryan, Dillon & Adley 2011), which together with 3rd generation cephalosporins, are the most used antimicrobials in the treatment of human salmonelosis. This result highlights the need for continuous monitoring of both nalidixic acid and ciprofloxacin resistance levels in *Salmonella* 1,4,[5],12:i:-, as also recommended by EFSA (EFSA 2010a, Ryan et al. 2011).

Additionally, these tetraresistant isolates should be submitted to genetic discrimination methods in order to determine strain relatedness, unravel epidemic patterns and confirm possible outbreaks. The PFGE technique is acknowledged to be highly discriminative and of epidemiological relevance making it one of the most reliable typing procedures (Wattiau et al. 2011).

Regarding the molecular epidemiology of the Portuguese R-type ASSuT isolates, their population structure is highly diverse. The genetic diversity revealed in this study maybe associated with extensive sampling period of 5 years, also related with the absence of relation with any *Salmonella* 1,4,[5],12:i:- outbreaks (Tavechio et al. 2009). This high diversity of clones was also detected in other European reports, even among isolates from a single country (Soyer et al. 2009, Mandilara et al. 2013). However, three predominant clones (STYM18; n=7, STYM23; n=7, STYM33; n=6) were identified and correspond approximately to 25% of the *Salmonella* 1,4,[5],12:i:- R-type ASSuT isolates.

The high genetic diversity does not relate to the low variability of the virulence gene repertoire of the *Salmonella* 1,4,[5],12:i:- isolates evaluated, since only four different virulotypes were observed (*gcp*A^{+}*spv*C^{-}, *gcp*A^{-}*spv*C^{+}, *gcp*A^{+}*spv*C^{+}, *gcp*A^{-}*spv*C^{-}). The low variability of virulence genes between isolates can be explained by the fact that most isolates were collected from humans and therefore, pointing out for the need of a common core of virulence genes to promote invasive salmonellosis (Suez et al. 2013).

The *inv*A and *sop*B genes, detected in all isolates, are genetic markers for the presence of the *Salmonella* Pathogenicity Islands (SPI) 1 and 5, respectively (Marcus et al. 2000). However, their detection does not necessarily imply the presence of the entire SPI. SPI are genes clusters present in the chromosome, usually associated with enhanced invasion and

intracellular survival. In particular, SPI-5 has been largely associated with the ability to promote enteritis (Marcus et al. 2000)

The *spv*C gene was detected in two-thirds of the isolates (67.1%). It is located at a highly conserved 8kb operon formed by five genes, designated *spv*RABCD, coding for *Salmonella* virulence plasmid (Marcus et al. 2000). A previous report (Guiney & Fierer 2011) showed that isolates collected from systemic infections of immunocompromised patients, frequently carry the *spv* locus. This gene is associated with an increased growth rate in host cells and with the downregulation of cytokine release from infected cells (Mazurkiewicz et al. 2008). The presence of this gene in the Portuguese *Salmonella* 1,4,[5],12:i:- isolates may be responsible for increased virulence, particularly relevant for immunocompromised patients.

The high frequency of *gcp*A (95.1%) may indicate a high ability of 1,4,[5],12:i:- serovar to form biofilm under low nutrient conditions, as already demonstrated for *Salmonella* Weltevreden (Bhowmick et al. 2011).

Biofilm formation in *Salmonella* 1,4,[5],12:i:- may be one of the reasons responsible for the expansion of this serovar in Portugal

It is well established that biofilm formation can play an important role during *Salmonella* infection and persistence, which may change completely the infection prognosis (Steenackers et al. 2012).

Until now, most studies regarding biofilm formation in *Salmonella* have mainly focused upon serovars such as *Salmonella* Typhimurium and *Salmonella* Enteritidis (Castelijn, van der Veen, Zwietering, Moezelaar & Abee 2012, Corcoran et al. 2013, De Oliveira et al. 2014). Biofilm-forming ability evaluation of *Salmonella* 1,4,[5],12:i:- using phenotypic and genotypic approaches has not yet been fully addressed, maybe due to the recent expansion of this serovar or difficulties in its identification.

Many bacteria species are able to form bacterial communities and produce a biofilm matrix, a group of extracellular components consisting of proteins, extracellular DNA and polysaccharides. In *Salmonella,* the most important components of this matrix are curli fimbriae and cellulose, which can be detected by the phenotypic expression of different morphotypes in agar plates (Malcova et al. 2008). Regarding *Salmonella* 1,4,[5],12:i:- Portuguese isolates, the RDAR (red, dry and rough) morphotype, which is able to coexpress curli fimbriae and cellulose is the most predominant one, following by the SAW (smooth and white) morphotype, which is not able to express any of these two components, and by the BDAR (brown, dry and rough) morphotype, able to express fimbriae but not cellulose. Although the RDAR morphotype in *Salmonella* 1,4,[5],12:i:- was described for the first time in this study, results are in accordance with other studies, which showed a higher occurence of

this morphotype in human clinical isolates of *Salmonella*, including *Salmonella* Typhimurium (Römling et al. 2003, Karaca, Akcelik & Akcelik 2013).

RDAR expression in *Salmonella*, and even in other Gram-negative bacteria, including *E.coli*, is associated to an increased environmental persistence capacity (Bokranz, Wang, Tschäpe & Römling 2005). In fact, pellicle formation, which is the formation of biofilm at air–liquid interfaces, characterized by a floating structure that requires a high organization due to the lack of a solid surface for initial attachment, is rapid and highly frequent in RDAR *Salmonella* 1,4,[5],12:i:- isolates, with the majority of the RDAR isolates being able to express pellicle at day 3 and 4 in a 8-day assay.

The ability for pellicle formation may vary between *Salmonella* serovars. One study revealed high frequencies of pellicle formation for serovars Agona (100%), Montevideo (100%) and Senftenberg (88%) and low frequency for serovar Typhimurium (55%). Although *Salmonella* Typhimurium demonstrated a lower ability to form pellicle, in our study the monophasic variant of this servoar revealed a higher ability to produce pellicle, with almost 80% of the isolates being able to express it. It is important to note that both studies were performed with the same conditions, allowing this comparison (Vestby et al. 2009). Additionally, serovar Agona and Montevideo are rapid pellicle producers being able to form pellicle between day 2 and 3, similar to *Salmonella* 1,4,[5],12:i:- isolates, while serovars Senftenberg and Typhimurium are slow pellicle producers (Vestby et al. 2009). Implications of biofilm formation at these interfaces is mainly important to the food processing industry, where pellicle may impair several industrial operations, in particular in water systems, decreasing the water velocity, carrying capacity and clogging of pipes (Scher, Romling & Yaron 2005).

Other authors (Römling et al. 2003, Solomon, Niemira, Sapers & Annous 2005, Steenackers et al. 2012) demonstrated that most isolates expressing SAW morphotype are more invasive, as the loss of abitlity to produce curli and cellulose may enhance bacteria's ability to evade host defenses and cause systemic infections. In this report, one third of isolates resistant to fluoroquinolones, including ciprofloxacin and 3rd generation cephalosporins as ceftazidime or cefotaxime, also expressed the SAW morphotype, which could be important in clinical settings, since they are associated with an enhanced invasiveness (Steenackers et al. 2012, Seixas, Machado, Bernardo, Vilela & Oliveira 2014b).

Biofilm-forming ability is frequently evaluated using a high-throughput experimental setup based on 96-well polystyrene microtiter plates wells (Stepanović et al. 2004, Vestby et al. 2009, Mishra et al. 2015). Biofilm formation by *Salmonella* 1,4,[5],12:i:- isolates evaluated by this method revealed a high ability to form biofilm on plastic surfaces, with all isolates being biofilm-positive at 24h. This was not surprising since other serovars, including *Salmonella* Typhimurium, are also able to form biofilm on plastic surfaces (Karaca et al. 2013, Keelara, Thakur & Patel 2016).

The Stepanović classification regarding biofilm formation using microtiter assays allowed to observe that more than half of the isolates exhibited moderate biofilm-producing ability at 24h. However, our assays were performed after 24h, 48h and 72h incubation period, which allowed *Salmonella* 1,4,[5],12:i:- to exhibit strong biofilms at the last time point (13.5% of all isolates). Although most studies aiming at evaluating biofilm formation by *Salmonella* serovars using microtiter plates only perform 24h incubations (Stepanović et al. 2004, Karaca et al. 2013, Mishra et al. 2015), once biofilm formation in *Salmonella* can proceed through different pathways and time ranges, its detection may differ according to the time-point of observation, being important to test biofilm formation using other incubation periods. The time required for biofilm formation varies between serovars and the 72h incubation period performed in this study demonstrated the ability of *Salmonella* 1,4,[5],12:i:- to express stronger biofilms at later time-points. In clinical or food settings, these differences must be taken into consideration for the establishment of adequate antibiotherapy or disinfection procedures (Garrett, Bhakoob & Zhanga 2008).

Moreover, *Salmonella* 1,4,[5],12:i:- also revealed the ability to form biofilm on glass slides, as evaluated by the direct observation of bacterial suspensions using FISH. The FISH protocol was performed after 24h, 48h and 72h incubation periods to allow biofilm formation. While at 24h approximately 40% of the isolates were biofilm-positive, it was necessary a 72h incubation for almost all isolates to be able to form biofilm on glass slides.

The results obtained by the microtiter plate and FISH methods after a 72h incubation period are very similar, but the differences observed may be explained by the differences between broth media used in both studies, as Muller Hinton Broth (MHB) was used in the microtiter plates assay and diluted Tryptic Soy Broth (TSB) was used in FISH. Although different media composition can influence biofilm formation in *Salmonella*, both are nutrient poor broths, which are most effective in promoting biofilm formation by *Salmonella* spp. (Stepanović et al. 2003, Stepanović et al. 2004). Therefore, differences between results by both methods may also be explained by the fact that *Salmonella* isolates adhere in higher numbers to hydrophobic materials, such as the polystyrene that constitutes the microtiter plates, in comparison with hydrophilic materials, such as the glass of the slides used in FISH (Garrett et al. 2008, Krasowska & Sigler 2014).

As evaluated by microtiter assays, biofilm production ability increased between the isolates collected in 2006 (0.743 ± 0.035) and in 2011 (0.981 ± 0.085). The improvement of biofilm-forming ability through the years may be responsible for an increased aptitude of this serovar to persist in the environment for longer periods of time. Therefore, biofilm-forming ability by this serovar could be one of the reasons accountable for its rise and dissemination, not only in Portugal but also worldwide. More studies are required to confirm this hypothesis, since data regarding biofilm formation is lacking in many EU countries (Hopkins et al. 2012, Wasyl & Hoszowski 2012, Mandilara et al. 2013).

Recently, a report from 2015 demonstrated other possible causes for the rise of this serovar, highlighting the detection of a high occurrence of genes responsible for copper and silver tolerance, among the two major *Salmonella* 1,4,[5],12:i:- clones circulating in Europe, which might enhance the adaptation or expansion of these strains in metal-contaminated environments (Mourão, Novais, Machado, Peixe & Antunes 2015).

Additionally, it was also observed that biofilm formation varied between the origin locations of *Salmonella* 1,4,[5],12:i:- isolates under study. Despite of the low number of samples available in some regions, differences found between isolates from each district may be responsible for distinct abilities to persist in the environment.

Conditions simulating those encountered *in vivo* can influence *in vitro* biofilm formation *Salmonella* 1,4,[5],12:i:-

Biofilm, as an important virulent factor, may play a role during colonization and infection of the intestinal tract, which is not fully understood due to technical constraints. Most studies aiming at evaluating biofilm-forming ability by bacterial isolates are performed using microtiter assays with conventional media (Stepanović et al. 2004, Karaca et al. 2013, Mishra et al. 2015). This technique is relatively easy to perform, but the media used in these assays are usually Luria Bertani or MHB, which composition does not correlate to the environment found in the gastrointestinal tract. To address this issue, a low cost adaption from the high-throughput screening microtiter assay method was develop to simulate the passage of *Salmonella* 1,4,[5],12:i:- through the gastrointestinal track, especially regarding pH, motility and the presence of digestive enzymes.

The passage of *Salmonella* through the stomach represents an important barrier to prevent intestinal colonization. Due to the gastric acid, bile salts and the short retention time of digestive components, microorganisms sparsely colonize the stomach. As confirmed by our study, following the simulation of the gastric passage, the number of *Salmonella* that survive these conditions is low (Macfarlane & Dillon 2007, Steenackers et al. 2012).

Also, human gastrointestinal conditions may decrease bacteria's ability to adhere and form biofilms. Two main observations may endorse this conclusion. First, intestinal conditions may promote the formation of weak biofilms. According to Stepanović classification, also applied in this study, a largest number of *Salmonella* 1,4,[5],12:i:- isolates forming weak biofilms was found when applying simulated intestinal fluid under static conditions, in comparison with incubation in conventional media, where the isolates were able to form moderate and strong biofilms. Secondly, dynamic conditions applied mimicking the human gastrointestinal motility may decrease bacteria's adhesion ability and impair the first step of biofilm formation. Biofilm production in simulated intestinal fluid under dynamic conditions decreased with incubation

time and was lower in comparison with incubation in the same medium under static conditions, at all three time points evaluated (24h, 48h and 72h). Moreover, the scarce reports available that evaluated biofilm formation under dynamics conditions are in accordance to our observations (Stepanović, Vukovic, Jezek, Pavlovic & Svabic-Vlahovic 2001, Stepanović et al. 2003). In fact, such conditions should be included as a parameter during biofilm evaluation, in particular of clinical isolates, where motility can deeply affect bacterial adhesion (Stepanović et al. 2003, Seixas et al. 2014a).

It is worth to mention that recent research in Gram-positive bacteria drew the same conclusions regarding the importance of mimicking conditions found *in vivo* to evaluate biofilm formation (Seixas, Varanda, Bexiga, Tavares & Oliveira 2015). In the report, biofilm-forming ability of isolates obtained from bovine clinical mastitis samples, namely *Staphylococcus aureus* and *S. epidermidis* were evaluated in conditions simulating the udder environment, which also showed that conditions found *in vivo* may influence *in vitro* results. Interestingly in our study, while biofilm formation by *Salmonella* clinical isolates is impaired under simulated *in vivo* conditions, in staphylococci isolates under whole milk, which was the medium used to simulate the udder environment, biofilm production increased, showing higher biofilm production in whole milk in comparison with conventional media (Seixas et al. 2015). Additionally, regarding biofilm-associated gene expression, a previous study showed that TSB medium supplemented with low concentrations of milk could up-regulate biofilm related genes (Xue, Chen & Shang 2014). On the other hand, expression of biofilm genes in *Salmonella* was demonstrated to be down-regulated during *in vivo* infection, and turned on again once the bacteria is shedded into the environment, which support our findings (White et al. 2008).

Taking these observations into account, it seems that by approximating experimental conditions to those found by bacteria in the human host, it may be possible to obtain a more clear insight into the real ability and importance of biofilm production.

CHAPTER 7

Conclusions and Future perspectives

7.1. Conclusions

Salmonella 1,4,[5],12:i:- is presently considered one of the most frequent *Salmonella* serovars in Portugal and in many EU countries, having important repercussions to public health.

This thesis represents the first report describing an increase incidence of *Salmonella* 1,4,[5],12:i:- from 2006 to 2011 in Portugal, and the presence of areas with a higher risk for human *Salmonella* 1,4,[5],12:i:- infections, in particular, at north in the coastland area, at center and at south of Portugal. Although this analysis was based on passive surveillance data, which may underestimate the real number of salmonellosis cases, it provided valuable information on incidence and trends that may contribute for the development of adequate control programs by the authorities, specifically in the districts mostly affected, avoiding extra health care costs.

This rapid expansion of *Salmonella* 1,4,[5],12:i:- R-type ASSuT in Portugal might be related with several factors, including the diversity of pulsotypes, the advantage offered by R-type ASSuT and the presence of a core of multiple virulence genes, also along with biofilm formation ability. In fact, biofilm-forming ability characterization of *Salmonella* 1,4,[5],12:i:- was not documented in the literature and may be an important advantage that may account for the rapid dissemination in our country. The ability to form multicellular communities strongly attached to surfaces is widely disseminated in the Portuguese isolates and it was also seemed to increase through the years.

Since biofilm formation is influenced by several factors, its evaluation by mimicking the intestinal human tract environment has shown that *in vivo* conditions may impair biofilm formation by *Salmonella* 1,4,[5],12:i:-. The modified high-throughput method developed may provide a better assessment, at a lower cost, of the influence of the gastrointestinal environment upon the ability of this serovar to form biofilm. It can be applied in the future to other bacteria related with foodborne diseases, providing reliable laboratory results that would be more helpful in clinical settings.

Due to its epidemic and pandemic behaviour, a close surveillance system of *Salmonella* 1,4,[5],12:i:- in Portugal, including its resistance profiling, is desirable. The need for continuous monitoring associated with the application of harmonized methods to identify this serovar may provide accurate knowledge of its epidemiology, contribute for the implementation of a national strategy to prevent outbreaks and provide useful information to the medical communities in order to rationalize salmonellosis antibiotherapy protocols.

7.2. Future Perspectives

Considering the importance of better understanding the rapid emergence of *Salmonella* 1,4,[5],12:i:- in Portugal, future work may include a deeper analysis using bayesian statistics in order to find possible associations with others factors, including the presence of animal farms. In many reports, pigs are referred as reservoirs for this serovar, and this analysis would allow determining a statistical significant link between pig production and the higher number of cases in some districts. However, other farm animals should also not be disregarded in this analysis.

Genomic fingerprint by MLST or DNA microarray analysis could also be performed, since certain *Salmonella* 1,4,[5],12:i:- isolates belong to multiple clones, which have emerged through independent deletion events, and can only be differentiated by highly sensitive molecular methods, providing a better understanding of serovar 1,4,[5],12:i:- molecular epidemiology.

Additionally, since biocides play an essential and effective role in limiting the spread of infection and disease, biocide susceptibility assays, also including their efficacy upon biofilm formation, may allow to reveal *Salmonella* 1,4,[5],12:i:- resistance to frequently used compounds, which can also be responsible for its increase persistence in the environment.

CHAPTER 8

References

8.1. References

Abruzzi, A. & Fried, B. (2011). Coinfection of Schistosoma (Trematoda) with bacteria, protozoa and helminths. *Advances in Parasitology*, 77(2011), 1-85.

Achtman, M., Wain, J., Weill, F.X., Nair, S., Zhou, Z., Sangal, V., Krauland, M.G., Hale, J.L., Harbottle, H., Uesbeck, A., Dougan, G., Harrison, L.H., Brisse, S. & S. Enterica MLST Study Group. (2012). Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica*. *PLoS Pathogens*, 8(6), 1-19.

Ahmer, B.M. (2004). Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. *Molecular Microbiology*, 52(4), 933-945.

Ahmer, B.M., van Reeuwijk, J., Timmers, C.D., Valentine, P.J. & Heffron, F. (1998). *Salmonella* Typhimurium encodes an SdiA homolog, a putative quorum sensor of the LuxR family, that regulates genes on the virulence plasmid. *Journal of Bacteriology*, 180(5), 1185-1193.

Aldridge, P.D., Wu, C., Gnerer, J., Karlinsey, J.E., Hughes, K.T. & Sachs, M.S. (2006). Regulatory protein that inhibits both synthesis and use of the target protein controls flagellar phase variation in *Salmonella enterica*. *Proceedings of the National Academy of Sciences of the United States of America*, 103(30), 11340-11345.

Almeida, J. (2008). *Produção intensiva de suínos - Projecto de exploração*. Universidade Técnica de Lisboa, Portugal.

Andrews-Polymenis, H.L., Bäumler, A.J., McCormick, B.A. & Fang, F.C. (2010). Taming the elephant: *Salmonella* biology, pathogenesis, and prevention. *Infection and Immunity*, 78(6), 2356-2369.

Anjum, R. & Krakat, N. (2016). Detection of multiple resistances, biofilm formation and conjugative transfer of *Bacillus cereus* from contaminated soils. *Current Microbiology*, 72(3), 321-328.

Anselme, K., Davidson, P., Popa, A.M., Giazzon, M., Liley, M. & Ploux, L. (2010). The interaction of cells and bacteria with surfaces structured at the nanometre scale. *Acta Biomaterialia*, 6(10), 3824-3846.

Antunes, P., Machado, J. & Peixe, L. (2007). Dissemination of *sul*3-containing elements linked to class 1 integrons with an unusual 3' conserved sequence region among *Salmonella* isolates. *Antimicrobial Agents and Chemotherapy*, 51(4), 1545-1548.

Antunes, P., Mourão, J., Pestana, N. & Peixe, L. (2011). Leakage of emerging clinically relevant multidrug-resistant *Salmonella* clones from pig farms. *Journal of Antimicrobial Chemotherapy*, 66(9), 2028-2032.

Arnold, J.W. & Yates, I.E. (2009). Interventions for control of *Salmonella*: clearance of microbial growth from rubber picker fingers. *Poultry Science*, 88(6), 1292-1298.

Backer, H.D., Mohle-Boetani, J.C., Werner, S.B., Abbott, S.L., Farrar, J. & Vugia, D.J. (2000). High incidence of extra-intestinal infections in a *Salmonella* Havana outbreak associated with alfalfa sprouts. *Public Health Reports*, 115(4), 339-345.

Bakowski, M.A., Braun, V. & Brumell, J.H. (2008). *Salmonella*-containing vacuoles: directing traffic and nesting to grow. *Traffic*, 9(12), 2022-2031.

Barco, L., Lettini, A.A., Ramon, E., Longo, A., Saccardin, C., Pozza, M.C. & Ricci, A. (2011). A rapid and sensitive method to identify and differentiate *Salmonella enterica* serotype Typhimurium and *Salmonella enterica* serotype 4,[5],12:i:- by combining traditional serotyping and multiplex polymerase chain reaction. *Foodborne Pathogens and Disease*, 8(6), 741-743.

Bäumler, A.J., Tsolis, R.M. & Heffron, F. (1996). The *lpf* fimbrial operon mediates adhesion of *Salmonella* Typhimurium to murine Peyer's patches. *Proceedings of the National Academy of Sciences of the United States of America*, 93(1), 279-283.

Bearson, S.M., Bearson, B.L. & Rasmussen, M.A. (2006). Identification of *Salmonella enterica* serovar Typhimurium genes important for survival in the swine gastric environment. *Applied and Environmental Microbiology*, 72(4), 2829-2836.

Bernbom, N., Ng, Y.Y., Jørgensen, R.L., Arpanaei, A., Meyer, R.L., Kingshott, P., Vejborg, R.M., Klemm, P. & Gram, L. (2009). Adhesion of food-borne bacteria to stainless steel is reduced by food conditioning films. *Journal of Applied Microbiology*, 106(4), 1268-1279.

Bhowmick, P.P., Devegowda, D., Darshanee Ruwandeepika, H.A., Srikumar, S., Karunasagar, I. & Karunasaga, I. (2011). *gcp*A (stm1987) is critical for cellulose production and biofilm formation on polystyrene surface by *Salmonella enterica* serovar Weltevreden in both high and low nutrient medium. *Microbial Pathogenesis*, 50(2011), 114-122.

Birmingham, C.L., Jiang, X., Ohlson, M.B., Miller, S.I. & Brumell, J.H. (2005). *Salmonella*-induced filament formation is a dynamic phenotype induced by rapidly replicating *Salmonella enterica* serovar Typhimurium in epithelial cells. *Infection and Immunity*, 73(2), 1204–1208.

Bjarnsholt, T., Jensen, P., Fiandaca, M.J., Pedersen, J., Hansen, C.R., Andersen, C.B., Pressler, T., Givskov, M. & Høiby, N. (2009). *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. *Pediatric Pulmonology*, 44(6), 547-558.

Blair, J.M., Webber, M.A., Baylay, A.J., Ogbolu, D.O. & Piddock, L.J. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology*, 13(1), 42-51.

Blaser, M.J. & Newman, L.S. (1982). A review of human salmonellosis: I. Infective dose. *Reviews of infectious diseases*, 4(6), 1096-1106.

Bokranz, W., Wang, X., Tschäpe, H. & Römling, U. (2005). Expression of cellulose and curli fimbriae by *Escherichia coli* isolated from the gastrointestinal tract. *Journal of Medical Microbiology*, 54(Pt 12), 1171-1182.

Borriello, G., Lucibelli, M.G., Pesciaroli, M., Carullo, M.R., Graziani, C., Ammendola, S., Battistoni, A., Ercolini, D., Pasquali, P. & Galiero, G. (2012). Diversity of *Salmonella* spp. serovars isolated from the intestines of water buffalo calves with gastroenteritis. *BMC Veterinary Research*, 8(201), 1-9.

Bowe, F., Lipps, C.J., Tsolis, R.M., Groisman, E., Heffron, F. & Kusters, J.G. (1998). At least four percent of the *Salmonella* Typhimurium genome is required for fatal infection of mice. *Infection and Immunity*, 66(7), 3372-3377.

Brennan, D.F. & Barford, D. (2009). Eliminylation: a post-translational modification catalyzed by phosphothreonine lyases. *Trends in Biochemical Sciences*, 34(3), 108-114.

Bugarel, M., Vignaud, M.L., Moury, F., Fach, P. & Brisabois, A. (2012). Molecular identification in monophasic and nonmotile variants of *Salmonella enterica* serovar Typhimurium. *Microbiologyopen*, 1(4), 481-489.

Burkholder, K.M. & Bhunia, A.K. (2009). *Salmonella enterica* serovar Typhimurium adhesion and cytotoxicity during epithelial cell stress is reduced by *Lactobacillus rhamnosus* GG. *Gut Pathogens*, 1(1), 1-14.

Cain, R.J., Hayward, R.D. & Koronakis, V. (2008). Deciphering interplay between *Salmonella* invasion effectors. *PLoS Pathogens*, 4(4), 1-16.

Capuano, F., Mancusi, A., Capparelli, R., Esposito, S. & Proroga, Y.T. (2013). Characterization of drug resistance and virulotypes of *Salmonella* strains isolated from food and humans. *Foodborne Pathogens and Disease*, 10(11), 963-968.

Carpentier, B. & Cerf, O. (1993). Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology*, 75(6), 499-511.

Carrie, A., Sheila, P., Fedorka-Cray, P. & Isaacson, R. (2003). Type 1 Fimbriae of *Salmonella enterica* serovar Typhimurium bind to enterocytes and contribute to colonization of swine *in vivo*. *Infection and Immunity*, 71(11), 6446–6452.

Castelijn, G.A., van der Veen, S., Zwietering, M.H., Moezelaar, R. & Abee, T. (2012). Diversity in biofilm formation and production of curli fimbriae and cellulose of *Salmonella* Typhimurium strains of different origin in high and low nutrient medium. *Biofouling*, 28(1), 51-63.

CDC. (2013). Antibiotic resistance threats in the United States, 2013. Accessed 18 December, 2015, available at http://www.cdc.gov/drugresistance/threat-report-2013

Chambers, H.F. & Deleo, F.R. (2009). Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nature Reviews Microbiology*, 7(9), 629-641.

Chen, D., Zhao, T. & Doyle, M.P. (2015). Control of pathogens in biofilms on the surface of stainless steel by levulinic acid plus sodium dodecyl sulfate. *International Journal of Food Microbiology*, 207(2015), 1-7.

Chiu, C.H., Tang, P., Chu, C., Hu, S., Bao, Q., Yu, J., Chou, Y.Y., Wang, H.S. & Lee, Y.S. (2005). The genome sequence of *Salmonella enterica* serovar Choleraesuis, a highly invasive and resistant zoonotic pathogen. *Nucleic Acids Research*, 33(5), 1690-1698.

Chu, C., Lu, C., Lee, C. & Tsai, C. (2003). Effects of chlorine level on the growth of biofilm in water pipes. *Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering*, 38(7), 1377-1388.

Cirillo, V.J. (2006). "Winged sponges": houseflies as carriers of typhoid fever in 19th- and early 20th-century military camps. *Perspectives in Biology and Medicine*, 49(1), 52-63.

Cito, F., Baldinelli, F., Calistri, P., Di Giannatale, E., Scavia, G., Orsini, M., Iannetti, S., Sacchini, L., Mangone, I., Candeloro, L., Conte, A., Ippoliti, C., Morelli, D., Migliorati, G., Barile, N.B., Marfoglia, C., Salucci, S., Cammà, C., Marcacci, M., Ancora, M., Dionisi, A.M., Owczartek, S., Luzzi, I. & outbreak investigation group. (2016). Outbreak of unusual *Salmonella enterica* serovar Typhimurium monophasic variant 1,4 [5],12:i:-, Italy, June 2013 to September 2014. *Euro surveillance*, 21(15), 1-10.

Clemente, L., Correia, I., Themudo, P., Neto, I., Caniça, M. & Bernardo, F. (2014). Antimicrobial susceptibility of *Salmonella enterica* isolates from healthy breeder and broiler flocks in Portugal. *Veterinary Journal*, 200(2), 276-281.

Clinical and Laboratory Standards Institute. (2012). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. CLSI document M100-S22. *Wayne, PA*.

Corcoran, M., Morris, D., De Lappe, N., O'Connor, J., Lalor, P., Dockery, P. & Cormican, M. (2013). *Salmonella enterica* biofilm formation and density in the Centers for Disease Control and Prevention's biofilm reactor model is related to serovar and substratum. *Journal of Food Protection*, 76(4), 662-667.

Corcoran, M., Morris, D., De Lappe, N., O'Connor, J., Lalor, P., Dockery, P. & Cormican, M. (2014). Commonly used disinfectants fail to eradicate *Salmonella* enterica biofilms from food contact surface materials. *Applied and Environmental Microbiology*, 80(4), 1507-1514.

Cross, A.S. (2008). What is a virulence factor? *Critical Care*, 12(196), 1-2.

Crum-Cianflone, N.F. (2008). Salmonellosis and the gastrointestinal tract: more than just peanut butter. *Current Gastroenterology Reports*, 10(4), 424-431.

Davey, M.E. & O'toole, G.A. (2000). Microbial biofilms: from ecology to molecular genetics. *Microbiology and Molecular Biology Reviews*, 64(4), 847-867.

de la Torre, E., Zapata, D., Tello, M., Mejía, W., Frías, N., García Peña, F.J., Mateu, E.M. & Torre, E. (2003). Several *Salmonella enterica* subsp. *enterica* serotype 4,5,12:i:- phage types isolated from swine samples originate from serotype Typhimurium DT U302. *Journal of Clinical Microbiology*, 41(6), 2395-2400.

De Lappe, N., Doran, G., O'Connor, J., O'Hare, C. & Cormican, M. (2009). Characterization of bacteriophages used in the *Salmonella enterica* serovar Enteritidis phage-typing scheme. *Journal of Medical Microbiology*, 58(Pt 1), 86-93.

De Oliveira, D.C., Fernandes Júnior, A., Kaneno, R., Silva, M.G., Araújo Júnior, J.P., Silva, N.C. & Rall, V.L. (2014). Ability of *Salmonella* spp. to produce biofilm is dependent on temperature and surface material. *Foodborne Pathogens and Disease*, 11(6), 478-483.

del Solar, G., Giraldo, R., Ruiz-Echevarría, M.J., Espinosa, M. & Díaz-Orejas, R. (1998). Replication and control of circular bacterial plasmids. *Microbiology and Molecular Biology Reviews*, 62(2), 434-464.

Delgado, M.A., Mouslim, C. & Groisman, E.A. (2006). The PmrA/PmrB and RcsC/YojN/RcsB systems control expression of the *Salmonella* O-antigen chain length determinant. *Molecular Microbiology*, 60(1), 39-50.

Diário do Governo-Presidência da República: Lei 2:036 de 19 de Agosto (1949), Volume I Série - Número 175. Acessed 10th January, 2016, available at https://dre.pt/application/file/264804

Dionisi, A.M., Graziani, C., Lucarelli, C., Filetici, E., Villa, L., Owczarek, S., Caprioli, A. & Luzzi, I. (2009). Molecular characterization of multidrug-resistant strains of *Salmonella enterica* serotype Typhimurium and Monophasic variant (S. 4,[5],12:i:-) isolated from human infections in Italy. *Foodborne Pathogens and Disease*, 6(6), 711-717.

Donlan, R.M. (2002). Biofilms: microbial life on surfaces. *Emerging Infectious Diseases*, 8(9), 881-890.

Donlan, R.M. & Costerton, J.W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15(2), 167-193.

Dougan, G., John, V., Palmer, S. & Mastroeni, P. (2011). Immunity to salmonellosis. *Immunological Reviews*, 240(1), 196–210.

Drahovská, H., Mikasová, E., Szemes, T., Ficek, A., Sásik, M., Majtán, V. & Turna, J. (2007). Variability in occurrence of multiple prophage genes in *Salmonella* Typhimurium strains isolated in Slovak Republic. *FEMS Microbiology Letters*, 270(2), 237-244.

Drecktrah, D., Knodler, L.A., Ireland, R. & Steele-Mortimer, O. (2006). The mechanism of *Salmonella* entry determines the vacuolar environment and intracellular gene expression. *Traffic*, 7(1), 39-51.

Duguid, J.P., Anderson, E.S. & Campbell, I. (1966). Fimbriae and adhesive properties in *Salmonellae*. *The Journal of Pathology and Bacteriology*, 92(1), 107-138.

Echeita, M.A., Aladueña, A., Cruchaga, S. & Usera, M.A. (1999). Emergence and spread of an atypical *Salmonella enterica* subsp. *enterica* serotype 4,5,12:i:- strain in Spain. *Journal of Clinical Microbiology*, 37(10), 3425.

Echeita, M.A., Herrera, S. & Usera, M.A. (2001). Atypical, fljB-negative *Salmonella enterica* subsp. *enterica* strain of serovar 4,5,12:i:- appears to be a monophasic variant of serovar Typhimurium. *Journal of Clinical Microbiology*, 39(8), 2981-2983.

EFSA. (2010a). Manual for reporting on zoonoses, zoonotic agents and antimicrobial resistance in the framework of Directive 2003/99/EC and of some other pathogenic microbiological agents for information derived from the reporting year 2009. *EFSA Journal*, 8(4), 1579-1676.

EFSA. (2010b). Scientific Opinion on monitoring and assessment of the public health risk of "*Salmonella* Typhimurium-like" strains. *EFSA Journal*, 8(10), 1826-1874.

EFSA and ECDC. (2014). The European Union Summary Report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2012. *EFSA Journal*, 12(3), 336.

EFSA and ECDC. (2015). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2013. *EFSA Journal*, 13(1), 3991.

Eswarappa, S.M., Janice, J., Nagarajan, A.G., Balasundaram, S.V., Karnam, G., Dixit, N.M. & Chakravortty, D. (2008). Differentially evolved genes of *Salmonella* pathogenicity islands: insights into the mechanism of host specificity in *Salmonella*. *PLoS One*, 3(12), 1-12.

European Comission. (2004). Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. *Official Journal of the European Union*, 47(L165).

Fang, H., Xu, J., Ding, D., Jackson, S.A., Patel, I.R., Frye, J.G., Zou, W., Nayak, R., Foley, S., Chen, J., Su, Z., Ye, Y., Turner, S., Harris, S., Zhou, G., Cerniglia, C. & Tong, W.

(2010). An FDA bioinformatics tool for microbial genomics research on molecular characterization of bacterial foodborne pathogens using microarrays. *BMC Bioinformatics*, 11(Suppl 6), 1-11.

Figueira, R. & Holden, D.W. (2012). Functions of the *Salmonella* pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology*, 158(Pt 5), 1147-1161.

Fink, S.L. & Cookson, B.T. (2007). Pyroptosis and host cell death responses during *Salmonella* infection. *Cellular Microbiology*, 9(11), 2562-2570.

Flemming, H.C. & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, 8(9), 623-633.

Foley, S.L., Johnson, T.J., Ricke, S.C., Nayak, R. & Danzeisen, J. (2013). *Salmonella* pathogenicity and host adaptation in chicken-associated serovars. *Microbiology and Molecular Biology Reviews*, 77(4), 582-607.

Frasson, I., Bettanello, S., De Canale, E., Richter, S.N. & Palù, G. (2016). Serotype epidemiology and multidrug resistance patterns of *Salmonella enterica* infecting humans in Italy. *Gut Pathogens*, 8(26), 1-7.

Garaizar, J., Porwollik, S., Echeita, A., Rementeria, A., Herrera, S., Wong, R.M., Frye, J., Usera, M.A. & McClelland, M. (2002). DNA microarray-based typing of an atypical monophasic *Salmonella enterica* serovar. *Journal of Clinical Microbiology*, 40(6), 2074-2078.

García, P., Guerra, B., Bances, M., Mendoza, M.C. & Rodicio, M.R. (2011). IncA/C plasmids mediate antimicrobial resistance linked to virulence genes in the Spanish clone of the emerging *Salmonella enterica* serotype 4,[5],12:i:-. *Journal of Antimicrobial Chemotherapy*, 66(33), 543-549.

García, P., Malorny, B., Rodicio, M.R., Stephan, R., Hächler, H., Guerra, B. & Lucarelli, C. (2016). Horizontal acquisition of a multidrug-resistance module (R-type ASSuT) is responsible for the monophasic phenotype in a widespread clone of *Salmonella* serovar 4,[5],12:i:. *Frontiers in Microbiology*, 7(680), 1-7.

Garrett, T.R., Bhakoob, M. & Zhanga, Z. (2008). Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science*, 18(9), 1049–1056.

Gerlach, R.G., Jäckel, D., Geymeier, N. & Hensel, M. (2007a). *Salmonella* pathogenicity island 4-mediated adhesion is coregulated with invasion genes in *Salmonella enterica*. *Infection and Immunity*, 75(10), 4697-4709.

Gerlach, R.G., Jäckel, D., Stecher, B., Wagner, C., Lupas, A., Hardt, W.D. & Hensel, M. (2007b). *Salmonella* Pathogenicity Island 4 encodes a giant non-fimbrial adhesin and the cognate type 1 secretion system. *Cellular Microbiology*, 9(7), 1834-1850.

Gerstel, U. & Römling, U. (2001). Oxygen tension and nutrient starvation are major signals that regulate agfD promoter activity and expression of the multicellular morphotype in *Salmonella* Typhimurium. *Environmental Microbiology*, 3(10), 638-648.

Gerstel, U. & Römling, U. (2003). The csgD promoter, a control unit for biofilm formation in *Salmonella* Typhimurium. *Research in Microbiology*, 154(10), 659-667.

Giacomodonato, M.N., Llana, M.N., Castañeda Mdel, R., Buzzola, F., García, M.D., Calderón, M.G., Sarnacki, S.H. & Cerquetti, M.C. (2014). Dam methylation regulates

the expression of SPI-5-encoded *sop*B gene in *Salmonella enterica* serovar Typhimurium. *Microbes and Infection*, 16(8), 615-622.

Giaouris, E., Chapot-Chartier, M.P. & Briandet, R. (2009). Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. *International Journal of Food Microbiology*, 131(1), 2-9.

Giaouris, E., Chorianopoulos, N. & Nychas, G.J. (2005). Effect of temperature, pH, and water activity on biofilm formation by *Salmonella enterica* enteritidis PT4 on stainless steel surfaces as indicated by the bead vortexing method and conductance measurements. *Journal of Food Protection*, 68(10), 2149-2154.

Goller, C.C. & Romeo, T. (2008). Environmental influences on biofilm development. *Current Topics in Microbiology and Immunology*, 2008(322), 37-66.

Gonçalves, G., Gouveia, E. & Prasad, L. (2014). The response of a local health authority to reported cases of salmonellosis in a Portuguese municipality, 2007 to 2011. *BMC Research Notes*, 7(161), 1-7.

Gondwe, E.N., Molyneux, M.E., Goodall, M., Graham, S.M., Mastroeni, P., Drayson, M.T. & MacLennan, C.A. (2010). Importance of antibody and complement for oxidative burst and killing of invasive nontyphoidal *Salmonella* by blood cells in Africans. *Proceedings of the National Academy of Sciences of the United States of America*, 107(7), 3070–3075.

Gonzalez-Escobedo, G. & Gunn, J.S. (2013). Gallbladder epithelium as a niche for chronic *Salmonella* carriage. *Infection and Immunity*, 81(8), 2929-2930.

Gorvel, J.P. & Méresse, S. (2001). Maturation steps of the *Salmonella*-containing vacuole. *Microbes and Infection*, 3(14-15), 1299-1303.

Götz, F. (2002). *Staphylococcus* and biofilms. *Molecular Microbiology*, 43(6), 1367-1378.

Grimont, P.A. & Weill, F.X. (2007). *Antigenic formulae of the Salmonella serovars*. WHO-Collaborating Centre for Reference and Research on *Salmonella*. Accessed 20 November, 2016, from Institute Pasteur, Paris, available at http://www.serotest-thailand.com/upload/news/download/9-8310-0.pdf.

Groisman, E.A. & Ochman, H. (1997). How *Salmonella* became a pathogen. *Trends in Microbiology*, 5(9), 343-349.

Guerra, B., Junker, E., Miko, A., Helmuth, R. & Mendoza, M.C. (2004). Characterization and localization of drug resistance determinants in multidrug-resistant, integron-carrying *Salmonella enterica* serotype Typhimurium strains. *Microbial Drug Resistance*, 10(2), 83-91.

Guerra, B., Soto, S.M., Argüelles, J.M. & Mendoza, M.C. (2001). Multidrug resistance is mediated by large plasmids carrying a class 1 integron in the emergent *Salmonella enterica* serotype [4,5,12:i:-]. *Antimicrobial Agents and Chemotherapy*, 45(4), 1305-1308.

Guibourdenche, M., Roggentin, P., Mikoleit, M., Fields, P.I., Bockemühl, J., Grimont, P.A. & Weill, F.X. (2010). Supplement 2003-2007 (No. 47) to the White-Kauffmann-Le Minor scheme. *Research in Microbiology*, 161(1), 26-29.

Guiney, D.G. & Fierer, J. (2011). The role of the spv genes in *Salmonella* pathogenesis. *Frontiers in Microbiology*, 2(129), 1-10.

Guo, X., Chen, J., Brackett, R.E. & Beuchat, L.R. (2002). Survival of *Salmonella* on tomatoes stored at high relative humidity, in soil, and on tomatoes in contact with soil. *Journal of Food Protection*, 65(2), 274-279.

Gyles, C., Prescott, J., Songer, G. & Thoen, C. (2010). *Pathogenesis of bacterial infections in animals* (4th edition ed.): Blackwell Publishing.

Hackett, J., Kotlarski, I., Mathan, V., Francki, K. & Rowley, D. (1986). The colonization of Peyer's patches by a strain of *Salmonella* Typhimurium cured of the cryptic plasmid. *Journal of Infectious Diseases*, 153(6), 1119-1125.

Haneda, T., Ishii, Y., Shimizu, H., Ohshima, K., Iida, N., Danbara, H. & Okada, N. (2012). *Salmonella* type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection. *Cellular Microbiology*, 14(4), 485-499.

Hapfelmeier, S., Ehrbar, K., Stecher, B., Barthel, M., Kremer, M. & Hardt, W.D. (2004). Role of the *Salmonella* pathogenicity island 1 effector proteins SipA, SopB, SopE, and SopE2 in *Salmonella enterica* subspecies 1 serovar Typhimurium colitis in streptomycin-pretreated mice. *Infection and Immunity*, 72(2), 795-809.

Hara-Kudo, Y. & Takatori, K. (2011). Contamination level and ingestion dose of foodborne pathogens associated with infections. *Epidemiology and Infection*, 139(10), 1505-1510.

Haraga, A., Ohlson, M.B. & Miller, S.I. (2008). *Salmonellae* interplay with host cells. *Nature Reviews Microbiology*, 6(1), 53-66.

Hauser, E., Tietze, E., Helmuth, R., Junker, E., Blank, K., Prager, R., Rabsch, W., Appel, B., Fruth, A. & Malorny, B. (2010). Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:, an emerging health risk for humans. *Applied and Environmental Microbiology*, 76(14), 4601-4610.

Hayward, R.D. & Koronakis, V. (2002). Direct modulation of the host cell cytoskeleton by *Salmonella* actin-binding proteins. *Trends in Cell Biology*, 12(1), 15-20.

Hinton, A.J., Buhr, R.J. & Ingram, K.D. (2000). Physical, chemical, and microbiological changes in the ceca of broiler chickens subjected to incremental feed withdrawal. *Poultry Science*, 79(4), 483-488.

Hohmann, E.L. (2001). Nontyphoidal salmonellosis. *Clinical Infectious Diseases*, 32(2), 263-269.

Høiby, N. (2014). A personal history of research on microbial biofilms and biofilm infections. *Pathogens and Disease*, 70(3), 205-211.

Høiby, N., Bjarnsholt, T., Givskov, M., Molin, S. & Ciofu, O. (2010). Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents*, 35(4), 322-332.

Høiby, N., Bjarnsholt, T., Moser, C., Bassi, G.L., Coenye, T., Donelli, G., Hall-Stoodley, L., Holá, V., Imbert, C., Kirketerp-Møller, K., Lebeaux, D., Oliver, A., Ullmann, A.J., Williams, C. & Zimmerli., E.S.G.f.B.a.C.E.E.W. (2015). ESCMID guideline for the diagnosis and treatment of biofilm infections. *Clinical Microbiology and Infection*, 21(Suppl 1), 1-25.

Hopkins, K.L., de Pinna, E. & Wain, J. (2012). Prevalence of *Salmonella enterica* serovar 4,[5],12:i:- in England and Wales, 2010. *Euro surveillance*, 17(37), pii=20275.

Hopkins, K.L., Kirchner, M., Guerra, B., Granier, S.A., Lucarelli, C., Porrero, M.C., Jakubczak, A., Threlfall, E.J. & Mevius, D.J. (2010). Multiresistant *Salmonella enterica* serovar 4,[5],12:i:- in Europe: a new pandemic strain? *Euro surveillance*, 15(22), 1-9.

Hueffer, K. & Galán, J.E. (2004). *Salmonella*-induced macrophage death: multiple mechanisms, different outcomes. *Cellular Microbiology*, 6(11), 1019-1025.

Hugas, M. & Beloeil, P. (2014). Controlling *Salmonella* along the food chain in the European Union - progress over the last ten years. *Euro surveillance*, 19(19), pii: 20804.

Humphrey, T. (2004). *Salmonella*, stress responses and food safety. *Nature Reviews Microbiology*, 2(6), 504-509.

Humphries, A.D., Raffatellu, M., Winter, S., Weening, E.H., Kingsley, R.A., Droleskey, R., Zhang, S., Figueiredo, J., Khare, S., Nunes, J., Adams, L.G., Tsolis, R.M. & Bäumler, A.J. (2003). The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. *Molecular Microbiology*, 48(5), 1357-1376.

Ido, N., Iwabuchi, K., Sato'o, Y., Sato, Y., Sugawara, M., Yaegashi, G., Konno, M., Akiba, M., Tanaka, K., Omoe, K. & Uchida, I. (2015). Molecular typing of *Salmonella enterica* serovar 4,[5],12:i:- isolates from humans, animals and river water in Japan by multilocus variable-number tandem repeat analysis and pulsed-field gel electrophoresis. *The Journal of Veterinary Medical Science*, 77(5), 609-613.

Ikeda, J.S., Schmitt, C.K., Darnell, S.C., Watson, P.R., Bispham, J., Wallis, T.S., Weinstein, D.L., Metcalf, E.S., Adams, P., O'Connor, C.D. & O'Brien, A.D. (2001). Flagellar phase variation of *Salmonella enterica* serovar Typhimurium contributes to virulence in the murine typhoid infection model but does not influence *Salmonella*-induced enteropathogenesis. *Infection and Immunity*, 69(5), 3021-3030.

Johnson, E.M., Krauskopf, B. & Baron, L.S. (1965). Genetic mapping of Vi and somatic antigenic determinants in *Salmonella*. *Journal of Bacteriology*, 90(2), 302-308.

Jones, B.D. (2005). *Salmonella* invasion gene regulation: a story of environmental awareness. *Journal of Microbiology*, 43(1), 110-117.

Jones-Carson, J. & Vazquez-Torres, A. (2007). Innate host defenses in Salmonelosis. In M. M. Mikael Rhen, Pietro Mastroeni, John Threfall (Ed.), *Salmonella: Molecular Biology and Pathogenesis*. Norfolk, UK: Horizon Bioscience.

Joseph, B., Otta, S.K., Karunasagar, I. & Karunasagar, I. (2001). Biofilm formation by salmonella spp. on food contact surfaces and their sensitivity to sanitizers. *International Journal of Food Microbiology*, 64(3), 367-372.

Kaniga, K., Uralil, J., Bliska, J.B. & Galán, J.E. (1996). A secreted protein tyrosine phosphatase with modular effector domains in the bacterial pathogen *Salmonella* Typhimurium. *Molecular Microbiology*, 21(3), 633-641.

Karaca, B., Akcelik, N. & Akcelik, M. (2013). Biofilm-producing abilities of *Salmonella* strains isolated from Turkey. *Biologia*, 68(1), 1-10.

Ke, B., Sun, J., He, D., Li, X., Liang, Z. & Ke, C. (2014). Serovar distribution, antimicrobial resistance profiles, and PFGE typing of *Salmonella enterica* strains isolated from 2007–2012 in Guangdong, China. *BMC Infectious Diseases*, 14(338), 1-10.

Keelara, S., Thakur, S. & Patel, J. (2016). Biofilm Formation by Environmental Isolates of *Salmonella* and Their Sensitivity to Natural Antimicrobials. *Foodborne Pathogens and Disease*, 13(9), 509-516.

Kendall, M.M. & Sperandio, V. (2014). Cell-to-Cell Signaling in *Escherichia coli* and *Salmonella*. *EcoSal Plus*, 6(1), 1-22.

Klausen, M., Gjermansen, M., Kreft, J.U. & Tolker-Nielsen, T. (2006). Dynamics of development and dispersal in sessile microbial communities: examples from *Pseudomonas aeruginosa* and *Pseudomonas putida* model biofilms. *FEMS Microbiology Letters*, 261(1), 1-11.

Klerks, M.M., van Gent-Pelzer, M., Franz, E., Zijlstra, C. & van Bruggen, A.H. (2007). Physiological and molecular responses of *Lactuca sativa* to colonization by *Salmonella enterica* serovar Dublin. *Applied and Environmental Microbiology*, 73(15), 4905-4914.

Knodler, L.A., Finlay, B.B. & Steele-Mortimer, O. (2005). The *Salmonella* effector protein SopB protects epithelial cells from apoptosis by sustained activation of Akt. *Journal of Biological Chemistry*, 280(10), 9058-9064.

Knodler, L.A. & Steele-Mortimer, O. (2003). Taking possession: biogenesis of the *Salmonella*-containing vacuole. *Traffic*, 4(9), 587-599.

Kothary, M.H. & Babu, U.S. (2001). Infective dose of foodborne pathogens in volunteers: a review. *Journal of Food Safety*, 21(2001), 49-73.

Krasowska, A. & Sigler, K. (2014). How microorganisms use hydrophobicity and what does this mean for human needs? *Frontiers in Cellular and Infection Microbiology*, 4(112), 1-7.

Kuhle, V., Abrahams, G.L. & Hensel, M. (2006). Intracellular *Salmonella* enterica redirect exocytic transport processes in a *Salmonella* pathogenicity island 2-dependent manner. *Traffic*, 7(6), 716–730.

Kuhle, V. & Hensel, M. (2002). SseF and SseG are translocated effectors of the type III secretion system of *Salmonella* pathogenicity island 2 that modulate aggregation of endosomal compartments. *Cellular Microbiology*, 4(12), 813-824.

Kumar, C.G. & Anand, S.K. (1998). Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology*, 42(1-2), 9-27.

Kutsukake, K., Nakashima, H., Tominaga, A. & Abo, T. (2006). Two DNA invertases contribute to flagellar phase variation in *Salmonella enterica* serovar Typhimurium strain LT2. *Journal of Bacteriology*, 188(3), 950-957.

Kwiecinski, J., Kahlmeter, G. & Jin, T. (2015). Biofilm formation by *Staphylococcus aureus* isolates from skin and soft tissue infections. *Current Microbiology*, 70(5), 698-703.

Lailler, R., Grout, J., Marault, M., Oudart, C., Moury, F. & Brisabois, A. (2013). Molecular confirmation method for monophasic and non-motile variant strains of *Salmonella* serovar Typhimurium. *Euro Reference*, 11(14-17).

Lambert, M.A. & Smith, S.G. (2008). The PagN protein of *Salmonella enterica* serovar Typhimurium is an adhesin and invasin. *BMC Microbiology*, 8(142), 1-11.

LaRock, D.L., Brzovic, P.S., Levin, I., Blanc, M.P. & Miller, S.I. (2012). A *Salmonella* typhimurium-translocated glycerophospholipid:cholesterol acyltransferase promotes virulence by binding to the RhoA protein switch regions. *Journal of Biological Chemistry*, 287(35), 29654-29663.

LaRock, D.L., Chaudhary, A. & Miller, S.I. (2015). *Salmonellae* interactions with host processes. *Nature Reviews Microbiology*, 13(4), 191-205.

Ledeboer, N.A., Frye, J.G., McClelland, M. & Jones, B.D. (2006). *Salmonella enterica* serovar Typhimurium requires the Lpf, Pef, and Tafi fimbriae for biofilm formation on HEp-2 tissue culture cells and chicken intestinal epithelium. *Infection and Immunity*, 74(6), 3156-3169.

Lee, C.A., Silva, M., Siber, A.M., Kelly, A.J., Galyov, E. & McCormick, B.A. (2000). A secreted *Salmonella* protein induces a proinflammatory response in epithelial cells, which promotes neutrophil migration. *Proceedings of the National Academy of Sciences of the United States of America*, 97(22), 12283-12288.

Levy, S.B. & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature Medicine*, 10(12 Suppl), 122-129.

Libby, S.J., Goebel, W., Ludwig, A., Buchmeier, N., Bowe, F., Fang, F.C., Guiney, D.G., Songer, J.G. & Heffron, F. (1994). A cytolysin encoded by *Salmonella* is required for survival within macrophages. *Proceedings of the National Academy of Sciences of the United States of America*, 91(2), 489-493.

Linehan, S.A., Rytkönen, A., Yu, X.J., Liu, M. & Holden, D.W. (2005). *Sly*A regulates function of *Salmonella* pathogenicity island 2 (SPI-2) and expression of SPI-2-associated genes. *Infection and Immunity*, 73(7), 4354-4362.

Lonescu, A., Wutscher, E., Brambilla, E., Schneider-Feyrer, S., Giessibl, F.J. & Hahnel, S. (2012). Influence of surface properties of resin-based composites on in vitro *Streptococcus mutans* biofilm development. *European Journal of Oral Sciences*, 120(5), 458-465.

López, F.E., Pescaretti, M.M., Morero, R. & Delgado, M.A. (2012). *Salmonella* Typhimurium general virulence factors: A battle of David against Goliath? *Food Research International*, 45(2), 842–851.

Lucarelli, C., Dionisi, A.M., Torpdahl, M., Villa, L., Graziani, C., Hopkins, K., Threlfall, J., Caprioli, A. & Luzzi, I. (2010). Evidence for a second genomic island conferring multidrug resistance in a clonal group of strains of *Salmonella enterica* serovar Typhimurium and its monophasic variant circulating in Italy, Denmark, and the United Kingdom. *Journal of Clinical Microbiology*, 48(6), 2103-2109.

Ludwig, A., Tengel, C., Bauer, S., Bubert, A., Benz, R., Mollenkopf, H.J. & Goebel, W. (1995). *Sly*A, a regulatory protein from *Salmonella* typhimurium, induces a haemolytic and pore-forming protein in *Escherichia coli*. *Molecular Genetics and Genomics*, 249(5), 474-486.

Macfarlane, S. & Dillon, J.F. (2007). Microbial biofilms in the human gastrointestinal tract. *Journal of Applied Microbiology*, 102(5), 1187-1196.

Machado, J. & Bernardo, F. (1990). Prevalence of *Salmonella* in chicken carcasses in Portugal. *Journal of Applied Microbiology*, 69(4), 477-480.

Madajczak, G., Wołkowicz, T., Dera-Tomaszewska, B., Wasiak, M., Chróst, A. & Szych, J. (2014). Occurrence and characterization of monophasic *Salmonella enterica* subsp. *enterica* with antigenic formula 1,4, [5], 12: i:-isolated in Poland in 2007-2012. *Medycyna Doświadczalna i Mikrobiologia*, 66(2), 65-78.

Madsen, J.S., Burmølle, M., Hansen, L.H. & Sørensen, S.J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunology & Medical Microbiology*, 65(2), 183-195.

Magiorakos, A.P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T. & Monnet, D.L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection*, 18(3), 268-281.

Mahon, B.E., Pönkä, A., Hall, W.N., Komatsu, K., Dietrich, S.E., Siitonen, A., Cage, G., Hayes, P.S., Lambert-Fair, M.A., Bean, N.H., Griffin, P.M. & Slutsker, L. (1997). An international outbreak of *Salmonella* infections caused by alfalfa sprouts grown from contaminated seeds. *Journal of Infectious Diseases*, 175(4), 876-882.

Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, S.J., Jones, T.F., Fazil, A., Hoekstra, R.M. & International Collaboration on Enteric Disease Burden of Illness Studies. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clinical Infectious Diseases*, 50(6), 882-889.

Majtan, V., Majtanova, L. & Majtan, J. (2011). Phenotypic and molecular characterization of human *Salmonella enterica* serovar 4,[5],12:i:- isolates in Slovakia. *Current Microbiology*, 63(5), 491-495.

Malcova, M., Hradecka, H., Karpiskova, R. & Rychlik, I. (2008). Biofilm formation in field strains of *Salmonella enterica* serovar Typhimurium: identification of a new colony morphology type and the role of SGI1 in biofilm formation. *Veterinary Microbiology*, 129(2008), 360-366.

Mandilara, G., Lambiri, M., Polemis, M., Passiotou, M. & Vatopoulos, A. (2013). Phenotypic and molecular characterisation of multiresistant monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) in Greece, 2006 to 2011. *Euro surveillance*, 18(22), 1-8.

Marcus, S.L., Brumell, J.H., Pfeifer, C.G. & Finlay, B.B. (2000). *Salmonella* pathogenicity islands: big virulence in small packages. *Microbes and Infection*, 2(2), 145-156.

Marineli, F., Tsoucalas, G., Karamanou, M. & Androutsos, G. (2013). Mary Mallon (1869-1938) and the history of typhoid fever. *Annals of Gastroenterology*, 26(2), 132-134.

Maurischat, S., Baumann, B., Martin, A. & Malorny, B. (2015). Rapid detection and specific differentiation of *Salmonella enterica* subsp. *enterica* Enteritidis, Typhimurium and its monophasic variant 4,[5],12:i:- by real-time multiplex PCR. *International Journal of Food Microbiology*, 193(8-14).

Mazurkiewicz, P., Thomas, J., Thompson, J.A., Liu, M., Arbibe, L., Sansonetti, P. & Holden, D.W. (2008). SpvC is a *Salmonella* effector with phosphothreonine lyase activity on host mitogen-activated protein kinases. *Molecular Microbiology*, 67(6), 1371-1383.

McGhie, E.J., Brawn, L.C., Hume, P.J., Humphreys, D. & Koronakis, V. (2009). *Salmonella* takes control: effector-driven manipulation of the host. *Current Opinion in Microbiology*, 12(1), 117-124.

McLaughlin, L.M., Govoni, G.R., Gerke, C., Gopinath, S., Peng, K., Laidlaw, G., Chien, Y.H., Jeong, H.W., Li, Z., Brown, M.D., Sacks, D.B. & Monack, D. (2009). The *Salmonella* SPI2 effector SseI mediates long-term systemic infection by modulating host cell migration. *PLoS Pathogens*, 5(11), 1-15.

Miao, E.A., Alpuche-Aranda, C.M., Dors, M., Clark, A.E., Bader, M.W., Miller, S.I. & Aderem, A. (2006). Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nature Immunology*, 7(6), 569-575.

Michael, B., Smith, J.N., Swift, S., Heffron, F. & Ahmer, B.M. (2001). SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. *Journal of Bacteriology*, 183(19), 5733-5742.

Miller, M.B. & Bassler, B.L. (2001). Quorum sensing in bacteria. *Annual Review of Microbiology*, 2001(55), 165-169.

Mishra, S.K., Basukala, P., Basukala, O., Parajuli, K., Pokhrel, B.M. & Rijal, B.P. (2015). Detection of biofilm production and antibiotic resistance pattern in clinical isolates from indwelling medical devices. *Current Microbiology*, 70(1), 128-134.

Misselwitz, B., Kreibich, S.K., Rout, S., Stecher, B., Periaswamy, B. & Hardt, W.D. (2011). *Salmonella enterica* serovar Typhimurium binds to HeLa cells via Fim-mediated reversible adhesion and irreversible type three secretion system 1-mediated docking. *Infection and Immunity*, 79(1), 330-341.

Molin, S. & Tolker-Nielsen, T. (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology*, 14(3), 255-261.

Moretro, T., Vestby, L.K., Nesse, L.L., Storheim, S.E., Kotlarz, K. & Langsrud, S. (2009). Evaluation of efficacy of disinfectants against *Salmonella* from the feed industry. *Journal of Applied Microbiology*, 106(3), 1005−1012.

Morgan, E., Campbell, J.D., Rowe, S.C., Bispham, J., Stevens, M.P., Bowen, A.J., Barrow, P.A., Maskell, D.J. & Wallis, T.S. (2004). Identification of host-specific colonization factors of *Salmonella enterica* serovar Typhimurium. *Molecular Microbiology*, 54(4), 994-1010.

Morser, K., Puskoor, R. & Zubay, G. (2005). Salmonella. In G. Zubay (Ed.), *Agents of Bioterrorism: Pathogens and Their Weaponization* (pp. 277). New York, USA: Columbia University Press.

Mossong, J., Marques, P., Ragimbeau, C., Huberty-Krau, P., Losch, S., Meyer, G., Moris, G., Strottner, C., Rabsch, W. & Schneider, F. (2007). Outbreaks of monophasic *Salmonella enterica* serovar 4,[5],12:i:- in Luxembourg, 2006. *Euro surveillance*, 12(6), 156-158.

Moulopoulos, S.D. (1998). A Mysterious Death. *New England Journal of Medicine*, 339, 1248-1249.

Mourão, J., Novais, C., Machado, J., Peixe, L. & Antunes, P. (2015). Metal tolerance in emerging clinically relevant multidrug-resistant *Salmonella enterica* serotype

4,[5],12:i:- clones circulating in Europe. *International Journal of Antimicrobial Agents*, 45(6), 610-616.

Mouslim, C., Delgado, M. & Groisman, E.A. (2004). Activation of the RcsC/YojN/RcsB phosphorelay system attenuates *Salmonella* virulence. *Molecular Microbiology*, 54(2), 386-395.

Mukhtar MS, Carvunis AR, Dreze M, Epple P, Steinbrenner J, Moore J, Tasan M, Galli M, Hao T, Nishimura MT, Pevzner SJ, Donovan SE, Ghamsari L, Santhanam B, Romero V, Poulin MM, Gebreab F, Gutierrez BJ, Tam S, Monachello D, Boxem M, Harbort CJ, McDonald N, Gai L, Chen H, Y;, H., European Union Effectoromics Consortium, Vandenhaute J, Roth FP, Hill DE, Ecker JR, Vidal M, Beynon J, Braun P & Dangl JL. (2011). Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science*, 333(6042), 596-601.

Mulvey, M.R., Finley, R., Allen, V., Ang, L., Bekal, S., El Bailey, S., Haldane, D., Hoang, L., Horsman, G., Louie, M., Robberts, L., Wylie, J., McCracken, M., Langner, S., Ahmed, R., Tabor, H. & Gilmour, M. (2013). Emergence of multidrug-resistant *Salmonella enterica* serotype 4,[5],12:i:- involving human cases in Canada: results from the Canadian Integrated Program on Antimicrobial Resistance Surveillance (CIPARS), 2003-10. *Journal of Antimicrobial Chemotherapy*, 68(9), 1982-1986.

O'Mahony, M., Cowden, J., Smyth, B., Lynch, D., Hall, M., Rowe, B., Teare, E.L., Tettmar, R.E., Rampling, A.M., Coles, M., Gilbert, R.J., Kingcott, E. & Bartlett, C.L.R. (1990). An outbreak of *Salmonella* saint-paul infection associated with beansprouts. *Epidemiology and Infection*, 104(2), 229-235.

O'Toole, G., Kaplan, H.B. & Kolter, R. (2000). Biofilm formation as microbial development. *Annual Review of Microbiology*, 2000(54), 49-79.

Regulation (EC) No 2160/2003 of the European Parliament and of the Council of 17 November 2003 on the control of *Salmonella* and other specified food-borne zoonotic agents (2003). Acessed available at

Oliveira, K., Oliveira, T., Teixeira, P., Azeredo, J., Henriques, M. & Oliveira, R. (2006). Comparison of the adhesion ability of different *Salmonella* enteritidis serotypes to materials used in kitchens. *Journal of Food Protection*, 69(19), 2352-2356.

Oni, R.A., Sharma, M. & Buchanan, R.L. (2015). Survival of *Salmonella enterica* in Dried Turkey Manure and Persistence on Spinach Leaves. *Journal of Food Protection*, 78(10), 1791-1799.

Paesold, G., Guiney, D.G., Eckmann, L. & Kagnoff, M.F. (2002). Genes in the *Salmonella* pathogenicity island 2 and the *Salmonella* virulence plasmid are essential for *Salmonella*-induced apoptosis in intestinal epithelial cells. *Cellular Microbiology*, 4(11), 771-781.

Parsons, B.N., Crayford, G., Humphrey, T.J. & Wigley, P. (2013). Infection of chickens with antimicrobial-resistant *Salmonella enterica* Typhimurium DT193 and monophasic *Salmonella* Typhimurium-like variants: an emerging risk to the poultry industry? *Avian Pathology*, 42(5), 443-446.

Pontes, M.H., Lee, E.J., Choi, J. & Groisman, E.A. (2015). *Salmonella* promotes virulence by repressing cellulose production. *Proceedings of the National Academy of Sciences of the United States of America*, 112(16), 5183-5188.

Prouty, A.M. & Gunn, J.S. (2003). Comparative analysis of *Salmonella enterica* serovar Typhimurium biofilm formation on gallstones and on glass. *Infection and Immunity*, 71(12), 7154-7158.

Ranieri, M.L., Shi, C., Moreno Switt, A.I., den Bakker, H.C. & Wiedmann, M. (2013). Comparison of typing methods with a new procedure based on sequence characterization for *Salmonella* serovar prediction. *Journal of Clinical Microbiology*, 51(6), 1786-1797.

Reeves, P.P. & Wang, L. (2013). Genomic organization of LPS-specific loci. *Current Topics in Microbiology and Immunology*, 264(2013), 109-135.

Rendueles, O. & Ghigo, J.M. (2015). Mechanisms of competition in biofilm communities. *Microbiology Spectrum*, 3(3), 1-18.

Roberts, T. (1988). Salmonellosis control: estimated economic costs. *Poultry Science*, 67(6), 936-943.

Rodrigues, D., Teixeira, P., Oliveira, R. & Azeredo, J. (2011). *Salmonella enterica* Enteritidis biofilm formation and viability on regular and triclosan-impregnated bench cover materials. *Journal of Food Protection*, 74(1), 32-37.

Rodríguez, I., Jahn, S., Schroeter, A., Malorny, B., Helmuth, R. & Guerra, B. (2012). Extended-spectrum β-lactamases in German isolates belonging to the emerging monophasic *Salmonella enterica* subsp. *enterica* serovar Typhimurium 4,[5],12:i:- European clone. *Journal of Antimicrobial Chemotherapy*, 67(2), 505-508.

Römling, U. (2002). Molecular biology of cellulose production in bacteria. *Research in Microbiology*, 153(4), 205-212.

Römling, U., Bokranz, W., Rabsch, W., Zogaj, X., Nimtz, M. & Tschape, H. (2003). Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. *International Journal of Medical Microbiology*, 293(4), 273−285.

Römling, U., Rohde, M., Olsén, A., Normark, S. & Reinköster, J. (2000). AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella* typhimurium regulates at least two independent pathways. *Molecular Microbiology*, 36(1), 10-23.

Rotger, R. & Casadesús, J. (1999). The virulence plasmids of *Salmonella*. *International Microbiology*, 2(3), 177-184.

Ruiz-Albert, J., Yu, X.J., Beuzón, C.R., Blakey, A.N., Galyov, E.E. & Holden, D.W. (2002). Complementary activities of SseJ and SifA regulate dynamics of the *Salmonella* typhimurium vacuolar membrane. *Molecular Microbiology*, 44(3), 645-661.

Ryan, M.P., Dillon, C. & Adley, C.C. (2011). Nalidixic acid-resistant strains of *Salmonella* showing decreased susceptibility to fluoroquinolones in the midwestern region of the Republic of Ireland due to mutations in the gyrA gene. *Journal of Clinical Microbiology*, 49(5), 2077-2079.

Rychlik I, Karasova D, Sebkova A, Volf J, Sisak F, Havlickova H, Kummer V, Imre A, Szmolka A & Nagy B. (2009). Virulence potential of five major pathogenicity islands (SPI-1 to SPI-5) of *Salmonella enterica* serovar *Enteritidis* for chickens. *BMC Microbiology*, 9(268), 1-9.

Rychlik, I., Gregorova, D. & Hradecka, H. (2006). Distribution and function of plasmids in *Salmonella enterica*. *Veterinary Microbiology*, 112(1), 1-10.

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

Sanderson, K.E., Sciore, P., Liu, S.L. & Hessel, A. (1993). Location of IS200 on the genomic cleavage map of *Salmonella* typhimurium LT2. *Journal of Bacteriology*, 175(23), 7624-7628.

Saroj, S.D., Shashidhar, R., Karani, M. & Bandekar, J.R. (2008). Distribution of *Salmonella* pathogenicity island (SPI)-8 and SPI-10 among different serotypes of *Salmonella*. *Journal of Medical Microbiology*, 57(Pt 4), 424-427.

Scher, K., Romling, U. & Yaron, S. (2005). Effect of heat, acidification, and chlorination on *Salmonella enterica* serovar Typhimurium cells in a biofilm formed at the air-liquid interface. *Applied and Environmental Microbiology*, 71(3), 1163-1168.

Seixas, R., Gabriel, M., Machado, J., Tavares, L., Bernardo, F. & Oliveira, M. (2014a). Effect of simulated gastrointestinal conditions on biofilm formation by *Salmonella* 1,4,[5],12:i:-. *The ScientificWorldJournal*, 2014(153956), 1-5.

Seixas, R., Machado, J., Bernardo, F., Vilela, C. & Oliveira, M. (2014b). Biofilm formation by *Salmonella enterica* serovar 1,4,[5],12:i:- Portuguese isolates: a phenotypic, genotypic, and socio-geographic analysis. *Current Microbiology*, 68(5), 670-677.

Seixas, R., Varanda, D., Bexiga, R., Tavares, L. & Oliveira, M. (2015). Biofilm-formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolates from subclinical mastitis in conditions mimicking the udder environment. *Polish Journal of Veterinary Sciences*, 18(4), 787-792.

Shah, D.H., Lee, M.J., Park, J.H., Lee, J.H., Eo, S.K., Kwon, J.T. & Chae, J.S. (2005). Identification of *Salmonella* Gallinarum virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis. *Microbiologyopen*, 151(Pt12), 3957-3968.

Silveira, L., Marques, A. & Machado, J. (2013). Infecções por *Salmonella enterica* no período entre 2000–2012. *Boletim Epidemiológico do Instituto Nacional de Saúde Dr Ricardo Jorge 2013*, Artigos breves n.6, 14-16.

Smith, A.C., Cirulis, J.T., Casanova, J.E., Scidmore, M.A. & Brumell, J.H. (2005). Interaction of the *Salmonella*-containing vacuole with the endocytic recycling system. *Journal of Biological Chemistry*, 280(26), 24634-24641.

Smith, J.L., Fratamico, P.M. & Yan, X. (2011). Eavesdropping by bacteria: the role of SdiA in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium quorum sensing. *Foodborne Pathogens and Disease*, 8(2), 169-178.

Smith, J.N. & Ahmer, B.M. (2003). Detection of other microbial species by *Salmonella*: expression of the SdiA regulon. *Journal of Bacteriology*, 185(4), 1357-1366.

Snellings NJ, J.E., Baron LS,. (1977). Genetic basis of Vi antigen expression in *Salmonella* Paratyphi C. *Journal of Bacteriology*, 131(1), 57-62.

Solano, C., García, B., Valle, J., Berasain, C., Ghigo, J.M., Gamazo, C. & Lasa, I. (2002). Genetic analysis of *Salmonella* Enteritidis biofilm formation: critical role of cellulose. *Molecular Microbiology*, 43(3), 793-808.

Solomon, E.B., Niemira, B.A., Sapers, G.M. & Annous, B.A. (2005). Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. *Journal of Food Protection*, 68(5), 906-912.

Song, F., Koo, H. & Ren, D. (2015). Effects of material properties on bacterial adhesion and biofilm formation. *Journal of Dental Research*, 94(8), 1027-1034.

Soper, G.A. (1907). The work of a chronic typhoid germ distributor. *Journal of the American Medical Association*, 48, 2019–2022.

Soyer, Y., Moreno Switt, A., Davis, M.A., Maurer, J., McDonough, P.L., Schoonmaker-Bopp, D.J., Dumas, N.B., Root, T., Warnick, L.D., Gröhn, Y.T. & Wiedmann, M. (2009). *Salmonella enterica* serotype 4,5,12:i:-, an emerging *Salmonella* serotype that represents multiple distinct clones. *Journal of Clinical Microbiology*, 47(11), 3546-3556.

Spadoni, I., Zagato, E., Bertocchi, A., Paolinelli, R., Hot, E., Di Sabatino, A., Caprioli, F., Bottiglieri, L., Oldani, A., Viale, G., Penna, G., Dejana, E. & Rescigno, M. (2015). A gut-vascular barrier controls the systemic dissemination of bacteria. *Science*, 350(6262), 830-834.

Stanley, T.L., Ellermeier, C.D. & 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. *Journal of Bacteriology*, 182(16), 4406-4413.

Steele-Mortimer, O. (2008). The *Salmonella*-containing vacuole: moving with the times. *Current Opinion in Microbiology*, 11(1), 38-45.

Steele-Mortimer, O., Méresse, S., Gorvel, J.P., Toh, B.H. & Finlay, B.B. (1999). Biogenesis of *Salmonella* typhimurium-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cellular Microbiology*, 1(1), 33-49.

Steenackers, H., Hermans, K., Vanderleyden, J. & De Keersmaecke, S.C.J. (2012). *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. *Food Research International*, 45(2012), 502-531.

Stepanović, S., Cirkovic, I., Mijac, V. & Svabic-Vlahovic, M. (2003). Influence of the incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. *Food Microbiology*, 20(3), 339-343.

Stepanović, S., Cirković, I., Ranin, L. & Svabić-Vlahović, M. (2004). Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Letters in Applied Microbiology*, 38(5), 428-432.

Stepanović, S., Vukovic, D., Jezek, P., Pavlovic, M. & Svabic-Vlahovic, M. (2001). Influence of dynamic conditions on biofilm formation by staphylococci. *European Journal of Clinical Microbiology & Infectious Diseases*, 20(7), 502-504.

Stupack, D.G., Cho, S.Y. & Klemke, R.L. (2000). Molecular signaling mechanisms of cell migration and invasion. *Immunologic Research*, 21(2-3), 83-88.

Suez, J., Porwollik, S., Dagan, A., Marzel, A., Schorr, Y.I., Desai, P.T., Agmon, V., McClelland, M., Rahav, G. & Gal-Mor, O. (2013). Virulence gene profiling and pathogenicity characterization of non-typhoidal *Salmonella* accounted for invasive disease in humans. *PLoS One*, 8(3), 1-21.

Switt, A.I.M., Soyer, Y., Warnick, L.D. & Wiedmann, M. (2009). Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i:-. *Foodborne Pathogens and Disease*, 6(4), 407-415.

Swofford, C.A., Van Dessel, N. & Forbes, N.S. (2015). Quorum-sensing *Salmonella* selectively trigger protein expression within tumors. *Proceedings of the National Academy of Sciences of the United States of America*, 112(11), 3457-3462.

Szeto, J., Namolovan, A., Osborne, S.E., Coombes, B.K. & Brumell, J.H. (2009). Salmonella-Containing Vacuoles display centrifugal movement associated with cell-to-cell transfer in epithelial cells. *Infection and Immunity*, 77(3), 996-1007.

Tavechio, A.T., Ghilardi, A.C.R. & Fernandes, S.A. (2004). Multiplex PCR" identification of the atypical and monophasic *Salmonella* enterica subsp. *enterica* serotype 1,4,[5],12:i:- in São Paulo State, Brazil: frequency and antibiotic resistance patterns. *Revista do Instituto de Medicina Tropical de São Paulo*, 46(2), 115-117.

Tavechio, A.T., Sueli, A.F., GhilardiI, A.C.R., Soule, G., Ahmed, R. & Melles, C.E.A. (2009). Tracing lineage by phenotypic and genotypic markers in *Salmonella enterica* subsp. *enterica* serovar 1,4,[5],12:i:- and *Salmonella* Typhimurium isolated in state of São Paulo, Brazil. *Memórias do Instituto Oswaldo Cruz*, 104(7), 1042-1046.

Tennant, S.M., Hartland, E.L., Phumoonna, T., Lyras, D., Rood, J.I., Robins-Browne, R.M. & van Driel, I.R. (2008). Influence of gastric acid on susceptibility to infection with ingested bacterial pathogens. *Infection and Immunity*, 76(2), 639–645.

Teughels, W., Van Assche, N., Sliepen, I. & Quirynen, M. (2006). Effect of material characteristics and/or surface topography on biofilm development. *Clinical Oral Implants Research*, 17(2), 68-81.

Thornbrough, J.M. & Worley, M.J. (2012). A naturally occurring single nucleotide polymorphism in the *Salmonella* SPI-2 type III effector srfH/sseI controls early extraintestinal dissemination. *PLoS One*, 7(9), 1-7.

Tu, L.T., Hoang, N.V., Cuong, N.V., Campbell, J., Bryant J E, Hoa, N.T., Kiet, B.T., Thompson, C., Duy, D.T., Phat, V.V., Hien, V.B., Thwaites, G., Baker, S. & Carrique-Mas, J.J. (2015). High levels of contamination and antimicrobial-resistant non-typhoidal *Salmonella* serovars on pig and poultry farms in the Mekong Delta of Vietnam. *Epidemiology and Infection*, 143(14), 3074-3086.

van Asten, A.J. & van Dijk, J.E. (2005). Distribution of "classic" virulence factors among *Salmonella* spp. *FEMS Immunology and Medical Microbiology*, 44(3), 251-259.

van der Velden, A.W., Velasquez, M. & Starnbach, M.N. (2003). *Salmonella* rapidly kill dendritic cells via a caspase-1-dependent mechanism. *Journal of Immunology*, 171(12), 6742-6749.

Van Houdt, R. & Michiels, C.W. (2010). Biofilm formation and the food industry, a focus on the bacterial outer surface. *Journal of Applied Microbiology*, 109(4), 1117-1131.

Vazquez-Torres, A., Jones-Carson, J., Bäumler, A.J., Falkow, S., Valdivia, R., Brown, W., Le, M., Berggren, R., Parks, W.T. & Fang, F.C. (1999). Extraintestinal dissemination of *Salmonella* by CD18-expressing phagocytes. *Nature*, 401(6755), 804-808.

Vestby, L.K., Moretro, T., Langsrud, S., Heir, E. & Nesse, L.L. (2009). Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal- and feed factories. *BMC Veterinary Research*, 5(20), 1-6.

Vuong, C. & Otto, M. (2002). *Staphylococcus epidermidis* infections. *Microbes and Infection*, 4(4), 481-489.

Wagner, C. & Hensel, M. (2011). Adhesive mechanisms of *Salmonella* enterica. *Advances in Experimental Medicine and Biology*, 2011(715), 17-34.

Wasyl, D. & Hoszowski, A. (2012). Occurrence and characterization of monophasic *Salmonella enterica* serovar Typhimurium (1,4,[5],12:i:-) of non-human origin in Poland. *Foodborne Pathogens and Disease*, 9(11), 1037-1043.

Watson, K.G. & Holden, D.W. (2010). Dynamics of growth and dissemination of *Salmonella in vivo*. *Cellular Microbiology*, 12(10), 1389-1397.

Wattiau, P., Boland, C. & Bertrand, S. (2011). Methodologies for *Salmonella enterica* subsp. *enterica* subtyping: gold standards and alternatives. *Applied and Environmental Microbiology*, 77(22), 7877-7885.

White, A.P., Gibson, D.L., Grassl, G.A., Kay, W.W., Finlay, B.B., Vallance, B.A. & Surette, M.G. (2008). Aggregation via the red, dry, and rough morphotype is not a virulence adaptation in *Salmonella enterica* serovar Typhimurium. *Infection and Immunity*, 76 (3), 1048−1058.

WHO Global Foodborne Infections Network. (2010). Laboratory protocol: isolation of *Salmonella* spp. from food and animal faeces. 5th Edition. Accessed 10th January, 2016, available at http://www.antimicrobialresistance.dk/232-169-215-protocols.htm

Wilson, M. (2001). Bacterial biofilms and human disease. *Science Progress*, 84(Pt 3), 235-254.

Wise, M.G., Siragusa, G.R., Plumblee, J., Healy, M., Cray, P.J. & Seal, B.S. (2009). Predicting *Salmonella enterica* serotypes by repetitive sequence-based PCR. *Journal of Microbiological Methods*, 76(1), 18-24.

Wood, M.W., Jones, M.A., Watson, P.R., Hedges, S., Wallis, T.S. & Galyov, E.E. (1998). Identification of a pathogenicity island required for *Salmonella* enteropathogenicity. *Molecular Microbiology*, 29(3), 883-891.

Worley, M.J., Nieman, G.S., Geddes, K. & Heffron, F. (2006). *Salmonella* Typhimurium disseminates within its host by manipulating the motility of infected cells. *Proceedings of the National Academy of Sciences of the United States of America*, 103(47), 17915-17920.

Xue, T., Chen, X. & Shang, F. (2014). Short communication: Effects of lactose and milk on the expression of biofilm-associated genes in *Staphylococcus aureus* strains isolated from a dairy cow with mastitis. *Journal of Dairy Science*, 97(10), 6129-6134.

Yamamoto, S. & Kutsukake, K. (2006). FljA-mediated posttranscriptional control of phase 1 flagellin expression in flagellar phase variation of *Salmonella enterica* serovar Typhimurium. *Journal of Bacteriology*, 188(3), 958-967.

Yang, X., Wu, Q., Zhang, J., Huang, J., Guo, W. & Cai, S. (2015). Prevalence and characterization of monophasic *Salmonella* serovar 1,4,[5],12:i:- of food origin in China. *PLoS One*, 10(9), 1-10.

Yoon, H., Ansong, C., Adkins, J.N. & Heffron, F. (2011a). Discovery of *Salmonella* virulence factors translocated via outer membrane vesicles to murine macrophages. *Infection and Immunity*, 79(6), 2182-2192.

Yoon, H., Ansong, C., Adkins, J.N. & Heffron, F. (2011b). Discovery of *Salmonella* virulence factors translocated via outer membrane vesicles to murine macrophages. *Infection and Immunity*, 79(6), 2182-2192.

Zakikhany, K., Harrington, C.R., Nimtz, M., Hinton, J.C. & Römling, U. (2010). Unphosphorylated CsgD controls biofilm formation in *Salmonella enterica* serovar Typhimurium. *Molecular Microbiology*, 77(3), 771–786.

Zamperini, K., Soni, V., Waltman, D., Sanchez, S., Theriault, E.C., Bray, J. & Maurer, J.J. (2007). Molecular characterization reveals *Salmonella enterica* serovar 4,[5],12:i:− from poultry is a variant Typhimurium serovar. *Avian Diseases*, 51(4), 958-964.

Zhou, D., Chen, L.M., Hernandez, L., Shears, S.B. & 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. *Molecular Microbiology*, 39(2), 248-259.

Zottola, E.A. & Sasahara, K.C. (1994). Microbial biofilms in the food processing industry--should they be a concern? *International Journal of Food Microbiology*, 23(2), 125-148.