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Natural compounds as new antibacterials to control *Campylobacter* spp.

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"Be the change that you wish to see in the world."

Mahatma Gandhi

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Resumo alargado

As doenças de origem alimentar continuam a ser um problema comum em todo o mundo. Ainda que possam ser provocadas por diversos agentes, são as bactérias, os vírus ou os parasitas a principal causa das infeções alimentares. O consumo ou processamento de alimentos contaminados têm sido descritos como as principais vias de transmissão para os humanos. *Campylobacter jejuni* e *Campylobacter coli* têm sido descritos como a principal causa de gastroenterite bacteriana em seres humanos em todo o mundo e em 2013, na União Europeia, a campilobacteriose, infeção causada por *Campylobacter*, foi a zoonose mais relatada. Nos seres humanos, as espécies de *Campylobacter* têm sido associadas a uma variedade de condições gastrointestinais, tais como gastroenterite, doenças inflamatórias do intestino, cancro colon-rectal, síndrome do intestino irritável, entre outras e também podem provocar manifestações extra-gastrointestinais, como bacteremia, infeções pulmonares e abcessos. As complicações pós-infeção por este microrganismo incluem a artrite reativa e podem conduzir a doenças auto-imunes, tais como a síndrome de Guillain-Barré. A maioria das infeções por *Campylobacter* não necessita de intervenção terapêutica sendo apenas necessária reidratação. No entanto, em pacientes imunodeprimidos, pacientes cujos sintomas são severos ou persistentes e aqueles com infeções extra-intestinais é utilizado tratamento antimicrobiano, sendo os antibióticos mais utilizados a eritromicina e a ciprofloxacina. No entanto, em todo o Mundo tem-se verificado que as estirpes de *Campylobacter* são cada vez mais resistentes a antibióticos, incluindo os usados em humanos. Para além disso também têm sido descritas estirpes de *Campylobacter* resistentes a desinfetantes, o que se deve, principalmente, à sua capacidade de formar biofilmes. Estes biofilmes são um problema emergente na indústria alimentar, aumentando a possibilidade de contaminações ao longo da cadeia alimentar. Assim, e como os estudos relativos a *Campylobacter* são escassos, é da maior relevância estudar a epidemiologia de espécies de *Campylobacter* isoladas em Portugal, a sua resistência a antibióticos e procurar alternativas, aos antimicrobianos convencionais, para o seu controlo.

Devido à escassez de estudos relativos à epidemiologia de *Campylobacter* em Portugal procedeu-se neste trabalho ao estudo da distribuição epidemiológica de isolados de *Campylobacter* de seres humanos, entre 2009 e 2012, em Portugal. Para isso, foram analisadas 837 estirpes obtidas através do Instituto Nacional de Saúde Dr. Ricardo Jorge. Destas 837 estirpes, 84,5% foram identificados como *C. jejuni*, 14,8% como *C. coli*, 0,2% como *C. upsaliensis*, 0,1% como *C. concisus* e 0,2% das amostras foram identificadas como *Arcobacter butzleri*. Em relação à sua distribuição por faixas etárias, observou-se que 61,5% das estirpes pertenciam ao grupo com idades entre 1 e 15 anos. Após este estudo preliminar, a partir deste grupo de isolados humanos, escolheram-se aleatoriamente 125 estirpes de *Campylobacter* (*C. jejuni* e *C. coli*) isoladas de seres humanos. A este grupo de estirpes,

adicionaram-se 39 isolados de retalho alimentar e 32 de animais. As 196 amostras foram então caracterizadas através da tipagem por sequenciamento multilocus e da tipagem do gene *flaA*. Através destes métodos de tipagem, observou-se que as estirpes de *C. coli* eram geneticamente mais conservadas do que *C. jejuni* e que dentro de cada espécie, existiam isolados geneticamente relacionados provenientes de fontes diferentes. Em seguida, o fenótipo de resistência ao ácido nalidíxico, amoxicilina, ciprofloxacina, eritromicina, gentamicina e tetraciclina foi avaliado pelo método de diluição em agar. Observaram-se elevadas taxas de resistência para todos os antibióticos com exceção à gentamicina, incluindo para os antibióticos utilizados no tratamento de campilobacteriose grave em humanos. Além disso, observou-se um fenótipo de resistência a múltiplos antibióticos (resistência a 3 ou mais classes de antibióticos) em 86% dos isolados. Perante os elevados níveis de resistência observados, estudaram-se os mecanismos moleculares subjacentes a essas resistências. Verificou-se que todos os isolados resistentes à ciprofloxacina possuíam a mutação Thr-Ile-86 na região que determina resistências às quinolonas no gene *gyrA*. Para a resistência à eritromicina apenas foi detetada a mutação A2075G no gene 23S rRNA. Em relação à resistência à gentamicina, observou-se que as três estirpes resistentes à gentamicina possuíam o marcador de resistência aos aminoglicosídeos *aphA-3*, sendo que uma das estirpes tinha uma mutação neste marcador. Foi ainda evidenciado neste estudo, que as bombas de efluxo cmeABC também podem desempenhar um papel na resistência a múltiplas drogas e no fenótipo da resistência a gentamicina. Assim, neste estudo foi possível obter uma visão geral da epidemiologia de *Campylobacter* em Portugal e descrever pela primeira vez a elevada taxa de multirresistência a antibióticos, assim como realçar o surgimento de estirpes de *Campylobacter* resistentes aos antibióticos de uso humano. Com base nos resultados deste estudo foram selecionadas estirpes com diferentes perfis genéticos e de resistência a antibióticos para serem utilizadas no decurso deste trabalho.

Como o género *Campylobacter* é a principal causa de gastroenterite bacteriana e a via alimentar é a principal via de contaminação, a elevada percentagem de estirpes resistentes a antibióticos aumenta o potencial zoonótico da infeção com estirpes multirresistentes. Logo, torna-se necessário controlar o crescimento de *Campylobacter* nas vias mais comuns de contaminação, que são os alimentos. Assim, o objetivo seguinte deste trabalho foi avaliar o potencial do resveratrol para controlar as estirpes de *Campylobacter* previamente caracterizadas. Apesar do resveratrol possuir várias propriedades biológicas, incluindo antimicrobianas, a sua baixa solubilidade em água e alta instabilidade comprometem a sua aplicação. Assim, para ultrapassar estes problemas, estudou-se o encapsulamento do resveratrol com metil-ß-ciclodextrina. Verificou-se que a complexação do resveratrol com esta ciclodextrina provocou um aumento de 400 vezes na sua solubilidade. Em seguida o complexo de inclusão foi caracterizado através de Espectroscopia de Infravermelho por Transformada de Fourier (FTIR), Calorimetria Exploratória Diferencial (DSC), Difração de Raios-X (XRD) e Microscopia Eletrónica de Varrimentos (SEM), confirmando-se efetivamente a

sua formação. Seguidamente foram avaliadas algumas propriedades biológicas do resveratrol e do seu complexo de inclusão com metil-ß-ciclodextrina, tendo-se verificado que ambos os compostos tinham atividade antioxidante muito forte, baixa toxicidade e ainda capacidade de reduzir a viabilidade das células Caco-2 (linha celular constituída por células epiteliais de adenocarcinoma colorectal heterogéneo). Além disso, também foi demonstrada a sua atividade antibacteriana contra estirpes de *Campylobacter* previamente selecionadas: duas estirpes de referência (*C. jejuni ATCC* 33560 e *C. coli* ATCC 33559), duas estirpes isoladas de fezes de pacientes com gastroenterite aguda (*C. coli* 53 e *C. coli* 873) e duas estirpes isoladas de carne de aves fresca (*C. jejuni* 225421 e *C. coli* 219872). Estes resultados sugerem que o resveratrol e o seu complexo de inclusão podem ser usados para controlar *Campylobacter* e que o resveratrol encapsulado em metil-ß-ciclodextrina mantem as suas propriedades antioxidantes e antibacterianas. Uma vez que os resultados com o resveratrol e o complexo de inclusão foram bastante promissores, estudou-se ainda outro complexo de inclusão (resveratrol-hidroxipropil-γ-ciclodextrina), previamente formado e caraterizado pelo grupo de investigação, que revelou ainda ter um potencial antimicrobiano mais elevado contra *Campylobacter* e *A. butzleri*, um patogéneo de origem alimentar relacionado a *Campylobacter*. Dado isto, inicialmente avaliou-se a atividade antibacteriana em células planctónicas e mostrou-se que tanto o resveratrol como o complexo de inclusão têm um efeito bactericida contra as estirpes multirresistentes *C. coli* 873 e *C. jejuni* 225421. Com o objetivo de esclarecer o potencial mecanismo de ação do complexo de inclusão do resveratrol em hidroxipropil-γ-ciclodextrina sobre as estirpes de *Campylobacter*, começou por se avaliar a despolarização das membranas celulares e a atividade metabólica por citometria de fluxo. Através desta técnica, observou-se que complexo de inclusão pode atuar induzindo a despolarização da membrana e afetando a atividade metabólica das células. Dado que os biofilmes bacterianos são um problema emergente na indústria alimentar, também foi avaliado o potencial destes dois compostos para inibir a formação de biofilmes e eliminar biofilmes estabelecidos. Tanto o resveratrol como o complexo de inclusão foram capazes de inibir a formação de biofilmes e diminuir biofilmes previamente estabelecidos, mesmo em concentrações sub-inibitórias. O sistema *quorum sensing* (QS) tem sido associado à resistência antimicrobiana e formação de biofilmes, portanto, o potencial anti-QS destes dois compostos também foi estudado através da utilização de uma estirpe biossensor (*Chromobacterium violaceum* ATCC 12472). Verificou-se que ambos, o resveratrol e o complexo de inclusão, foram capazes de inibir o sistema QS, o que pode explicar o efeito anti-biofilme destes compostos. Assim, nestes estudos foram demonstradas as propriedades antimicrobianas e anti-biofilme do resveratrol e complexo de inclusão em estirpes de *Campylobacter*. Este aspeto associado ao facto de o resveratrol ser um composto de origem natural, e de também apresentar forte atividade antioxidante, encorajam futuros estudos com vista à sua aplicação como potencial conservante alimentar.

Finalmente, uma vez que tem havido um interesse crescente na utilização de compostos naturais para aplicação em produtos alimentares, também foi avaliado o potencial do óleo essencial de coentros (*Coriandrum sativum* L.) e do seu principal composto, o linalool, para controlar *Campylobacter*. Ambos os compostos exibiram um efeito bactericida contra as quatro estirpes testadas (*C. jejuni* ATCC 33560, *C. coli* ATCC 33559, *C. jejuni* 225421 e *C. c*oli 873) com valores de concentração mínima inibitória entre 0,5 e 1 µL/mL e observou-se que os compostos voláteis do óleo essencial de coentros também inibiram o crescimento de *Campylobacter*. Observou-se ainda que ambos os compostos inibiram a formação de biofilmes e promoveram a dispersão de biofilmes de *Campylobacter*. Como descrito anteriormente, também foi estudado o efeito destes compostos sobre o sistema QS. Foi demonstrada a atividade anti-QS do óleo essencial de coentros e do linalool através da inibição da produção de violaceína pela estirpe biossensor *C. violaceum*. Assim, mais uma vez, esta atividade anti-QS pode estar associada com a atividade anti-biofilme dos compostos, uma vez que o QS tem um papel importante na regulação da formação e desenvolvimento de biofilmes. Para além do potencial anti-bacteriano e anti-biofilme do óleo essencial e do linalool, também se avaliou a sua atividade antioxidante, uma vez que o processo de oxidação está relacionado com a perda da qualidade dos alimentos. Assim, observou-se que ambos os compostos têm uma elevada capacidade para inibir a peroxidação de lípidos. Em suma, os resultados demonstraram que estes compostos naturais podem ser utilizado para controlar *Campylobacter* e também como agentes antioxidantes para melhorar a qualidade dos alimentos.

Em conclusão, neste trabalho, foram apresentados, dados recentes referentes à epidemiologia de *Campylobacter* em Portugal, bem como a sua diversidade genética e respetivos perfis de resistência a antibióticos. Diante disto, são necessários novos agentes antimicrobianos para controlar este patogéneo emergente de origem alimentar. Neste trabalho mostrou-se que o resveratrol e o óleo essencial de coentros, ambos compostos naturais, têm a capacidade de reduzir células planctónicas e biofilmes de *Campylobacter*, possuindo também várias atividades biológicas, incluindo propriedades antioxidantes. Este trabalho permitiu alargar o conhecimento sobre a epidemiologia e taxas resistência de estirpes de *Campylobacter* isoladas em Portugal e desenvolver novas estratégias de controlo deste microrganismo baseadas na utilização de compostos de origem natural.

Palavras-chave

Campylobacter, epidemiologia, resistência a antibióticos, resveratrol, óleo essencial de coentros, atividade antibacteriana, atividade anti-biofilmes, atividade anti-*quorum sensing*, potencial antioxidante.

Abstract

Foodborne diseases remain common around the world and can be caused by a variety of agents, being bacteria, viruses or parasites the main causes of such infections. Moreover, the major cause of foodborne diseases is consumption or handling of contaminated food. *Campylobacter jejuni* and *Campylobacter coli* are the major cause of bacterial gastroenteritis in humans worldwide and are increasingly resistant to antimicrobial agents mainly due to its ability to form biofilms. Furthermore, campylobacteriosis was the most commonly reported zoonosis in the European Union in 2013. So, the main objective of this work was to study the epidemiology of *Campylobacter* species in Portugal and to explore the potential of natural compounds as new antibacterials to control this foodborne pathogen and to increase the shelf life of food products.

We started this work with an epidemiological study of *Campylobacter* in Portugal. Firstly we studied the epidemiological distribution of *Campylobacter* isolates from humans, between 2009 and 2012, in Portugal. For this, 837 strains from the National Institute of Health Dr Ricardo Jorge were analyzed, of which 84.5% were identified as *C. jejuni*, 14.8% as *C. coli*, 0.2% as *C. upsaliensis*, 0.1% as *C. concisus* and 0.2% of the samples were identified as *Arcobacter butzleri*. Concerning the distribution per age groups, we observed that 61.5% of the strains belonged to the group aged between 1 and 15 years. Afterward, from the group of 837 strains, we randomly choose 125 *Campylobacter* isolates from humans. In addition to these isolates, we added strains isolated from different sources: 39 from retail food and 32 from animals. All the 196 strains were then characterized by multilocus sequence typing (MLST) and *flaA* typing. We found that the *C. coli* isolates were genetically more conserved than *C. jejuni*, and within each species, genetically related isolates were recovered from different sources. Then, the resistance phenotype to six antibiotics was evaluated by the agar dilution method. We observed high resistance rates to several antibiotics, including the ones used in the treatment of severe campylobacteriosis. We also identified a multidrug resistance phenotype in 86% of the isolates. Once this high resistance to antibiotics was confirmed, we decided to study the underlying molecular mechanisms. In all the ciprofloxacin resistant isolates we found the Thr-86-Ile mutation in the quinolone-resistance-determining regions (QRDR) in the DNA gyrase gene (*gyrA*). For the erythromycin resistance only the mutation A2075G was detected. Regarding gentamicin resistance, we found the three gentamicin-resistant isolates harboured the *aphA-3* aminoglycoside resistance marker, with one strain having a point mutation. In addition, we showed that cmeABC efflux pumps may also play a role in the multidrug resistance phenotype and in the gentamicin resistance. In sum, the results obtained in this first part of the study gave an overview of the *Campylobacter* epidemiology in Portugal and worrying antibiotic multi-resistance rate. This part also highlighted the emergence of *Campylobacter* strains resistant to antibiotics commonly used in

humans. Then, we select a group of strains with different genetic and antibiotic profiles to be used in the subsequent steps of our work.

Since the main source of *Campylobacter* infection is through contaminated food, it is necessary to find new strategies to control the growth of *Campylobacter* in the most common way of contamination, which are foods. So, in the next step we assessed the potential of resveratrol to control the strains of *Campylobacter* that were characterized previously. However, despite resveratrol having several biological properties, its low aqueous solubility and high instability compromise its application. So, to overcome these limitations, we studied the encapsulation of resveratrol with a methyl-ß-cyclodextrin. We found that resveratrol complexation caused a 400 fold improvement in its solubility. The inclusion complex was characterized by several techniques. After the formation of the inclusion complex, we compared the biological properties of resveratrol and its inclusion complex. We showed that both compounds had very strong antioxidant activity and low toxicity, together with the ability to reduce the viability of Caco-2 cells (heterogeneous human epithelial colorectal adenocarcinoma cell line). In addition, we also demonstrated their antibacterial activity against the previously selected *Campylobacter* strains. These results suggest that resveratrol and its inclusion complex can be used to control *Campylobacter*, since the biological properties are maintained. Due to the very good results obtained with resveratrol and this inclusion complex, we decided to study another inclusion complex (resveratrol-hydroxypropyl-γ-cyclodextrin) and its potential to control *Campylobacter* and *A. butzleri*, a closely related foodborne pathogen. Firstly we evaluated the antibacterial activity against planktonic cells, demonstrating that both resveratrol and inclusion complex have a bactericidal effect against the two microorganisms. The inclusion complex may act by inducing membrane depolarization and by affecting the metabolic activity of the cells. Since bacterial biofilms are an emerging problem in the food industry, we also evaluated the potential of these two compounds against biofilms. We showed that resveratrol and the inclusion complex inhibit biofilm formation and diminish established biofilms, even at sub-inhibitory concentrations. Since the quorum sensing (QS) system has been associated with the antimicrobial resistance and biofilm formation, we also evaluated the potential anti-QS effect of these two compounds by using a biosensor strain. We found that both resveratrol and inclusion complex were able to inhibit the QS system, which could explain their anti-biofilm effect. The results showed that resveratrol could be used as antibacterial and anti-biofilm agent in the food industry, allowing an improve shelf life and an increase in food safety.

Finally, since there has been a growing interest in the use of natural compounds for application in food products, we also evaluated the potential of coriander (*Coriandrum sativum* L.) essential oil and its major compound linalool to control *Campylobacter*. Both compounds showed a bactericidal effect against all the tested strains and we observed that the volatile compounds of the coriander essential oil also inhibited the growth of

Campylobacter. Then, since biofilms are a growing problem in the food industry, we also evaluated the anti-biofilm activity of these compounds. Both coriander oil and linalool inhibited biofilm formation and promoted biofilm dispersion of *Campylobacter* biofilms. As previously described, we also studied the effect of these compounds on the QS system and we observed an anti-QS activity by inhibiting the violacein production. So, once more, this anti-QS activity could be associated with the anti-biofilm activity, since QS has been described to regulate the biofilm formation and development. Moreover, in addition to the antibacterial and anti-biofilm potential, we evaluated the antioxidant activity of coriander oil and linalool, since the oxidation process is also associated with the loss of food quality. We observed that both compounds showed an exceptional ability to inhibit lipid peroxidation. Those results demonstrate that these natural compounds could be used to control *Campylobacter* and as antioxidant to enhance food quality.

In sum, in this thesis, we described the recent epidemiology of *Campylobacter* in Portugal, as well as its genetic diversity and worrying antibiotic resistance rates. In addition, we also demonstrated that resveratrol and coriander essential oil, which are both natural compounds, have the ability to reduce planktonic cells and biofilms of *Campylobacter*, including the multiresistant strains characterized in the first part of the work. Clearly, the results obtained in this work encourage the future use of these natural compounds to control *Campylobacter*.

Keywords

Campylobacter, epidemiology, antibiotic resistance, resveratrol, coriander essential oil, antibacterial activity, anti-biofilm activity, anti-quorum sensing activity, antioxidant potential.

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List of Abbreviations and Acronyms

AHL	Acyl homoserine lactones
AI	Autoinducers
AIDP	Acute inflammatory demyelinating polyneuropathy
AMAN	Acute motor axonal neuropathy
APC	Antigen-presenting cell
Caco-2	Heterogeneous human epithelial colorectal adenocarcinoma cell line
CapA	*Campylobacter* adhesion protein A
CC	Clonal complex
CCDA	Charchoal cefoperazone deoxycholate agar
CD	Cyclodextrin
CDT	Cytolethal distending toxin
Cia	*Campylobacter* invasive antigens
CPS	Capsular polysaccharide
DNA	Deoxyribonucleic Acid
DSC	Differential scanning calorimetry
EFSA	European Food Safety Authority
EO	Essential oil
EPS	Exopolymeric substance
flaA	Flagellin A gene
FRET	Fluorescence resonance energy transfer
FTIR	Fourier transform infrared spectroscopy
GBS	Guillain-Barré syndrome
GRAS	Generally recognized as safe
gyrA	Gyrase gene
IBD	Inflammatory bowel diseases
IBS	Irritable bowel syndrome
INSARJ	National Institute of Health Dr. Ricardo Jorge
IR	Inverted repeat
LOS	Lipooligosaccharides
MDR	Multidrug-resistant
MLST	Multilocus sequence typing
MOMP	Major outer membrane porin
MW	Molecular weight
PCR	Polymerase chain reaction
PEB	Periplasmic binding protein
PFGE	Pulsed-field gel electrophoresis

pVir	Virulence plasmid
QRDR	Quinolone-resistance-determining regions
QS	Quorum sensing
RFLP	Restriction fragment length polymorphism
rRNA	Ribossomal Ribonucleic acid
SEM	Scanning electron microscopy
ST	Sequence type
SVR	Short variable region
T3SS	Type III secretory system
T4SS	Type IV secretion system
T6SS	Type VI secretory system
tRNA	Transfer Ribonucleic acid
WGS	Whole genome sequencing
XRD	X-ray diffraction

List of Publications

Papers included in this Thesis:

I. A infeção humana por *Campylobacter* em Portugal – alguns dados epidemiológicos.
Duarte A., Santos A., Benoliel J., Domingues F.C., Oleastro M.
Boletim epidemiológico "Observações" - Instituto Nacional de Saúde Dr. Ricardo Jorge. 2013; Volume 2 - Número Especial 1: Doenças Infeciosas. ISSN: 0874-2928 | ISSN: 2182-8873.

II. Human, Food and Animal *Campylobacter* spp. isolated in Portugal: high genetic diversity and antibiotic resistance rate.
Duarte A., Santos A., Manageiro V., Martins A., Fraqueza M.J., Caniça M., Domingues F.C., Oleastro M.
International Journal of Antimicrobial Agents. 2014; 44:306-313.
http://dx.doi.org/10.1016/j.ijantimicag.2014.06.012

III. Resveratrol encapsulation with methyl-β-cyclodextrin for antibacterial and antioxidant delivery applications.
Duarte A., Martinho A., Luís Â., Figueiras A., Oleastro M., Domingues F.C., Silva F.
LWT-Food Science and Technology. 2015; 63:1254-1260.
http://dx.doi.org/10.1016/j.lwt.2015.04.004

IV. Resveratrol inclusion complexes: antibacterial and anti-biofilm activity against *Campylobacter* spp. and *Arcobacter butzleri*.
Duarte A., Alves A., Ferreira S., Silva F., Domingues F.C.
Food Research International. 2015; 77:244-250.
http://dx.doi.org/10.1016/j.foodres.2015.05.047.

V. Antioxidant properties of Coriander essential oil and Linalool and their potential to control *Campylobacter* spp.
Duarte A., Luís Â., Oleastro M., Domingues F.C.
Food Control. 2016; 61:115-122.
http://dx.doi.org/10.1016/j.foodcont.2015.09.033

Papers not included in this Thesis:

I. Antifungal activity of *Coriandrum sativum* essential oil, its mode of action against *Candida* species and potential synergism with amphotericin B.
Silva F., Ferreira S., **Duarte A.**, Mendonça D.I., Domingues F.C.
Phytomedicine. 2011; 19:42-47.
http://dx.doi.org/10.1016/j.phymed.2011.06.033

II. Screening of antimicrobial activity of *Cistus ladanifer* and *Arbutus unedo* extracts.
Ferreira S., Santos J., **Duarte A.**, Duarte A.P., Queiroz J.A., Domingues F.C.
Natural Product Research. 2012; 26:1558-1560.
http://dx.doi.org/10.1080/14786419.2011.569504

III. Synergistic activity of coriander oil and conventional antibiotics against *Acinetobacter baumannii*.
Duarte A., Ferreira S., Silva F., Domingues F.C.
Phytomedicine. 2012; 19:236-238.
http://dx.doi.org/10.1016/j.phymed.2011.11.010

IV. Effect of coriander oil (*Coriandrum sativum* L.) on Planktonic and Biofilm Cells of *Acinetobacter baumannii*.
Duarte A.F., Ferreira S., Oliveira R., Domingues F.C.
Natural Product Communications. 2013; 8:673-678.

V. Clinical isolates of *Acinetobacter baumannii* from a Portuguese hospital: PFGE characterization, antibiotic susceptibility and biofilm-forming ability.
Duarte A., Ferreira S., Almeida S., Domingues F.C.
Comparative Immunology, Microbiology and Infectious Diseases. 2016; 45:29-33.

VI. Study of major *Coriandrum sativum* essential oil compounds against *A. baumannii* and effect of Linalool on adhesion, biofilms and quorum-sensing.
Alves S., **Duarte A.**, Sousa S., Domingues F.C.
Biofouling. 2016; 32:155-165.
http://dx.doi.org/10.1080/08927014.2015.1133810

VII. Characterization and antimicrobial activity of cellulose derivatives films incorporated with a resveratrol inclusion complex.
Silva A., **Duarte A.**, Sousa S., Ramos A., Domingues F.C.
Journal of Food Science and Technology. 2016 (submitted for publication)

VIII. Chemical composition, antioxidant, antibacterial and anti-quorum sensing activities of *Eucalyptus globulus* and *Eucalyptus radiata* essential oils.
Luís Â., **Duarte A.**, Gominho J., Domingues F., Duarte A.P.
Industrial Crops and Products. 2016; 79:274-282.
http://dx.doi.org/10.1016/j.indcrop.2015.10.055

List of Scientific Communications

Oral scientific communications:

- High antibiotic resistance of *Campylobacter* spp. isolated in Portugal.
  Duarte A., Domingues F.C., Oleastro M.
  I-IC2AR - Ist Internacional Caparica Conference in Antibiotic Resistance. Caparica, Portugal. 26-28 January, 2015.

- Coriander oil antimicrobial activity: A Flow Cytometric Study.
  Silva F., Ferreira S., **Duarte A.**, Queiroz J.A., Domingues F.C.
  43rd International Symposium on Essential Oils. Lisbon, Portugal. 5-8 September, 2012.

Poster presentations:

- *Coriandrum sativum* essential oil and its major compound linalool to control the foodborne pathogen *Campylobacter* spp.
  Duarte A., Silva Â., Luís Â, Oleastro M., Domingues F.C.
  5th MoniQA International Conference 2015. Porto, Portugal. 16-18 September 2015.

- Characterization and antimicrobial activity of cellulose derivatives films incorporated with a resveratrol inclusion complex
  Silva Â., **Duarte A.**, Sousa S., Domingues F.C.
  5th MoniQA International Conference 2015. Porto, Portugal. 16-18 September 2015.

- Characterization and anti-*Campylobacter* activity of cellulose derivatives films incorporated with resveratrol.
  Silva A., **Duarte A.**, Sousa S., Domingues F.C.
  9th World Congress on Polyphenols Applications. St Julian's, Malta. 3-5 June, 2015.

- The antimicrobial effect of resveratrol on *Listeria monocytogenes*.
  Ferreira S., **Duarte A.**, Domingues F.C.
  I-IC2AR - Ist Internacional Caparica Conference in Antibiotic Resistance. Caparica, Portugal. 26-28 January, 2015.

- Anti-*Campylobacter* activity of resveratrol and its inclusion complex with hydroxypropyl-γ-cyclodextrin: a potential preservative for the food industry.
  Duarte A., Silva F., Oleastro M., Domingues F.C.
  ICAR 2014 - II International Conference on Antimicrobial Research. Madrid, Spain. 1-3 October, 2014.

- Linalool: a natural strategy to control biofilms of *Acinetobacter baumannii*.
  Alves S., **Duarte A.**, Sousa S., Domingues F.C.
  ICAR 2014 - II International Conference on Antimicrobial Research. Madrid, Spain. 1-3 October, 2014.

- Inhibitory effect of resveratrol encapsulated in hydroxypropyl-γ-cyclodextrin against *Arcobacter butzleri*.
  Alves A.C., **Duarte A.**, Domingues F.C., Ferreira S., Silva F.
  ICAR 2014 - II International Conference on Antimicrobial Research. Madrid, Spain. 1-3 October, 2014.

- Antibiotic resistance and genetic diversity of Human, Food and animal origin *Campylobacter* spp. isolates from Portugal.
  Duarte A., Ferreira S., Santos A., Benoliel J., Domingues F.C., Oleastro M.
  CHRO 2013 - 17th International Workshop on *Campylobacter*, *Helicobacter* and Related Organisms. Aberdeen, Scotland. 15-19 September, 2013.

- High rate of antibiotic-resistance and genetic diversity of Human *Campylobacter* spp. isolates from Portugal.
  Duarte A., Santos A., Benoliel J., Domingues F.C., Oleastro M.
  FEMS 2013 - 5th Congress of European Microbiologists. Leipzig, Germany. 21-25 July, 2013.

- Genetic diversity, antibiotic resistance and biofilm-forming ability of *Acinetobacter baumannii* isolated from a Portuguese hospital.
  Duarte A., Ferreira S., Domingues F.C.
  ICAR 2012 - II International Conference on Antimicrobial Research. Lisbon, Portugal. 21-23 November, 2012.

- Effect of coriander oil (*Coriandrum sativum* L.) against biofilm of multiresistant *Acinetobacter baumannii*.
  Duarte A., Ferreira S., Domingues F.C.
  CESAR2012 - Central European Symposium on Antimicrobials and Antimicrobial Resistance. Primošten, Crotia. 23-26 September, 2012.

Chapter 1 - General Introduction

1. Introduction

Despite advances in food safety, foodborne illnesses represent a substantial health burden worldwide (Braden & Tauxe, 2013). Foodborne illness, also known as foodborne infection or foodborne disease, can be caused by a variety of agents that a person contacts with or ingests via contaminated food products (Kalyoussef & Feja, 2014). The ingestion of toxins or chemicals can lead to some foodborne disease; however, bacteria, viruses or parasites are the main cause of such infections (Newell *et al.*, 2010). These infections present a wide variety of symptoms and can lead to hospitalization and even death, particularly in high-risk patients. World Health Organization estimates that annually 2.2 million people die from foodborne and waterborne diarrheal diseases across the world (http://www.who.int/foodsafety/areas_work/foodborne-diseases/en/, accessed: July 2015). Foodborne diseases are responsible for about 48 million illnesses each year in the United States, of which about 9.4 million are caused by known pathogens (CDC, 2015). In the European Union, over 320,000 human cases are reported each year, but the real number is likely to be much higher (http://www.efsa.europa.eu/en/topics/topic/foodbornezoonoticdiseases.htm, accessed: July 2015). If *Salmonella*, Enterohaemorrhagic *Escherichia coli* and *Campylobacter* are the most common foodborne pathogens that affect millions of people annually in United States (CDC, 2015), in the European Union, *Campylobacter* is the most commonly reported pathogen associated with foodborne disease, with over 214,000 human cases annually (EFSA & ECDC, 2015a). In addition to the foodborne pathogens, oxidation is a well-known non-microbial cause of deterioration of food that considerably limits its shelf life (Falowo *et al.*, 2014; Sanches-Silva *et al.*, 2014). Therefore, finding compounds that have both antimicrobial and antioxidant properties has a great potential for application in food systems to help prevent food contamination and spoilage and consequently, foodborne illness (Braden & Tauxe, 2013; Sanches-Silva *et al.*, 2014). Recently, natural compounds have gained a significant interest for application in food products, mainly due to the preference of consumers for natural ingredients and the concerns about the toxic effects of synthetic compounds (Falowo *et al.*, 2014).

2. The genus *Campylobacter*

The implications of *Campylobacter* infection in public health were already described a century ago (Silva *et al.*, 2011c; Skirrow, 1977). In 1886, Theodor Escherich observed *Campylobacter*-like organisms in stool samples of children with diarrhea. At the time, due to the spiral morphology, it was believed to be a related *Vibrio* specie and because of its association with aborted fetuses in sheep and cattle it was referred as *Vibrio fetus* (Ketley, 1997). In 1963, the genus *Campylobacter* was established by Sebald and Véron, distinguishing it from the "real" *Vibrio* spp., based on their low DNA base composition, its non-fermentative metabolism and growing microaerophilic needs (Sebald & Veron, 1963). The *Campylobacter* genus belongs to the family *Campylobacteraceae*, the order *Campylobacterales*, the class *Epsilonproteobacteria*, and the phylum *Proteobacteria* (Vandamme & De Ley, 1991; Vandamme *et al.*, 1991, 2005).

2.1. Physiology and Structure

Members of the genus *Campylobacter* are typically Gram-negative, non-sporeforming, small (0.2–0.8μm wide and 0.5–5μm long) and slender spirally curved rods. They also appear to be S-shaped and gull-winged when two or more bacterial cells are grouped together. Cells in old cultures may form spherical or coccoid bodies. The majority of the species are motile with a characteristic corkscrew-like motion by means of a single polar unsheathed flagellum at one or both ends of the cell. Some species are non-motile (*C. gracilis*) or have multiple flagella (*C. showae*). These bacteria require microaerophilic conditions with an oxygen concentration between 3% and 15% and a CO_2 concentration of 10% to 35%, although some strains can grow either aerobically or anaerobically. Since they neither ferment nor oxidize carbohydrates, the energy is obtained from amino acids or tricarboxylic acid cycle intermediates. Serum or blood enhances growth, but is not essential. Oxidase activity is present in all species except for *C. gracilis* (Vandamme *et al.*, 2005).

2.2. *Campylobacter* species

The *Campylobacter* genus consists of a large and diverse group of bacteria and comprises 27 known species and 2 provisional species (up to July 2015) (Fitzgerald, 2015; Kaakoush *et al.*, 2015; http://www.bacterio.net/campylobacter.html, accessed: July 2015). Table 1 describes the known *Campylobacter* species, authors and year of discovery, sources and human disease associated.

Table 1. *Campylobacter* species, author and year of discovery, sources of isolation or detection and their clinical relevance to humans. Adapted from (Fitzgerald, 2015; Kaakoush *et al.*, 2015; Man, 2011).

***Campylobacter* species**	**Authors and year**	**Sources**	**Human diseases associated**
C. avium	Rossi et al. 2009	Poultry	None
C. canadensis	Inglis et al. 2007	Whooping cranes	None
C. coli	Véron and Chatelain, 1973	Pigs, poultry, sheep, wild birds, cattle, monkeys and dogs	Gastroenteritis, septicemia, meningitis and acute cholecystitis
C. concisus	Tanner et al. 1981	Humans and domestic pets	Gastroenteritis, periodontal disease, abscesses, IBD (Crohn's disease and ulcerative colitis) and Barrett's esophagitis.
C. corcagiensis	Koziel et al. 2014	Lion-tailed macaques	None
C. cuniculorum	Zanoni et al. 2009	Rabbits	None
C. curvus	Vandamme et al. 1991	Humans and dogs	Gastroenteritis, periodontal disease, abscesses, ulcerative colitis and Barrett's esophagitis.
C. fetus	Sebald and Véron, 1963		Gastroenteritis, septicemia, abscesses, cellulitis, endocarditis and peritonitis.
C. fetus* subsp. *fetus	Véron and Chatelain, 1973	Cattle, sheep, horse, kangaroo and reptiles	
C. fetus* subsp. *testudinum	Fitzgerald et al. 2014	Reptiles	
C. fetus* subsp. *venerealis	Véron and Chatelain, 1973	Cattle and sheep	
C. gracilis	Vandamme et al. 1995	Humans and dogs	Periodontal disease, abscesses, IBD, head and neck infection and abscesses.

Table 1 (cont.). *Campylobacter* species, author and year of discovery, sources of isolation or detection and their clinical relevance to humans. Adapted from (Fitzgerald, 2015; Kaakoush *et al.*, 2015; Man, 2011).

***Campylobacter* species**	**Authors and year**	**Sources**	**Human diseases associated**
C. helveticus	Stanley et al. 1993	Dogs and cats	Gastroenteritis
C. hominis	Lawson et al. 2001	Humans	IBD (possibly a commensal in the intestine)
C. hyointestinalis	Gebhart et al. 1985		Gastroenteritis
C. hyointestinalis* subsp. *hyointestinalis	On et al. 1995	Pigs and cattle	
C. hyointestinalis* subsp. *lawsonii	On et al. 1995	Pigs	
C. iguaniorum	Gilbert et al. 2015	Reptiles	None
C. insulaenigrae	Foster et al. 2004	Marine mammals	Gastroenteritis
C. jejuni	Véron and Chatelain, 1973		Gastroenteritis, septicemia, IBD, post-infectious IBS, celiac disease, Guillain-Barré syndrome, Miller Fisher syndrome, Bell's palsy (unilateral facial paralysis), reactive arthritis; myocarditis, meningitis, acute cholecystitis and urinary tract infections
C. jejuni* subsp. *doylei	Steele and Owen, 1988	Humans	
C. jejuni* subsp. *jejuni	Véron and Chatelain, 1973	Poultry, cattle, sheep, wild birds and pigs	
C. lanienae	Logan et al. 2000	Cattle, pigs and sheep	Gastroenteritis
C. lari	Benjamin et al. 1984		Gastroenteritis and septicemia
C. lari* subsp. *concheus	Debruyne et al. 2009	Shellfish	
C. lari* subsp. *lari	Debruyne et al. 2009	Wild birds, poultry, dogs and cats	

Table 1 (cont.). *Campylobacter* species, author and year of discovery, sources of isolation or detection and their clinical relevance to humans. Adapted from (Fitzgerald, 2015; Kaakoush *et al.*, 2015; Man, 2011).

***Campylobacter* species**	**Authors and year**	**Sources**	**Human diseases associated**
C. mucosalis	Roop et al. 1985	Pigs and dogs	Gastroenteritis
C. peloridis	Debruyne et al. 2009	Shellfish	Gastroenteritis
C. rectus	Tanner et al. 1981	Humans and dogs	Periodontal disease, gastroenteritis, IBD and abscesses
C. showae	Etoh et al. 1993	Humans and dogs	Periodontal disease, abscesses and IBD
C. sputorum	Véron and Chatelain, 1973		Abscesses and gastroenteritis
C. sputorum* bv *sputorum	Véron and Chatelain, 1973	Cattle and pigs	
C. sputorum* bv *faecalis	On et al. 1998	Sheep and bulls	
C. sputorum* bv *paraureolyticus	On et al. 1998	Cattle	
C. subantarcticus	Debruyne et al. 2010	Gray-headed and black-browed albatrosses and gentoo penguins	None
C. troglodytis	Kaur et al. 2011	Chimpanzees	None
C. upsaliensis	Sandstedt and Ursing 1991	Dogs and cats	Gastroenteritis, septicemia and abscesses
C. ureolyticus	Jackson and Goodman, 1978	Humans and horse	Gastroenteritis, septicemia, soft tissue abscesses and IBD
C. volucris	Debruyne et al. 2010	Black-headed gulls	None
Campylobacter sp. Dolphin DP (provisional)	Goldman et al. 2011	Dolphin	None
Campylobacter sp. Prairie Dog (provisional)	Beisele et al. 2011	Prairie dog	None

IBD, inflammatory bowel diseases; IBS, irritable bowel syndrome.

C. jejuni, *C. coli*, *C. lari*, *C. upsaliensis*, and *C. helveticus* form the genetically similar group of the thermophilic or thermotolerant campylobacters because they grow optimally at 42°C (Vandamme *et al.*, 2005). The remaining *Campylobacter* species are divided into three groups: (1) species associated with livestock animals and that rarely cause disease in humans (e.g. *C. fetus*, *C. sputorum* and *C. hyointestinalis*); (2) species isolated from humans and/or implicated in periodontal disease (e.g. *C. curvus*, *C. rectus*, *C. showae* and *C. concisus*) and (3) species which have not been isolated from food or water and are not associated with human disease (e.g. *C. canadensis*, *C. corcagiensis* and *C. subantarcticus*) (Fitzgerald, 2015).

Although various pathogenic *Campylobacter* species have been identified as causative agents in human diseases, *C. jejuni* and *C. coli* are nowadays the most common cause responsible for human infections and human campylobacteriosis (Gölz *et al.*, 2014; Janssen *et al.*, 2008; Kaakoush *et al.*, 2015; Man, 2011). As a result, these species of *Campylobacter* have been known as "Emerging *Campylobacter* species". Included in this group are also *C. concisus*, *C. lari*, *C. upsaliensis* and *C. ureolyticus* (Man, 2011).

2.3. Methods of detection and identification of *Campylobacter*

2.3.1. Isolation and identification

Laboratory isolation and detection of *Campylobacter* species can be challenging owing to their fastidious nature and the need for special atmospheric requirements for growth. The isolation process is further complicated by the presence of fast-growing commensal bacteria that competes with *Campylobacter* on the growth medium during the isolation process. To date, several methodologies have been used to isolate fastidious *Campylobacter* bacteria, however, the use of multiple methods in parallel could maximize the process of recovering emerging *Campylobacter* species from clinical, food or environmental samples (On, 2013). Several selective agar media, using blood-based agar or blood-free agar, have been used for the isolation of *Campylobacter* species, principally the thermotolerant ones (Vandamme *et al.*, 2005). However, given the variability in antibiotic susceptibilities among *Campylobacter* species, these methods are effective for only a subset of species. Several methods have been described to be able to isolate *Campylobacter* species, including conventional streaking on antibiotic-selective agar, the filtration technique also known as the 'Cape Town Protocol', magnetic separation, etc (On, 2013).

The sensitivity of *Campylobacter* species to oxygen has led to the development of several selective media containing one or more oxygen scavengers (e.g. blood, ferrous iron and pyruvate) and selective agents, particularly antibiotics. Most methods also involve a pre-enrichment in a liquid medium, before plating on agar selective medium. The isolation of

Campylobacter from clinical specimens, primarily fecal samples, involves the direct plating of the specimen onto selective media and the incubation on a microaerobic (5% O_2, 10% CO_2, 85% N_2) environment (On, 2013; Shin & Lee, 2009). Several selective broths such as Bolton broth, *Campylobacter* enrichment broth and Preston broth have been developed over the years and the incorporation of the enzyme Oxyrase in the selective broths has been demonstrated to be particularly effective in reducing the levels of oxygen and improving the isolation of *Campylobacter* species from naturally contaminated samples. Selective agar media have also been formulated and tested for their efficacy in isolating *Campylobacter* species (Goossens *et al.*, 1989; Hinton, 2006; Silva *et al.*, 2011c). The use of charchoal cefoperazone deoxycholate agar (CCDA) medium and incubation at 42°C is usually the methodology of choice since it allows for the isolation of more *Campylobacter* strains (Nielsen *et al.*, 2015; On, 2013). The "Cape Town protocol", which is a more robust method, requires filtration of homogenized clinical samples through membrane filters with a pore size of 0.45 or 0.65 µm onto blood agar media and then incubating the plates at 37°C under microaerobic conditions. This filtration method has been used successfully to isolate a range of *Campylobacter* species from fecal, intestinal biopsy, saliva and food matrix samples (Kaakoush *et al.*, 2015; Kulkarni *et al.*, 2002; Nielsen *et al.*, 2015).

Following the successful isolation of a strain it is necessary to proceed to its identification. Biochemical tests can be used to differentiate *Campylobacter* species from related genera and to identify the organisms up to the species level (Vandamme *et al.*, 2005). The more relevant biochemical tests include the growth of the isolate with or without supplementation of H_2, followed by an indoxyl acetate test, a hippurate test, growth on MacConkey agar, an aryl sulfatase test and production of H_2S (Lastovica, 2006). In addition, the detection of L-alanine aminopeptidase activity can be used to differentiate between *Campylobacter*, *Helicobacter* and *Arcobacter* species and other Gram-negative bacteria (Lastovica, 2006). However, the only biochemical test that distinguishes between *C. jejuni* and *C. coli* is the hippurate hydrolysis test, since *C. jejuni* isolates have the ability to hydrolyze hippurate, whereas *C. coli* isolates do not have that ability.

In addition to the biochemical tests, several alternative and rapid methods have been developed for detecting and confirming *Campylobacter* species, such as fluorescence *in situ* hybridization (Moreno *et al.*, 2003; Poppert *et al.*, 2008), latex agglutination (Hazeleger *et al.*, 1992), DNA oligonucleotide arrays (Quiñones *et al.*, 2007) and more recently, the laser optical scattering technology (He *et al.*, 2015). Perhaps the most effective confirmation methods are those based on the polymerase chain reaction (PCR), and the real-time PCR has become the method of choice due to its highly sensitive and quantitative detection (Ménard *et al.*, 2005; Yang *et al.*, 2004).

2.3.2. Molecular typing methods

Molecular typing of *Campylobacter* strains represents an important tool for epidemiological studies, in order to assess the genetic diversity and to trace sources of sporadic infections with *Campylobacter* species by providing information on the genetic subtypes in circulation (Dingle *et al.*, 2002; Taboada *et al.*, 2013).

Typing methods based on electrophoresis banding patterns or single loci analyses are successful in pointing out similarities among *Campylobacter* isolates from humans and animals. Among those methods, the most common include pulsed-field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP) analysis and *flaA* short variable region (SVR) typing (Colles & Maiden, 2012; Magnússon *et al.*, 2011). However, these typing schemes are not usually used to compare *Campylobacter* isolates in a larger scale due to the difficulties in the reproducibility of the methodology and comparison of results between laboratories (Wassenaar & Newell, 2000)

The application of sequence-based typing schemes to *Campylobacter*, such as multilocus sequence typing (MLST) provides the necessary tools for the reproducible and large range classification of the isolates (Taboada *et al.*, 2013; Urwin & Maiden, 2003). The widespread adoption of this typing method, along with the online databases that catalogue the extensive variation of *Campylobacter* (http://pubMLST.org/campylobacter), have allowed major advances in understanding the local, national and global epidemiology of *Campylobacter* (Colles & Maiden, 2012; Urwin & Maiden, 2003). Since *flaA*-SVR typing and MLST typing tools are extensively used, often in parallel, these methods will be discussed further.

flaA-SVR typing

Short regions of a highly variable gene are used to provide a "fingerprint" of the isolate of interest. For *Campylobacter*, the flagellin (*flaA*) gene has provided a useful target for discriminating among isolates (Meinersmann *et al.*, 1997; Taboada *et al.*, 2013). *flaA*, a gene encoding the primary structural flagellin protein of *C. jejuni*, has a SVR that has been shown to be conserved among outbreak-related strains (Meinersmann *et al.*, 1997). Analysis of the SVR sequences provides not only reproducible results but also a high level of discriminatory strain-typing results. In addition, the nucleotide sequences obtained by the *flaA*-SVR typing can be deposited in the *flaA*-SVR sequence database (http://pubMLST.org/campylobacter) and can be analysed by comparing against the existing sequences (Dingle *et al.*, 2002; Taboada *et al.*, 2013).

Several studies have described the successful use of *flaA-SVR* typing for epidemiological investigations. For example, Wassenaar *et al.* (2009), compared and contrasted the genetic diversity of *Campylobacter* isolates obtained from human and chicken hosts in three European regions, by *flaA*-SVR genotyping. Despite the widespread use of *flaA*-based typing methods,

doubts have been raised about the consistency of single-locus subtyping methods for *Campylobacter* species, since they are naturally transforming organisms capable of high rates of exogenous DNA uptake. In addition, due to recombination and intra species transfer, *flaA* alleles are not very stable, nor are species-specific (Dingle *et al.*, 2005). These properties make molecular typing using this locus unsuited for longer-term investigations. Nevertheless this method when used in combination with the typing of more conserved genes, like the MLST scheme, may be appropriate for distinguishing closely related strains (Dingle *et al.*, 2002; Kittl *et al.*, 2013; Price *et al.*, 2006).

MLST

MLST has become one of the most extensively used molecular typing methods for *Campylobacter* (Duarte *et al.*, 2014; Griekspoor *et al.*, 2010; Kittl *et al.*, 2013; Strachan *et al.*, 2012; Taboada *et al.*, 2013; Urwin & Maiden, 2003). The first MLST scheme was developed for *C. jejuni* and *C. coli* and requires the sequencing of short DNA fragments within seven housekeeping genes: *aspA* (aspatase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxy methyl transferase), *pgm* (phospho glucomutase), *tkt* (transketolase) and *uncA* (ATP synthase alpha subunit) (Dingle *et al.*, 2001, 2005). After sequencing of the PCR products for each loci, the sequences are assigned to an allele number based on a complete match to an allele in the global PubMLST database. Then, to the collection of the seven allele numbers is assigned a sequence type (ST) and STs that share four or more alleles belong to the same clonal complex (CC) (Dingle *et al.*, 2001). Actually, over 450,000 sequences and 7,800 STs have been identified within the PubMLST database.

The MLST approach, by indexing the nucleotide sequence variation at the seven housekeeping loci, which are subject to stabilizing selection for conservation of metabolic function, provides data that is highly discriminatory (Colles & Maiden, 2012). MLST has been a valuable tool in identifying major sources of human disease and is an important tool in both long and short term epidemiological studies (Denis *et al.*, 2009; Dingle *et al.*, 2008; Duarte *et al.*, 2014; Nielsen *et al.*, 2010; Sheppard *et al.*, 2009; Wilson *et al.*, 2008; Yabe *et al.*, 2010). For most applications, MLST alone has been valuable in the analysis of sporadic cases and in long-term epidemiology. In the context of short-term epidemiological investigations, such as the occurrence of epidemic strains with common sequence types, it may require the use of a second subtyping method to provide additional discrimination power (Taboada *et al.*, 2013). Variable regions within *flaA* gene have been used to allow further discrimination between matching STs (Dingle *et al.*, 2002). However, an ongoing challenge with MLST has been the high cost and time consuming nature of the analysis (On, 2013).

Moreover, with the decrease in the cost of the whole genome sequencing (WGS) the genomes of *Campylobacter* strains can now be fully sequenced, and many of these are completed or drafted. WGS provides a highest level of discriminatory power for epidemiologic typing than

MLST. However, since MLST data is entirely compatible with WGS data, while MLST can resolve many clinical and epidemiological questions, WGS data may be over discriminatory in some cases (Cody *et al.*, 2013; Colles & Maiden, 2012; Taboada *et al.*, 2013).

2.4. Epidemiology of *Campylobacter*

2.4.1. *Campylobacter* in humans

Campylobacter is a major cause of foodborne diarrheal illness in humans and is the bacteria that causes the most cases of gastroenteritis worldwide (Fitzgerald, 2015; WHO, 2013). This bacteria causes more cases of diarrhea than foodborne *Salmonella* in developed and developing countries (Kaakoush et al., 2015). Although there are many species within the genus *Campylobacter*, *C. jejuni* and *C. coli* are the most commonly isolated in reported cases of *Campylobacter* infections (Gölz et al., 2014; Man, 2011). The importance of other species in terms of burden of disease is not clear, but is considered unlikely compared to *C. jejuni* and *C. coli* (Man, 2011; WHO, 2013). In United States, among human *Campylobacter* isolates with species information, 89% are *C. jejuni*, 8% are *C. coli*, 2% are *C. upsaliensis* and the remaining 1% are other species (CDC, 2014). Regarding the European Union data, the most commonly reported species are *C. jejuni* (80.6%), *C. coli* (7.1%), *C. lari* (0.22%), *C. fetus* (0.1%) and *C. upsaliensis* (0.08%) (EFSA & ECDC, 2015a). Concerning the incidence of infection, the highest incidence is among children with less than 5 years, although incidence among persons aged 60 years and older seems to be increasing. In developing countries, *Campylobacter* infections in children under the age of 2 years are particularly frequent, sometimes resulting in death (Gölz et al., 2014; Kaakoush et al., 2015; WHO, 2013).

In the United States, ahead of *Salmonella*, *Campylobacter* is the second most common cause of bacterial foodborne illness. In 2014, FoodNet identified 6,486 cases of *Campylobacter* infection with an incidence rate of 13.45 per 100,000 population, resulting in 1,080 hospitalizations and 11 deaths (CDC, 2015; Crim *et al.*, 2015). In addition, many more cases are undiagnosed or unreported and it is estimated that campylobacteriosis affects over 2 million persons every year (Scallan *et al.*, 2011). In the European Union, *Campylobacter* continues to be the most commonly reported gastrointestinal bacterial pathogen since 2005 (EFSA & ECDC, 2015a; Kaakoush *et al.*, 2015). In 2013, the number of reported confirmed cases of human campylobacteriosis was 214,779 with an incidence rate of 64.8 per 100,000 population (EFSA & ECDC, 2015a). Among the European Union countries there is a large variation in the notification rate: the highest rates were observed in the Czech Republic (173.7 cases per 100,000 population), Luxembourg (125.7), Slovakia (108.0) and the United Kingdom (104.0), while the lowest rates were reported in Latvia, Romania, Poland and

Bulgaria (< 2.0 per 100,000). Unfortunately, for Portugal, no surveillance system is yet included in the EFSA reports (EFSA & ECDC, 2015a).

In Portugal, studies concerning campylobacteriosis are scarce. The first study was published by Cabrita *et al.* (1992a), addressing epidemiological features of human isolates. In this study, from 1984 to 1989, stool samples from 2811 gastroenteritis cases were examined for the presence of *C. jejuni* and *C. coli*, *Salmonella*, *Shigella* and *Yersinia species*. Concerning *Campylobacter*, 150 isolates were identified as *Campylobacter* species (5.3%), with predominant *C. jejuni* (77.2%) and *C. coli* (22.8%), and the incidence was highest among people with less than 5 years (83.4%) (Cabrita *et al.*, 1992a). In 2008, a total of 123 *Campylobacter*-positive stool cultures were analyzed and 110 of the 123 isolates were identified as *C. jejuni* (89.4%) and 13 were *C. coli* (10.6%), being 85 isolates (69.1%) obtained from children younger than 14 years old (Vicente *et al.*, 2008). Also, Duarte *et al.* (2013b), in an epidemiological study about the *Campylobacter* infection in Portugal between 2009 and 2012, analyzed 837 isolates and identified *C. jejuni* (84.5%), *C. coli* (14.8%), *C. upsaliensis* (0.2%), *C. concisus* (0.1%) and *Arcobacter butzleri* (0.2%). In this study it was also observed a higher incidence of *Campylobacter* in isolates from children aged between 26 days and 11 months (25.2%) and 1 and 15 years (61.5%) (Duarte *et al.*, 2013b). In 2014 the same authors identified 79 isolates as *C. jejuni* (63.2%) and 46 as *C. coli* (36.8%) from 125 strains isolated between 2009 and 2012 from fecal samples of patients presenting acute gastroenteritis, randomly selected from the collection of *Campylobacter* strains of the Department of Infectious Diseases of the National Institute of Health Dr. Ricardo Jorge (INSARJ, Lisbon) (Duarte *et al.*, 2014). In this last study 30.2% of the isolates were recovered from children aged between 1-11 months and the majority (75.6%) were from children between 1 to 15 years old (Duarte *et al.*, 2014). Soares *et al.* (2014), studied the incidence of *Campylobacter* species in the pediatric emergency department of a general district hospital in patients with acute gastroenteritis. Between 2011 and 2013, from 98 positive cultures 49 were identified as *Campylobacter* species (50%): 48 were *C. jejuni* (98%) and only one was *C. coli* (2%); 39 (79.6%) *Campylobacter* species were identified in children younger than 5 years old (Soares *et al.*, 2014). Other authors have found *Campylobacter* species in 95 (31.9%) of 298 stool samples of patients with diarrhea, being identified as *C. jejuni* (13.7%), *C. concisus* (7.4%), *C. coli* (1.0%) and *C. fetus* (0.3%) (Ferreira *et al.*, 2014a). Finally, Pereira *et al.* (2014) reported a clinical case of a renal transplant recipient with *C. jejuni* bacteremia presenting a brief diarrheal illness followed by cellulitis, and only 2 cases of *C. jejuni* bacteremia have been described worldwide in renal transplant recipient.

In sum, the Portuguese reports are in accordance with the ones described worldwide, with *C. jejuni* and *C. coli* being the most common species in reported cases of human campylobacteriosis and the incidence of *Campylobacter* infections being higher in children under 5 years old.

2.4.2. *Campylobacter* in animals and food

Campylobacter occurs almost ubiquitously in the environment and can be found in the intestinal tract of warm-blooded animals, usually without showing clinical symptoms (Fitzgerald, 2015; Kaakoush *et al.*, 2015; Lévesque *et al.*, 2008; Painter *et al.*, 2013). The gastrointestinal tracts of domestic and wild birds and animals are reservoirs of this bacterium (Corry & Atabay, 2001; EFSA & ECDC, 2015a; Jamali *et al.*, 2015; Sheppard *et al.*, 2009). Also, many farm animals and meat sources can harbor the organism, and pets, including dogs, cats, hamsters, and birds, are potential sources of infection (de Cesare *et al.*, 2003; Kittl *et al.*, 2013; Magnússon *et al.*, 2011).

Concerning the prevalence in animals, poultry is recognized as a primary source of *Campylobacter* species (Corry & Atabay, 2001; EFSA & ECDC, 2015a; Josefsen *et al.*, 2015). In the last European Food Safety Authority (EFSA) report, *Campylobacter* was found in 19.9% of the 11,475 broiler units analyzed, in 29.6% of the tested slaughter batches and in 15.1% of the tested flocks. In addition, 30.4% of the tested animals were *Campylobacter* positive (EFSA & ECDC, 2015a). Therefore, *Campylobacter* species are widely found in poultry animals, poultry farms and poultry production chains and their surrounding environment, including soil, water sources, dust, building surfaces and air (Ellis-Iversen *et al.*, 2012; Josefsen *et al.*, 2015; O'Mahony *et al.*, 2011). In addition to chickens, turkeys, ducks and geese can also serve as reservoirs of *C. jejuni* and *C. coli* (Carreira *et al.*, 2012; Jamali *et al.*, 2015; Magnússon *et al.*, 2011; Perko-Mäkelä *et al.*, 2011). The sources of *Campylobacter* entry into poultry flocks and poultry production are multiple and include drinking water, environment of the outdoor poultry, wild birds, rodents, flies and working staff (Nather *et al.*, 2009). Apart from poultry animals, *Campylobacter* has been also detected in pigs, cattle, goats, sheep, horses, cats, dogs and a range of wild animals (Cabrita *et al.*, 1992b; EFSA & ECDC, 2015a; de Haan *et al.*, 2013; Magnússon *et al.*, 2011; Pezzotti *et al.*, 2003). EFSA reported data on prevalence of *Campylobacter* in pigs ranging from 0% to 92.7% and from 0% to 50.4% in cattle (EFSA & ECDC, 2015a). Pet animals, mainly dogs and cats must also be considered as possible sources of *Campylobacter* species (Gölz *et al.*, 2014; Kittl *et al.*, 2013; Korczak *et al.*, 2009). In many cases, dogs and cats can asymptomatically carry thermophilic *Campylobacter* species, with prevalence ranging up to about 40% (Gölz *et al.*, 2014). The prevalence of *Campylobacter* species has also been reported in dogs with diarrhea, being as high as 97% (Chaban *et al.*, 2010). In the European Union the proportion of *Campylobacter*-positive cats and dogs is generally low, but in 2013, in two clinical investigations from the Netherlands and Norway, 40.4% and 31.2%, respectively, of the tested dogs were found to be *Campylobacter*-positive (EFSA & ECDC, 2015a). Recently, *Campylobacter* species were isolated from 47 cynomolgus monkeys (*Macaca fascicularis*) from China, Cambodia and Indonesia and two monkeys infected with *C. coli* and *C. jejuni* showed clinical symptoms of diarrhea and bloody feces (Koga *et al.*, 2015).

In food products, broiler meat is considered to be the main source of human campylobacteriosis and *C. jejuni* and *C. coli* are the most frequently isolated *Campylobacte*r species in chicken products (EFSA & ECDC, 2015a; Hardy *et al.*, 2011; Man, 2011; Mead, 2004). Emerging *Campylobacter* species have also been isolated from diverse food and drink products such as pork chop and ground beef (Thakur *et al.*, 2009), fresh broiler meat (Xavier *et al.*, 2014), pork and beef raw meat (Duarte *et al.*, 2014; Pezzotti *et al.*, 2003), quails (Fraqueza *et al.*, 2016), processed poultry products (Chicken cuts and Hamburgers) (Mena *et al.*, 2008), drinking water (de Haan *et al.*, 2013) and raw milk (Lévesque *et al.*, 2008).

2.4.3. Sources and transmission

The main source of human *Campylobacter* infection is generally considered to be foodborne, via undercooked meat and meat products, mainly poultry, as well as raw or contaminated milk (Sheppard *et al.*, 2009; WHO, 2013; Wilson *et al.*, 2008). Contaminated water is also a source of infection and other sources include sheep, pigs, bovine and wild birds (Abdollahpour *et al.*, 2015; Sheppard *et al.*, 2009; Silva *et al.*, 2011c). *Campylobacter* can also be recovered from seawater and bovine manure compost, indicating that the environment is also a potential reservoir for this group of organisms (Man, 2011). Contact with pet animals has also been proposed as a source of campylobacteriosis in humans (Kittl *et al.*, 2013; Parsons *et al.*, 2009). The transmission of *Campylobacter* to humans occurs by ingesting or handling of contaminated food or water or by direct contact with fecal material from infected animals or people (Figure 1). Although it is not as common, person-to-person transmission can also occur (fecal-oral or via fomites) (Fitzgerald, 2015; Wimalarathna *et al.*, 2013). In addition, cross-contamination may be particularly important due to the high prevalence of contamination in the poultry slaughtering process, retail establishments, raw poultry products and other foods (de Cesare *et al.*, 2003; Hansson *et al.*, 2015; Silva *et al.*, 2011c; Srey *et al.*, 2013; Zorman *et al.*, 2006).

Although *Campylobacter* species are generally considered to be sensitive to the external environment of the animals, they are in fact stronger than previously thought (Bolton, 2015; Silva *et al.*, 2011c). Still these pathogens are known to be sensitive to drying or even low humidity freezing and freeze-thaw stress, oxygen and other factors, therefore the control must focus on those aspects when relevant (Humphrey *et al.*, 2007; Silva *et al.*, 2011c). So, in domestic settings, working surfaces and utensils should be properly cleaned and sanitized and cooking temperatures and times should be adjusted as to be sufficient to eliminate *Campylobacter*. Cross-contaminations also need to be avoided. In addition, there is an urgent need for informing consumers and cooks about the best ways to handle chicken in households and catering in order to minimize the spread of *Campylobacter* and foodborne infections among the population (Humphrey *et al.*, 2007).

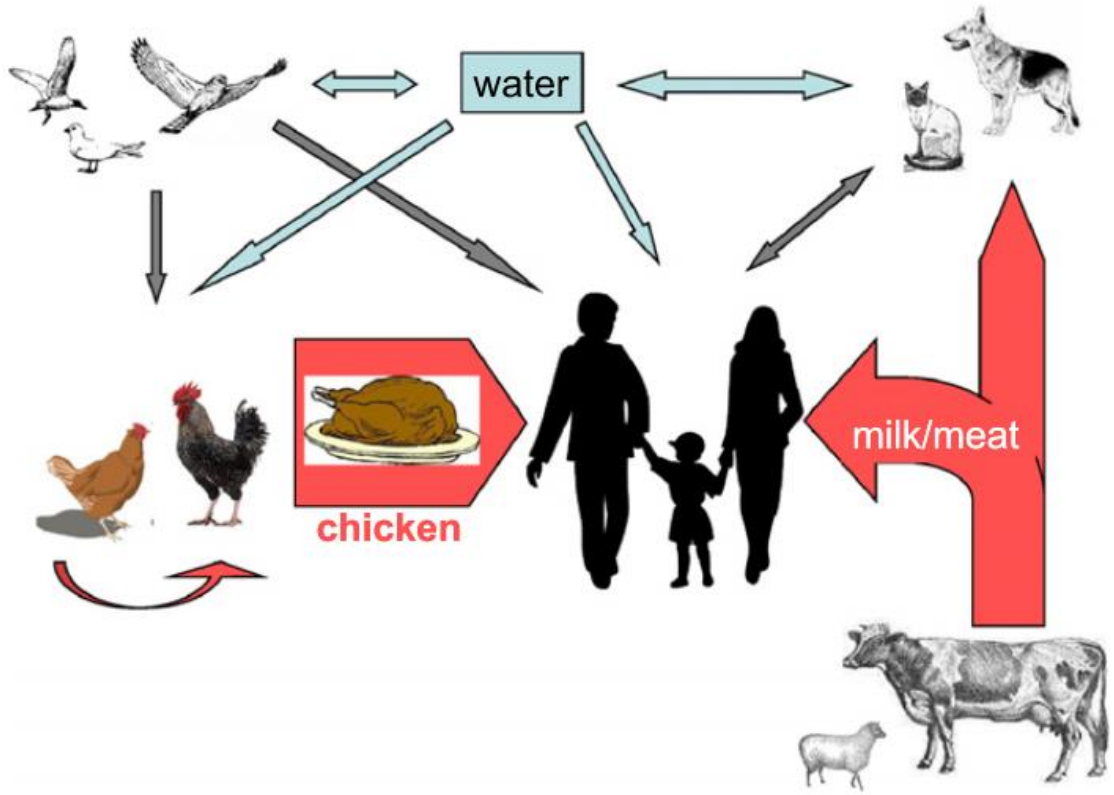

Figure 1. Most important routes of *Campylobacter* transmission for human infection (Dasti et al., 2010).

2.5. Clinical manifestations of *Campylobacter* infection

Campylobacteriosis is the disease caused by the infection with *Campylobacter*. The appearance of disease symptoms generally occurs two to five days after infection with the bacteria, but may vary from one to ten days (Man, 2011). The most common symptoms of *Campylobacter* infections include diarrhea (frequently with bloody feces), abdominal pain, fever, headache, nausea and/or vomiting. Death from campylobacteriosis is rare and is usually restricted to very young or elderly patients, or those already suffering from another serious illness (Feodoroff *et al.*, 2009; Man, 2011; Riddle *et al.*, 2012).

From all *Campylobacter* species, *C. jejuni* is the major cause of campylobacteriosis worldwide. In humans, *Campylobacter* species have also been associated with a range of gastrointestinal conditions, including gastroenteritis, inflammatory bowel diseases (IBD), Barrett's esophagus, periodontal diseases, colorectal cancer and irritable bowel syndrome (IBS) (Gradel *et al.*, 2009; Kaakoush *et al.*, 2015; Riddle *et al.*, 2012). They have also been reported to be involved in extragastrointestinal manifestations, including bacteremia, hepatitis, pancreatitis, lung infections, brain abscesses and meningitis (Kaakoush *et al.*, 2015). In addition, post-infection complications may include reactive arthritis and may lead to autoimmune conditions such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome

(Kaakoush *et al.*, 2015; Nyati & Nyati, 2013; Poropatich *et al.*, 2010). A summary of the clinical manifestations associated with *Campylobacter* infection is presented in Figure 2.

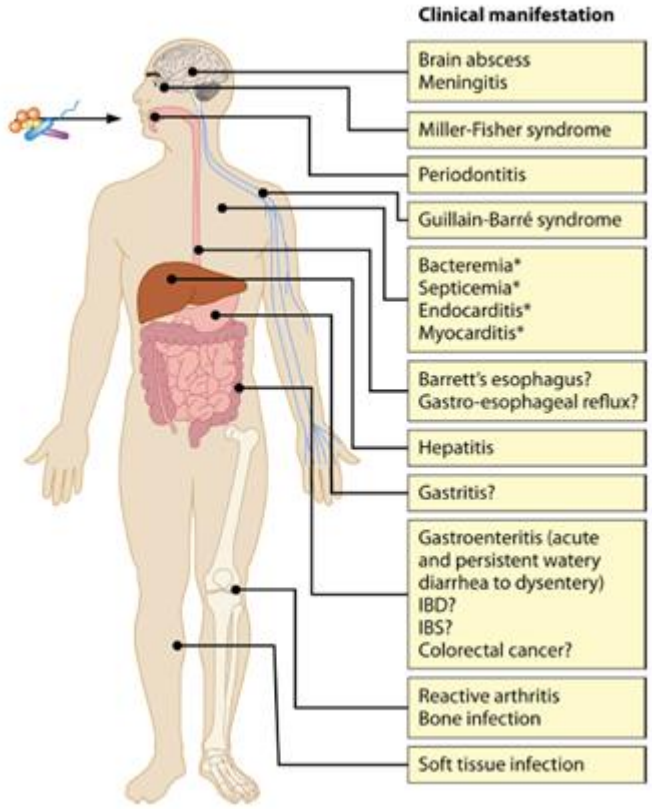

Figure 2. Principal clinical manifestations associated with *Campylobacter* species. Abbreviations: IBD, inflammatory bowel diseases; IBS, irritable bowel syndrome. "?" indicate conditions for which the role of *Campylobacter* is not certain; * Systematic manifestation may be acquired via extraintestinal routes (adapted from Kaakoush *et al.* (2015)).

2.5.1. Gastrointestinal manifestations

Gastroenteritis is the major clinical condition resulting from *Campylobacter* infection, however these organisms have also been associated with other serious conditions within the gastrointestinal tract, including IBD, esophageal diseases, periodontitis, celiac disease, colorectal cancer and the post-infection manifestation IBS (Kaakoush *et al.*, 2015; Man, 2011; Riddle *et al.*, 2012) (Figure 2).

Gastroenteritis

C. jejuni and *C. coli* are well known established causes of gastroenteritis in humans (Griffiths & Park, 1990; Man, 2011). The main symptoms of gastroenteritis caused by *C. jejuni* or *C. coli* are acute watery or bloody diarrhea, fever, weight loss, and abdominal pain which last on average 6 days (Zilbauer *et al.*, 2008). Gastroenteritis induced by *C. coli* is clinically indistinguishable from that by *C. jejuni* (Kaakoush *et al.*, 2015). The onset of symptoms

typically occurs 24 to 72 hours after ingestion and may depend on the infective dose. The peak of the disease can last 24 to 48 hours and may include abdominal pain that mimics appendicitis (Galanis, 2007; Zilbauer *et al.*, 2008). In the stool it is possible to observe polymorphonuclear leukocytes and blood and diffuse inflammatory colitis is present in colonic biopsy specimens from infected patients (Kaakoush *et al.*, 2015). While infection with *Campylobacter* can occur in patients of all ages, recent studies showed that infection is more prevalent in younger (< 5 years) persons than in other age groups (Kaakoush *et al.*, 2015; WHO, 2013).

The possible factors that aid in the adhesion, invasion, and consequently the clinical disease include: (1) the bacterial capsule resistance to host-innate immunity, which may play a role in immune evasion; (2) flagellar proteins, which are used as a secretory apparatus essential for invasion and adherence; (3) presence of a DNAse, cytolethal distending toxin (CDT) that causes apoptosis and (4) ability of infective strains for N- and O-glycosylation, which may also contribute to immune evasion (Man, 2011; Riddle *et al.*, 2012; Zilbauer *et al.*, 2008).

Inflammatory bowel diseases (IBD)

Epidemiological evidence for IBD after acute diarrheal infection and *Campylobacter* has grown (Kaakoush *et al.*, 2014; WHO, 2013). IBD are chronic inflammatory conditions of the gastrointestinal tract which include Crohn's disease and ulcerative colitis (Mas-Moya & Singhi, 2015). Crohn's disease is characterized by recurrent episodes of inflammation involving any site along the gastrointestinal tract, from the mouth to the anus. The inflammation is transmural and often causes strictures, fissures, fistulas, inter-loop adhesions and abscesses. Similarly, ulcerative colitis is characterized by recurrent episodes of submucosal inflammation restricted to the colon. The mucosal inflammation results in ulceration, hemorrhage, granularity and friability (Mas-Moya & Singhi, 2015). The exact etiology of IBD is unclear, however it is hypothesized to be due to a combination of factors that include (1) host genetic factors; (2) disruption of the gastrointestinal epithelium triggered by environmental factors (eg. smoking, dietary intake, etc.) and (3) dysregulated gastrointestinal microbial ecology, leading to alterations of the immune response that result in chronic inflammation (Kaakoush et al., 2015; Mas-Moya & Singhi, 2015).

The role of *Campylobacter* species in IBD has been investigated for the past decades and in 2009, an association between *C. jejuni* infection and an increased risk of IBD was reported (Gradel *et al.*, 2009). In addition, recent studies have investigated the role of other *Campylobacter* species in IBD and have demonstrated an association between *C. concisus* and these gastrointestinal disorders (Kaakoush *et al.*, 2014; Zhang *et al.*, 2009, 2014). The mechanism by which *C. concisus*, may trigger the onset of IBD remains unclear, however some mechanisms have been proposed. With the human oral cavity as the reservoir of *C. concisus*, the intestinal colonization of *C. concisus* may occur. This bacterium can attach to and invade

host intestinal epithelial cells and induce immune responses by host epithelial cells. However, some strains are efficiently eliminated from within the host cells by autophagy. Nonetheless, they secrete a toxin named Zot, which targets the tight junctions of host cells, resulting in barrier dysfunction which facilitates translocation. In genetically susceptible individuals, this may trigger the development of IBD. The damage to the intestinal epithelial tight junctions may also lead to the development of diarrhea (Kaakoush *et al.*, 2014, 2015; Zhang *et al.*, 2014).

Esophageal diseases

Esophageal diseases include gastroesophageal reflux disease, Barrett's esophagus and esophageal adenocarcinoma (Blackett *et al.*, 2013). Gastroesophageal reflux disease is a chronic disorder in which mucosal damage to the esophagus occurs due to stomach acid or, occasionally, stomach content flowing back into the esophagus, which over time increases the risk of Barrett's esophagus. In turn, Barrett's esophagus is a preneoplasic condition in which the normal squamous mucosa is replaced by metaplastic columnar mucosa in the distal esophagus. Both these conditions increase the predisposition for the development of esophageal adenocarcinoma (Kaakoush *et al.*, 2015; Macfarlane *et al.*, 2007).

Over the past two decades, there has been an increase in the incidence of both Barrett's esophagus and adenocarcinoma, however, the involvement of microorganisms was uncertain. Recently it has been described that the gastrointestinal tract hosts a large range of bacteria, which could be a cause of chronic inflammation and genome toxins, which can lead to the development of Barrett's esophagus or even esophageal adenocarcinoma (Macfarlane *et al.*, 2007). *Campylobacter* species and *C. concisus* in particular, are among the principal species present in patients with gastroesophageal reflux disease and Barrett's esophagus, suggesting a possible association between *C. concisus* colonization and reflux into the esophagus (Blackett *et al.*, 2013; Macfarlane *et al.*, 2007).

Periodontitis

In addition to the lower gastrointestinal tract, *Campylobacter* species also colonize the human oral cavity. *C. rectus*, *C. gracilis*, *C. showae* and *C. concisus* have been identified as potential oral pathogens, while other species, including *C. curvus*, *C. sputorum* and *C. ureolyticus* that despite have been isolated from the oral cavity its role on periodontal disease remains unclear (Macuch & Tanner, 2000; Man, 2011). Periodontitis is a severe condition characterized by a set of inflammatory diseases affecting the tissues that surround and support the teeth. It involves progressive reduction of the bone around the teeth, and ultimately, can lead to the tooth loss. These oral inflammatory conditions are induced by biofilms in the gingival margin and are reported to be initiated in periodontal tissue by bacteria, including *C. rectus* (Kaakoush *et al.*, 2015; Macuch & Tanner, 2000). In addition, the

possible etiological association between oral *Campylobacter* species and IBD has been investigated and it was found that periodontal disease and IBD share some common clinical features (Kaakoush *et al.*, 2014). For example, elevated levels of *C. concisus* can be found in periodontal lesions of IBD patients (Zhang *et al.*, 2014).

Celiac disease

Celiac disease is a digestive and autoimmune disorder that results in damage to the lining of the small intestine due to the abnormal reaction of the immune system to gluten. Celiac disease is an increasingly common disease and is estimated to affect approximately 1% of people worldwide (Kaakoush *et al.*, 2015; Riddle *et al.*, 2012). A case study described for the first time, in 2007, the development of celiac disease in a healthy woman following a *C. jejuni* infection (Verdu *et al.*, 2007) and recently, Riddle *et al.* (2013) also reported an increased risk of celiac disease following campylobacteriosis. However, the epidemiological data is insufficient to conclude whether campylobacteriosis is associated with celiac disease.

Colorectal cancer

Increasing evidence suggests that the gut microbiota may play an important role in the development of colorectal cancer (Wu *et al.*, 2013). Nonetheless, the evidence supporting the role of *Campylobacter* species in colorectal cancer is very limited. In a recent study, Warren *et al.* (2013) demonstrated that *Campylobacter* species, predominantly *C. showae*, co-aggregate with *Fusobacterium* in colorectal cancer tissues. Wu *et al.* (2013) also reported a specific microbial profile associated with colorectal cancer, characterized by significant increases in *Fusobacterium* and *Campylobacter* species. Although these studies provide an indication that *Campylobacter* species are present in patients with colorectal cancer, further studies are needed to determine whether there is any relationship between *Campylobacter* and the development of colorectal cancer.

Irritable bowel syndrome (IBS)

As a post-infectious manifestation, IBS has been increasingly linked to enteric *C. jejuni* and other *Campylobacter* species infection (Riddle *et al.*, 2012; Thabane *et al.*, 2007). Infectious gastroenteritis is the best characterized environmental risk factor for the development of IBS and studies reported that up to 36% of those with acute campylobacteriosis develop IBS in the 1–2 years after illness (Marshall *et al.*, 2006; Spiller & Garsed, 2009). IBS is characterized by abdominal pain discomfort, associated with altered bowel habits (diarrhea, constipation, or both) (Kaakoush *et al.*, 2015; Spiller & Garsed, 2009).

The underlying mechanisms of IBS are not well known but may include persistent changes in the intestinal flora, as well as in mucosal immunocytes, enterochromaffin cells, mast cells

and enteric nerves. In addition, host factors such as the female gender, depression, hypochondriasis, smoking and treatment with antibiotics, are also risk factors for the development of IBS (Spiller & Garsed, 2009). So, current evidence suggests that both bacterial and host factors play a crucial role in the predisposition to *C. jejuni*-associated post-infectious IBS. These include increased cytotoxic virulence of the *Campylobacter* strain, increased transcellular bacterial translocation and increased mucosal permeability in the host during acute gastroenteritis (Grover *et al.*, 2014). More recently, in addition to *C. jejuni*, other *Campylobacter* species have been identified to play a role in IBS, such as *C. coli* and *C. concisus* (Grover *et al.*, 2014; Kaakoush *et al.*, 2015). Overall, there is good evidence to suggest a link between *Campylobacter* infection and IBS.

2.5.2. Extragastrointestinal manifestations

In addition to gastrointestinal infection, *Campylobacter* species also cause a range of clinical manifestations in other parts of the body. These manifestations include GBS, Miller Fisher syndrome, reactive arthritis, bacteremia, sepsis, meningitis, abscesses and endocarditis and myocarditis (Figure 2) (Kaakoush *et al.*, 2015).

Guillain-Barré syndrome (GBS)

GBS is a neurologic condition characterized by a progressive ascending symmetrical paralysis in the limbs, with or without hyporeflexia. Motor paralysis affects the lower extremities more frequently than the upper extremities. Respiratory failure due to neuromuscular compromise may also occur, often requiring supportive ventilation (Ansar & Valadi, 2015; van den Berg *et al.*, 2014). The most common subtypes of GBS are acute motor axonal neuropathy (AMAN) and acute inflammatory demyelinating polyneuropathy (AIDP), each one displaying a distinct immunopathogenesis and response to treatment (van den Berg *et al.*, 2014; Kuwabara & Yuki, 2013). AMAN is characterized by a more rapid progress and is considered the major subtype in Asia and Central and South America, whereas AIDP is more prevalent in Europe and North America (van den Berg *et al.*, 2014; Kuwabara & Yuki, 2013). The incidence of GBS is approximately 1 to 2 cases per 100,000 persons and affects men and elderly more commonly than women or younger patients (Ansar & Valadi, 2015).

GBS is considered a post-infectious disease, and *C. jejuni* is now recognized as the most identifiable pathogen associated with the development of this disease (Drenthen *et al.*, 2011; Nyati & Nyati, 2013). Furthermore, several studies have associated GBS with outbreaks of *C. jejuni* infection (Heikema *et al.*, 2015; Islam *et al.*, 2009; Poropatich *et al.*, 2010). The molecular mimicry of the peripheral nerve gangliosides by *C. jejuni* lipooligosaccharide has been reported as the cause of the generation of autoreactive antibodies, causing inflammation and tissue damage and consequently leading to the GBS (van den Berg *et al.*,

2014). Briefly, after the infection with *C. jejuni*, the lipooligosaccharides on the *C. jejuni* outer membrane may trigger the production of antibodies that cross-react with gangliosides, such as GM1 and GD1a on peripheral nerves (Figure 3).

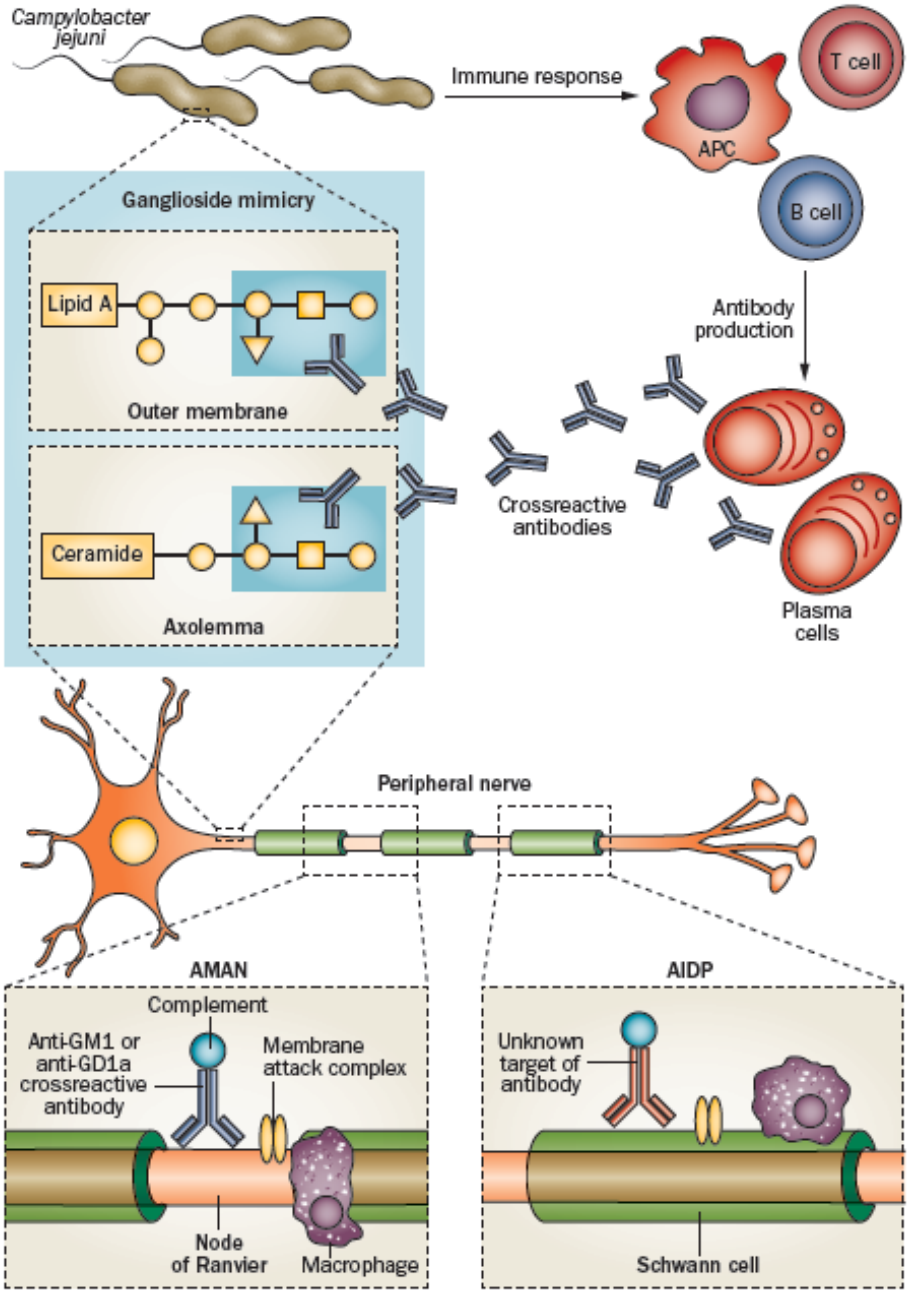

Figure 3. Immunopathogenesis of Guillain-Barré syndrome (GBS): *C. jejuni* strains contain lipooligosaccharides that mimic the carbohydrate moiety of gangliosides that are present in human peripheral nerves, leading to the production of antiganglioside antibodies that cross-react with gangliosides on peripheral nerves. Abbreviations: AIDP, acute inflammatory demyelinating polyneuropathy; AMAN, acute motor axonal neuropathy; APC, antigen-presenting cell (van den Berg *et al.*, 2014).

In AMAN, the antigens targeted are located at or near the node of Ranvier. Then, the anti-GM1 and anti-GD1a antibodies bind to the nodal axolemma, leading to complement activation followed by the formation of membrane attack complex and disappearance of voltage-gated sodium channels. This damage can lead to detachment of paranodal myelin and the failure in nerve conduction. Macrophages then move from the nodes into the periaxonal space, scavenging the injured axons. In AIDP, the antigens targeted are, presumably, located on the myelin sheath. The antibodies can activate complement, which leads to the formation of the membrane attack complex on the outer surface of Schwann cells, followed by the initiation of vesicular degeneration and invasion of myelin by macrophages (van den Berg *et al.*, 2014; Yuki *et al.*, 2004) (Figure 3). However, the production of crossreactive antibodies is only induced in susceptible individuals and only a subset of *C. jejuni* strains contain lipooligosaccharides that mimic the carbohydrate moiety of gangliosides that are present in human peripheral nerves. The synthesis of these ganglioside-mimicking carbohydrate structures depends on a set of polymorphic genes and enzymes that vary greatly between different *C. jejuni* strains (van den Berg *et al.*, 2014; Nyati & Nyati, 2013; Yuki *et al.*, 2004).

Miller Fisher syndrome

Miller Fisher syndrome is a clinical variant of GBS and is defined by acute-onset ophthalmoparesis, areflexia and ataxia (van den Berg *et al.*, 2014). This condition arises from the development of anti-GQ1b antibodies following exposure to lipooligosaccharides from certain bacteria and several pathogens have been linked to this molecular mimicry, being *C. jejuni* one of the most frequently associated with this condition (Kaakoush *et al.*, 2015; Koga *et al.*, 2005). Nevertheless, further studies are required to investigate the etiology and epidemiology of *Campylobacter*-associated Miller Fisher syndrome and the ability of other *Campylobacter* species to trigger this disease.

Reactive arthritis

Reactive arthritis is a form of arthritis which most commonly develops following gastrointestinal or genitourinary infections and normally occurs in patients between 30 and 40 years of age. This condition can affect joints, such as knees and ankles, the eyes and the genital, urinary and gastrointestinal systems. Symptoms can begin approximately 1 month after infection and be resolved within one year, although in some patients this condition can persist for up to 5 years (Batz *et al.*, 2013; Kaakoush *et al.*, 2015). The incidence of reactive arthritis associated with *Campylobacter* infection has been estimated to range from 1 to 13%, being *C. jejuni* and *C. coli* being the prevalent species causing this condition (Ajene *et al.*, 2013; Batz *et al.*, 2013; Pope *et al.*, 2010).

Bacteremia and septicemia

Bacteremia is one of the most common extragastrointestinal manifestations of *Campylobacter* species and is predominantly associated with *C. jejuni*, *C. coli* and *C. fetus* infections (Man, 2011). *Campylobacter*-associated bacteremia cases are often underreported and the majority of the cases occur in elderly or immunocompromised patients with one or more concurrent pathologies and among these patients about 10% die within 30 days of the diagnosis (Pacanowski *et al.*, 2008). In children, the disease generally results from a single gastroenteritis complication or as recurrent episodes in immunocompromised children without gastrointestinal symptoms (Pacanowski *et al.*, 2008). Furthermore, *C. jejuni*, *C. coli*, *C. fetus* and *C. upsaliensis*, have also been associated with septicemia in both immunocompetent and immunocompromised children and adults (Kaakoush *et al.*, 2015; Man, 2011).

Meningitis

Meningitis is an acute inflammation of the protective membranes covering the brain and spinal cord, known as meninges. This inflammation can be caused by microorganism and less commonly by certain drugs. *C. jejuni* and *C. fetus* have been implicated in the development of meningitis in humans (Man, 2011). *C. jejuni*-associated meningitis is rare and may affect both healthy and immunocompromised children and adults, while meningitis caused by *C. fetus* has been reported for immunocompromised adults, it is also rare (Kaakoush *et al.*, 2015).

Abscesses

Campylobacter species have been reported to be present in several types of abscesses. Most abscesses are polymicrobial, making it difficult to determine the contribution of a specific *Campylobacter* species to the clinical outcome (Kaakoush *et al.*, 2015). *C. rectus* has been associated with breast and vertebral abscesses, *C. gracilis* and *C. concisus* have been implicated in brain abscesses and *C. showae* was detected in an intraorbital abscess (Man, 2011).

Cardiovascular complications

C. jejuni and *C. fetus* have been associated with cardiovascular complications, such as endocarditis and myocarditis (Man, 2011). Myocarditis associated with bacterial enteritis is a rare but serious condition in immunocompetent individuals, which can lead to arrhythmia, dilated cardiomyopathy, congestive heart failure, and sudden cardiac death (De Cock *et al.*, 2012; Westling & Evengard, 2001). *Campylobacter*-associated endocarditis is also an infrequent condition in which both native and prosthetic valves can be affected. It has been demonstrated that individuals with *Campylobacter*-associated endocarditis have either *C.*

jejuni or *C. fetus* infection and one-third of the patients suffer from chronic diseases (Kaakoush *et al.*, 2015; Morrison *et al.*, 1990).

2.6. Mechanisms of virulence of *Campylobacter*

Several virulence mechanisms have been proposed for *Campylobacter* species. Flagella-mediated motility, bacterial adherence to intestinal mucosa and invasion, ability to produce toxins and ability to form biofilms have been identified as the main virulence factors (Bolton, 2015; Dasti *et al.*, 2010; Silva *et al.*, 2011c).

2.6.1. Motility

The motility system in *Campylobacter* requires flagella and a chemosensory system that drives flagellar movement based on the environmental conditions. This motility ability is essential for colonization of the small intestine (Guerry, 2007).

The unusual motility of *Campylobacter* in viscous substances has been attributed to the presence of one or two polar flagella and to the helical cell shape. Flagella provides propulsive torque and rotary cell movement, while the cell shape facilitates corkscrew rotation (Lertsethtakarn *et al.*, 2011). The flagellum is composed by two broad substructures, the hook-basal body and the extracellular filament. The hook-basal body is further divided into three structures: (1) a base embedded in the cytoplasm and inner membrane of the cell, (2) the periplasmic rod and associated ring structures and (3) the surface localized hook. The extracellular filament is composed mostly of the major flagellin protein, FlaA (coded by *flaA*) and the less abundant minor flagellin protein, FlaB (coded by *flaB*). While FlaA is essential for flagellation and motility, the requirement of FlaB varies between *Campylobacter* species (Guerry, 2007; Lertsethtakarn *et al.*, 2011; Nachamkin *et al.*, 1993). The *flaA* gene seems to be essential for the motility and invasion of epithelial cells, since it has been reported that a mutation in this gene leads to a truncated flagellar filament composed only by FlaB with a substantial reduction in motility (Guerry, 2007). The *flaA* gene expression is responsible for the adherence, colonization of the gastrointestinal tract and invasion of the host cells (Dasti *et al.*, 2010). In addition, it is believed that flagella also possess the ability to secrete non-flagellar proteins that may be associated with the virulence phenomenon itself (Baqar *et al.*, 2008; Guerry, 2007). Flagellin O-linked glycosylation is also important for successful colonization. *C. jejuni* possesses a polar flagellum that is composed of O-linked glycosylated flagellin (a two-component system comprised by the sensor FlgS and the response regulator FlgR) that is crucial for approaching attachment sites on intestinal epithelial cells. Defects of O-linked glycosylation lead to loss of motility, thereby decreasing adherence and invasion of host cells (Dasti *et al.*, 2010).

Chemotaxis is a mechanism by which motile bacteria sense and move in the direction of favourable conditions. It is known that *C. jejuni* colonises the avian gut as a commensal and uses chemotaxis to establish primary colonisation sites (Dasti *et al.*, 2010; Lertsethtakarn *et al.*, 2011). During colonisation, the main chemoattractants are the mucins and glycoproteins, which are the major constituent of mucus. Other chemoattractants have also been described and include metabolic substrates such as ketoglutamate, aspartate, asparagine, cysteine, glutamate, pyruvate and serine, electron donors including formate, malate, lactate and succinate and electron acceptors including fumarate, nitrite, nitrate and hydrogen peroxide (Bolton, 2015; Lertsethtakarn *et al.*, 2011). Chemotaxis has been extensively studied in *E. coli* and a similar mechanism has been suggested for *Campylobacter*: using chemoreceptors to sense certain environmental stimulus and transmit this information to a signal transduction cascade that affects flagellar rotation. The core signal transduction proteins include chemoreceptors, the CheA kinase, the CheY response regulator, and the CheW coupling protein, which physically links receptors to CheA. In some strains CheW might be replaced by the homologs CheV. CheA, when activated transfers a phosphoryl group to either CheY and the direction of flagellar rotation is influenced by the phosphorylation status of CheY. The amount of phosphorylated CheY (CheY-P) is regulated by ligand binding to the chemoreceptors, such that an attractant ligand squelches CheA kinase activity and non-phosphorylated CheY predominates. In this form, CheY fails to interact with the flagellar switch, the flagella rotate counter-clockwise, and the bacteria swim. In turn, when phosphorylated, CheY-P binds to the FliM component of the flagellar motor turning the flagellum clockwise, which promotes bacterial sideways tumbling and direction changes. The whole process is regulated by CheR and CheB enzymes which methylate and demethylate, respectively, chemoreceptor glutamyl residues to control adaptation responses to sustained ligand levels (Lertsethtakarn *et al.*, 2011). For *Campylobacter*, it has been described that some *C. jejuni* strains encode chemoreceptor-like proteins, that when mutated severely inhibit colonisation in chickens. Other genes associated with *Campylobacter* chemotaxis include *acfB*, *cetA* and *B* and *luxS*. The *acfB* gene encodes a putative chemoreceptor that may have a role in the persistence of *Campylobacter* in the cecum. The *cetA* and *B* gene products are involved in the *Campylobacter* energy taxis response, while mutation in *luxS* results in reduced chemotaxis toward organic acids and decreased chick colonisation (Bolton, 2015; Cordwell *et al.*, 2008; Lertsethtakarn *et al.*, 2011; Rathbun *et al.*, 2009).

Taken together, the bacterial flagella and chemotaxis system are crucial factors for the initial interaction of *Campylobacter* with its host and facilitate the colonization of the intestine. Nonetheless, signal transduction pathways that regulate motility and chemotaxis of *Campylobacter* still remain open areas for further investigation.

2.6.2. Adhesion and invasion

Several studies demonstrated the importance of *Campylobacter* adhesion in the colonization process, which is mediated by several adhesins on the bacterial surface (Bolton, 2015; Dasti *et al.*, 2010; Penn, 2001). *Campylobacter* adhesion to fibronectin, a glycoprotein found in gastrointestinal epithelial cells, is mediated by CadF, a fibronectin-binding outer membrane protein, encoded by the highly conserved chromosomal *cadF* gene (Cordwell *et al.*, 2008; Dasti *et al.*, 2010; Krause-Gruszczynska *et al.*, 2007a). Binding to fibronectin triggers a signalling process that leads to the activation of the GTPases Rac1 and Cdc42 which induce *Campylobacter* cell internalisation (Krause-Gruszczynska *et al.*, 2007b). The *Campylobacter* adhesion protein A (CapA), encoded by the *capA* gene, was originally thought to be an autotransporter lipoprotein, however it was observed that mutations in this gene result in a reduced capacity to adhere to and invade human epithelial cells (Bolton, 2015; Dasti *et al.*, 2010; Flanagan *et al.*, 2009). Also the phospholipase A (*pldA* gene) has been associated with the bacterial adhesion, since mutants of *pldA* have impaired ability to colonize the cecum, suggesting that this protein may have a role in maintaining the functional integrity of adhesins on the surface of the cell (Bolton, 2015; Penn, 2001). Periplasmic binding protein (PEB) may also have a function in the adhesion process. It includes PEB1, PEB4 and PEB3, commonly found in *C. jejuni* and *C. coli* (Penn, 2001). PEB4 was thought to be an adhesin of *C. jejuni*, but recent studies suggested that its main function was as a chaperone, playing a key role in exporting proteins such as CadF to the outer membrane. PEB3 is a transport protein involved in the utilisation of 3-phosphoglycerate (Asakura *et al.*, 2007; Bolton, 2015; Flanagan *et al.*, 2009; Penn, 2001; Rathbun & Thompson, 2009). FlpA, fibronectin-like protein A, is a protein capable of binding fibronectin and it has been proposed that CadF and FlpA act together during *Campylobacter* adhesion and subsequent invasion (Eucker & Konkel, 2012; Flanagan *et al.*, 2009). Other studies have suggested that *jlpA*, which encodes a lipoprotein, is also involved in adhesion to Hep-2 cells (Cordwell *et al.*, 2008; Zilbauer *et al.*, 2008). Also, the virulence plasmid (pVir) could be involved in the adhesion and invasion, since mutations in one of the plasmid genes encoding a component of a type IV secretion system (T4SS), significantly reduces adherence and invasion as compared to the wild type strain (Bacon *et al.*, 2000; Bolton, 2015; Dasti *et al.*, 2010).

Furthermore, the presence of intracellular bacteria in tissue biopsies from patients and several tissue culture models have demonstrated the invasion ability of *Campylobacter* (Byrne *et al.*, 2007; Chansiripornchai & Sasipreeyajan, 2009; Dasti *et al.*, 2010; Ganan *et al.*, 2010). It has been proposed that invasive *Campylobacter* strains enter gut intestinal cells by a microtubule-dependent and actin filament-independent invasion mechanism (Dasti *et al.*, 2010). As previously described, CadF plays a dual role, firstly it leads to the adhesion to host cells by binding specifically to fibronectin and then it triggers signalling processes leading to activation of the small GTPases, inducing its own internalisation. The invasion process is unique since *Campylobacter* enters the cell with its tip followed by the flagellar end (Krause-

Gruszczynska *et al.*, 2007a, b). Some other bacterial factors have also been studied to determine their role in *Campylobacter* invasion process, such as the capsular polysaccharide (CPS), sialylation of the lipooligosaccharides (LOS) outer core or *Campylobacter* invasive antigens (Cia) (Bolton, 2015; Dasti *et al.*, 2010; Louwen *et al.*, 2008; Zilbauer *et al.*, 2008). It is believed that flagella have a second function, in addition to motility, which is to serve as an export apparatus (type III secretory system; T3SS) in the secretion of non-flagellar proteins during host invasion (Bolton, 2015; Lertsethtakarn *et al.*, 2011). Mutants of the *flaA*, *flaB*, *flgB* and *flgE* genes have reduced invasive ability and the *flaC* and *cia* gene products are delivered into the cytoplasm of the host cells using this flagellar secretion system, being essential for colonisation and invasion (Dasti *et al.*, 2010; Konkel *et al.*, 2004). Cia are known to be synthesized in the presence of the bile component deoxycholate, however, their secretion mechanism and role in the invasion mechanism is not clear. CiaB is a 73 kDa protein that seems to enter host cells during the invasion process and *ciaB* mutants have reduced adherence and significantly reduced invasion potential. CiaC is required for full invasion of INT-407 cells, while CiaI has a reported role in intracellular survival (Bolton, 2015; Dasti *et al.*, 2010; Eucker & Konkel, 2012; Konkel *et al.*, 2004). Homologues of a T4SS on a large plasmid of *C. jejuni* have been identified, and the pVir has also been associated with the bacterial invasion (Bacon *et al.*, 2000). Although the knowledge of the factors related to bacterial invasion is growing, the precise role of invasiveness of *Campylobacter* in the pathogenesis of disease in humans still needs further clarification.

2.6.3. Type IV and VI secretion systems

The type IV secretion system (T4SS) is a system by which bacteria deliver effector proteins or DNA into host cells. In *C. jejuni*, the T4SS has been described to be involved in the invasion into host cells (Bacon *et al.*, 2000; Bolton, 2015).

The type VI secretion system (T6SS) is a functionally-diverse protein secretion system that has been described to play a role in promoting or controlling bacterial virulence in eukaryotic host cells, mediating competition between bacteria and adaptation to environmental perturbation (Ho *et al.*, 2014). A functional T6SS was recently identified in *C. jejuni* and found to exert several roles in virulence, influencing cell adhesion, cytotoxicity toward erythrocytes, and colonization of mice (Bleumink-Pluym *et al.*, 2013; Lertpiriyapong *et al.*, 2012). However, it is unclear whether T6SS contributes directly to human *Campylobacter* infection (Harrison *et al.*, 2014). Given that T6SS-associated genes seem to be present in *C. jejuni*, it would be worthwhile to investigate the potential role of this secretion system in the pathogenesis of other *Campylobacter* species.

2.6.4. Toxin production

Campylobacteriosis, characterized by watery diarrhea that can progress to a bloody diarrhea, is consistent with the idea that bacterial toxins may play a role in this disease. Several different cytotoxins have been reported to be produced by *Campylobacter*, however, the cytolethal distending toxin (CDT) is the only verified *Campylobacter* toxin identified so far (Dassanayake *et al.*, 2005; Johnson & Lior, 1988). Many Gram-negative bacteria have the ability to produce CDT, a tripartite toxin composed of three subunits encoded by the *cdtA*, *cdtB* and *cdtC* genes (Dassanayake *et al.*, 2005; Pickett & Whitehouse, 1999). All three gene products are required for the toxin to be functionally active. The *cdtA* and *cdtC* gene products are responsible for toxin binding to the cell membrane and for delivery of the CdtB, which is the enzymatically active subunit. After that, the CdtB active subunit, which has DNaseI-like activity, enters the nucleus of the host cell and acts like the enzyme deoxyribonuclease blocking the CDC2 kinase, inducing eukaryotic cells to arrest in the G2/M phase of the cell cycle, preventing them from entering mitosis and consequently leading to cell death (Ceelen *et al.*, 2006; Dasti *et al.*, 2010; Jain *et al.*, 2009; Pickett & Whitehouse, 1999).

2.6.5. Biofilm formation

Biofilms are microbial aggregates encapsulated in extracellular polymeric substances that are attached onto surfaces. They are particularly important for conferring resistance to antimicrobial agents and host immune defences (Donlan, 2002; Høiby *et al.*, 2010). During the biofilm formation process, it was observed that bacteria exhibit distinct phenotypic differences from their planktonic counterparts during each subsequent developmental stage. The biofilm development involves 5 stages (Figure 4): (1) reversible attachment, (2) irreversible attachment, (3) early development of biofilm architecture, (4) maturation and, finally (5) dispersion (Richards & Melander, 2009; Srey *et al.*, 2013).

Firstly, the planktonic bacteria identify a substrate suitable for attachment and to begin the biofilm growth cycle. This attachment is initially characterized as reversible, but once the bacteria begin to secrete an exopolymeric substance (EPS) it becomes irreversible. The EPS is one of the most significant components of bacterial biofilms and is composed of a variety of biomolecules originating from both the bacteria and the surrounding environment. EPS serves as a barrier to defend the gathered microorganisms from microbicides and to provide an enclosed space for the biofilm development. The biofilm maturation is observed when the structure becomes three dimensional in space. Then, the biofilm continues to develop and its morphology and topography becomes very distinct. Unique pillar shapes are projected from the biomass, allowing a maximization of nutrient adsorption and waste disposal. In addition, the formation of cavities or empty channels throughout the biofilm becomes evident, creating in the biofilm a transport system necessary to funnel water and planktonic bacteria

throughout the community. The last stage in the developmental cycle is the detachment of bacteria from the biofilm, that move on to further colonize distal sites and repeat the development cycle. The mechanism of how or why dispersion occurs is still not clear, but it can be attributed to a number of factors including various environmental pressures such as starvation conditions or overcrowding (Donlan, 2002; Flemming *et al.*, 2007; Høiby *et al.*, 2010; Richards & Melander, 2009; Srey *et al.*, 2013).

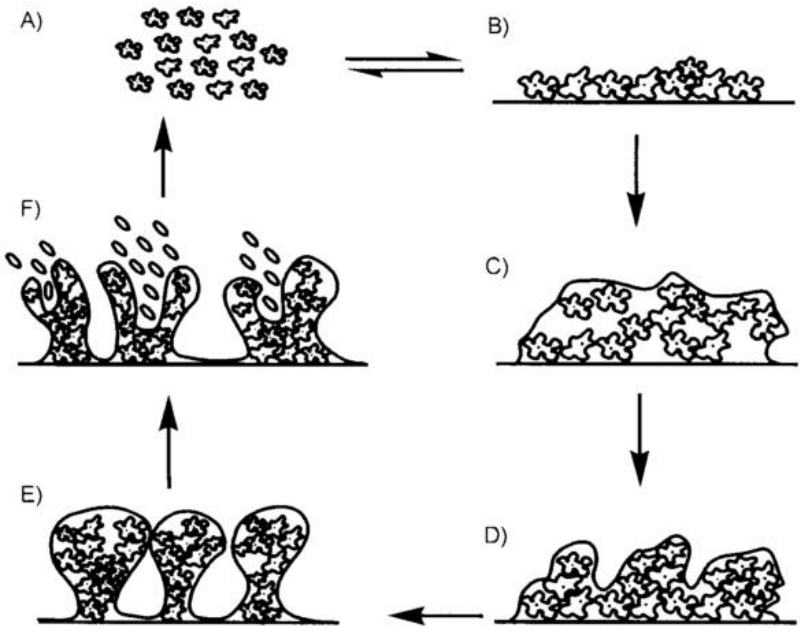

Figure 4. Biofilm development stages: A) planktonic bacteria; B) reversibly attach to a surface; C) attachment becomes irreversible due to bacteria secretion of the exopolymeric substance (EPS); D) development of the 3-dimensional shape of the mature biofilm; E) Mature biofilm with a complex architecture; F) bacteria disperse from the biofilm to reinitiate biofilm colonization of a distal surface (Richards & Melander, 2009).

The biofilm formation has been extensively studied for a number of bacteria and the cellular communication system "quorum sensing" (QS) has been associated with the development of biofilms (Deep *et al.*, 2011; Richards & Melander, 2009). QS is the mechanism by which the bacteria communicate with each other, attempting to in cooperation change the gene expression within the population (Deep *et al.*, 2011). In Gram-negative bacteria, small molecules such as acyl homoserine lactones (AHLs) have been identified as the major players in QS and the autoinducing peptides, which bind to receptor histidine kinases embedded within the cellular membranes are commonly used for the communication (Deep *et al.*, 2011; Richards & Melander, 2009). QS systems are also known to be involved in a range of microbial activities, such as extracellular enzyme biosynthesis, antibiotic resistance, biosurfactant production, EPS synthesis and extracellular virulence factors in Gram-negative bacteria (Deep *et al.*, 2011; Prashanth *et al.*, 2012; Que *et al.*, 2013; Shrout *et al.*, 2006; Simões *et al.*, 2010).

Campylobacter species have the capacity to form biofilms and consequently the ability to survive in the natural environment, such as in the food plants (Bridier *et al.*, 2015; Gunther IV & Chen, 2009; Murphy *et al.*, 2006; Srey *et al.*, 2013; Teh *et al.*, 2014). However, the precise role of biofilm formation in the pathogenesis of *Campylobacter* requires further investigation. It has been proposed that the microenvironment created within the biofilm may protect the *Campylobacter* cells from oxygen inactivation (Buswell *et al.*, 1998). The increased viability of *C. jejuni* in biofilms and the resistance to stress conditions may be caused by the fact that *C. jejuni* becomes viable but non-culturable in biofilms (Jackson *et al.*, 2009; Magajna & Schraft, 2015). In contrast, biofilm cells of *C. jejuni* have also been demonstrated to lack an adaptive stress resistance mechanism. Dykes *et al.* (2003), described an altered protein expression when cells were grown in biofilm mode, but they observed a significant reduction in survival of these cells to acid stress when compared with planktonic cells. *Campylobacter* biofilms may also have a role on antimicrobial resistance and recently it was demonstrated that *C. jejuni* transfers antibiotic resistance genes more frequently in biofilms than in planktonic cells by natural transformation (Bae & Jeon, 2013; Bae *et al.*, 2014). Biofilms may play a critical role in the prevalence of *Campylobacter* in food processing environments, although these biofilms are susceptible to common sanitizers and are completely inactivated by chlorine (Sarjit & Dykes, 2015; Simões *et al.*, 2010; Srey *et al.*, 2013; Teh *et al.*, 2014).

QS was also identified in *Campylobacter* and involves the synthesis, secretion and detection of extracellular signaling molecules termed autoinducers (AI) (Elvers & Park, 2002). *C. jejuni* is known to possess a LuxS/AI-2 mediated system that has been partially characterized over the last decade. AI-2 is formed as a byproduct of the activated methyl recycling pathway, specifically by the LuxS enzyme. Previous work has demonstrated that this gene is involved in a variety of physiologic pathways of *C. jejuni* including motility, CDT expression, flagellar expression, oxidative stress and animal colonization (Gölz *et al.*, 2012; Plummer, 2012; Plummer *et al.*, 2011). *C. jejuni* AI-2 activity was found in several food environments, such as milk, apple juice and chicken broth, suggesting that this could be a mechanism of adaptation to the environment (Cloak *et al.*, 2002).

Pathogenic microorganisms in biofilms formed in different food industry settings are a source of food contamination. As the demand for fresh, ready-to eat and processed foods increases, strategies to reduce/eradicate bacteria and their activity and to prevent/control the formation of biological deposits on the processing equipments are needed. Nowadays, the most efficient and practical resources for limiting microbial growth include good hygiene, a rational running of the process line and effective use of cleaning and disinfectant products. Due to the increased resistance of biofilms to conventional disinfection processes, novel means for their control are constantly required. In addition, those methods shall provide a desirable cost-effective result and not causing any adverse effect on human health as well as the environment. Therefore, the discovery of new biofilm control strategies based on the use

of biological-based solutions with high antimicrobial activity and specificity seems to be a step in the right direction to overcome biofilm resistance. Moreover, inhibiting biofilms and quorum sensing by natural antimicrobials could also be an alternative to control the appearance and growth of biofilms (Simões *et al.*, 2010; Srey *et al.*, 2013).

2.7. Antimicrobial resistance

Most *Campylobacter* infections are self-limiting and therapeutic intervention is not generally required, with the exception of electrolyte replacement and rehydration. However, antimicrobial treatment is employed in immunocompromised patients, patients whose symptoms are severe or persistent and those with extraintestinal infections (Allos, 2001). The most common antimicrobial agents used in the treatment of *Campylobacter* infections are macrolides, such as erythromycin, and fluoroquinolones, such as ciprofloxacin (Duarte *et al.*, 2014; Wieczorek & Osek, 2013). Tetracyclines have been suggested as an alternative in the treatment of these infections, but in practice they are not often used (Wieczorek & Osek, 2013). Nevertheless, the antibiotic resistance in *Campylobacter* has become in recent years a major public health concern in both developed and developing countries. Another problem related to antibiotic resistance in *Campylobacter* infections is the emergence of multidrug-resistant (MDR) strains, which have been isolated worldwide.

2.7.1. *Campylobacter* emerging resistance to antimicrobials

Campylobacter may encounter specific antibiotics during commensal carriage in animals or during infection in humans, nevertheless there is strong evidence linking the indiscriminate usage of antibiotics in animal production to the emergence and spread of antibiotic resistance in *Campylobacter* species (Aarestrup & Wegener, 1999; Luangtongkum *et al.*, 2009; Silva *et al.*, 2011c). Antimicrobial resistance can difficult the clinical treatment of *Campylobacter* infections. As previously described, macrolides, tetracyclines and fluoroquinolones are the antibiotics of choice to the therapy of *Campylobacter* infections, however, the efficacy of these treatments is currently compromised by the increased resistance of *Campylobacter* to these antibiotics (Bolton, 2015; Koluman & Dikici, 2013).

Antimicrobial resistance has become a major concern in public health. The latest EFSA report concerning the antimicrobial resistance in zoonotic bacteria described that, in 2013, a high proportion of *Campylobacter* human isolates were resistant to ciprofloxacin and tetracyclines, while resistance to erythromycin was low to moderate and the resistance to fluoroquinolones in some European countries was extremely high (EFSA & ECDC, 2015b). It was also described a high to extremely high resistance to ciprofloxacin, nalidixic acid and tetracyclines in

Campylobacter isolates from broiler meat, pigs and cattle, whereas much lower levels were observed for erythromycin and gentamicin (EFSA & ECDC, 2015b).

In recent years, a rapidly increasing proportion of *Campylobacter* strains worldwide have developed resistance to several antimicrobials. In a five-year study during 2009-2013 in Poland, Wieczorek & Osek (2015) showed that overall 81.6% of the poultry isolates were resistant to ciprofloxacin, 56.1% to tetracycline and 2.4% to erythromycin. Furthermore, the authors observed a significant increase in the percentage of *C. jejuni* resistant to ciprofloxacin, from 59.6% in 2009 to 85.9% in 2014, and to tetracycline from 23.2% to 70.4% (Wieczorek & Osek, 2015). A study that investigated the antimicrobial resistance of thermotolerant *Campylobacter* species isolated from different stages of the poultry meat supply chain in Argentina, also showed that almost all of the isolates were resistant to nalidixic acid (91.2%) and ciprofloxacin (88.2%), one strain was resistant to all the antibiotics analyzed and 46.1% of the isolates were resistant to three or more drug classes (Zbrun *et al.*, 2015). Moreover, 50% of the isolates were resistant to all quinolones tested (ciprofloxacin, nalidixic acid and enrofloxacin) and 13.2% were resistant to erythromycin in addition to all quinolones (Zbrun *et al.*, 2015). Torralbo *et al.* (2015), also reported worrying levels of antimicrobial resistance in *Campylobacter* species isolated from chicken meat. They reported that 100% of resistance was observed for ciprofloxacin and tetracycline, 73.3% of the *C. coli* isolates were resistant to erythromycin and 13.3% and 16.7% of *C. jejuni* and *C. coli* isolates, respectively, were resistant to gentamicin (Torralbo *et al.*, 2015). Similarly, Ghosh (2013) also reported an increasing antimicrobial resistance of *C. jejuni* isolated from pediatric diarrhea cases. In this study, a high degree of resistance to fluoroquinolones (100% to nalidixic acid and 86.7% to ciprofloxacin) was detected, in addition 33.3% of the isolates were resistant to tetracycline, 22.2% to erythromycin and 41.7% of the isolates were multiresistant (Ghosh *et al.*, 2013). Although the high resistance rates observed worldwide, the antimicrobial resistance may be very different from one country or world region to another (Wieczorek *et al.*, 2013). For example, in a study comparing the antimicrobial resistance among *Campylobacter* isolates from Australia, Poland and Malaysia, 18 of the 20 Australian isolates were susceptible to all the tested antimicrobials, while 54.5% of the Polish isolates were classified as multiresistant (Wieczorek *et al.*, 2013). Among Malaysian isolates more than 60% of the strains were resistant to each antimicrobial (Wieczorek *et al.*, 2013).

In Portugal, several studies have also investigated the antimicrobial resistance in *Campylobacter* strains. In the EFSA report, antimicrobial resistance levels among *C. coli* isolates from meat in Portugal were the highest described (EFSA & ECDC, 2015b). For ciprofloxacin, nalidixic acid and tetracyclines 100% of resistance was described and about 73% for erythromycin (EFSA & ECDC, 2015b). In one of the first reports in Portugal, Cabrita *et al.* (1992a), within 150 *Campylobacter*-positive stool samples from gastroenteritis cases, described low rates of antimicrobial resistance with 3.7% of the isolates being resistant to

ampicillin, 2.8% to tetracycline and all of the isolates being susceptible to erythromycin, gentamicin and nalidixic acid. The same author in the same year, also reported low antimicrobial resistance of *Campylobacter* isolated from wild and domestic animals from northeast Portugal (Cabrita *et al.*, 1992b). In this study, the antimicrobial susceptibility testing revealed that 5.5% of the strains were resistant to ampicillin, 5.5% to tetracycline, 12.6% to erythromycin and no isolates were resistant to gentamicin (Cabrita *et al.*, 1992b). Years later and unlike the older data, Vicente *et al.* (2008) described high rates (80%) of ciprofloxacin-resistant *Campylobacter* isolated from humans in Portugal. In the same year, it was observed that 90% of the isolates collected from poultry in retail establishments were resistant to tetracycline, nalidixic acid, erythromycin, vancomycin and penicillin and sensitive to gentamicin and chloramphenicol (Mena *et al.*, 2008). In 2012, in a national survey of zoonotic *Campylobacter* species isolated from broilers, the same high antimicrobial resistance rates were observed with 98% and 88% of resistance to ciprofloxacin and tetracycline, respectively, and 63% to erythromycin (Carreira *et al.*, 2012). Similarly, Duarte *et al.* (2014), in a study with human, food and animal *Campylobacter* isolates, reported a high rate of resistance for quinolones (100% to nalidixic acid, >90% to ciprofloxacin), 80% of resistance for tetracycline and most isolates (86%) were resistant to multiple antimicrobial families. In addition, Fraqueza *et al.* (2014) showed that *Campylobacter* isolated from poultry were resistant to the fluoroquinolones studied (80–98%). The same author, in a recent study, observed that among the studied *Campylobacter* isolates from quails, the fluoroquinolones resistance frequency was very high (96.7%-100%), the isolates were also very frequently resistant to tetracycline (96.7%) and ampicillin (91.2%) and a lower resistance to erythromycin (69.2%) was observed, while all the isolates presented a high susceptibility to chloramphenicol and gentamicin (Fraqueza *et al.*, 2016). In sum, a rapidly increasing in the resistance rates among *Campylobacter* strains in Portugal has been observed, in particular resistance to macrolides, fluoroquinolones and tetracyclines, which are the drugs of choice for serious campylobacteriosis treatment.

2.7.2. Mechanisms of antimicrobial resistance in *Campylobacter*

Campylobacter species use similar mechanisms for the acquisition of resistance as other Gram-negative bacteria. The main mechanisms of antimicrobial resistance for the main classes of antibiotics are described in Table 2.

Table 2. Antimicrobial resistance mechanism of *Campylobacter*.

Antibiotic class	Mechanism of action	Resistance mechanism
Aminoglycosides	Inhibiting the bacterial protein synthesis	Modification of the antibiotic by aminoglycoside-modifying enzymes (a*phA*, *aadE*, *sat*). Contribution of efflux is not clear.
ß-Lactams	Inhibiting cell wall biosynthesis	Enzymatic inactivation of the antibiotic by ß-lactamase (penicillinase, OXA-61). Efflux through CmeABC pump. Decreased membrane permeability.
Fluoroquinolones	Affecting DNA replication, transcription and repair	Modification of the DNA gyrase target (Thr-86-Ile and Thr-86-Ala; also Asp-90-Asn, Asp-90-Tyr and Ala-70-Thr). Efflux through CmeABC pump.
Macrolides	Inhibiting the bacterial protein synthesis	Mutations in 23S rRNA gene. Efflux through CmeABC pump. Decreased membrane permeability.
Tetracyclines	Inhibiting the bacterial protein synthesis	Modification of the target ribosomal A site by TetO binding. Efflux through CmeABC and possibly others.

Fluoroquinolones resistance

Fluoroquinolones are broad-spectrum antibiotics, effective against infections caused by both Gram-negative and Gram-positive pathogens. Ciprofloxacin is the most commonly used fluoroquinolone in human medicine, while enrofloxacin is widely used in animals. Some newer molecules, such as levofloxacin or moxifloxacin, have also been used recently (Iovine, 2013; Luangtongkum *et al.*, 2009). In general, fluoroquinolones bind to a specific region known as the quinolone-resistance-determining region (QRDR) in the DNA gyrase gene (*gyrA*) and affect DNA supercoiling, decreasing transcription and causing bacterial death (Changkwanyeun *et al.*, 2015; Luangtongkum *et al.*, 2009).

In *Campylobacter*, there are two well-described mechanisms that underlie the resistance to fluoroquinolones and can work synergistically: (1) inactivation of the target and (2) efflux mechanisms (Changkwanyeun *et al.*, 2015; Ge *et al.*, 2005; Wieczorek & Osek, 2013; Yan *et al.*, 2006). Mutations in the QRDR region are associated with resistance and the mutation in codon Thr-86-Ile is associated with high-level resistance to nalidixic acid and ciprofloxacin being the most commonly described mutation (Iovine, 2013; Jesse *et al.*, 2006; Payot *et al.*, 2006). The less common Thr-86-Ala mutation confers resistance only to nalidixic acid (Iovine, 2013; Jesse *et al.*, 2006; Payot *et al.*, 2006). Other mutations associated with increased

resistance are Asp-90-Asn, Asp-90-Tyr and Ala-70-Thr and these mutations cause an intermediate level of resistance (Payot *et al.*, 2006; Wieczorek & Osek, 2013). Another mechanism of fluoroquinolones-resistance is trough efflux pumps. The chromosomally-encoded CmeABC efflux pump which is responsible for the transport of physiologically important compounds, also plays a role in the acquisition of fluoroquinolones resistance (Guo *et al.*, 2010; Iovine, 2013; Lin *et al.*, 2002; Yan *et al.*, 2006). The CmeABC efflux pump is negatively regulated by the *CmeR* regulator, which also regulates a set of other activities in the bacterial cell making this regulator important for cell function as well as for regulating antimicrobial resistance. The transcriptional regulator *CmeR* binds specifically with an inverted repeat (IR) region upstream of the *CmeA* gene (Lin *et al.*, 2002, 2005). It has been shown that mutations in the repressing site lead to an overexpression of the efflux pump and consequently to enhanced resistance to several antimicrobials. Studies have identified mutations in the IR region which could change the affinity for *CmeR* binding (Iovine, 2013; Perez-Boto *et al.*, 2010). An additional putative efflux pump, CmeG, has also recently been described as conferring resistance to fluoroquinolones. Mutations in the *cmeG* gene led to a 4-fold reduction in ciprofloxacin resistance vs. the wild-type, while an 8- to 32-fold increase in resistance to ciprofloxacin is observed when the *cmeG* is overexpressed (Iovine, 2013).

Macrolides resistance

Erythromycin (a macrolide antibiotic) is the treatment of choice for campylobacteriosis. Other members of the macrolides class of antibiotics include clarithromycin, azithromycin, telithromycin used in human medicine and tylosin and tilmicosin approved for veterinary use and the most commonly used in food production animals (European Medicines Agency, 2013; McEwen & Fedorka-Cray, 2002). Macrolides are large molecules (MW > 500) that inhibit protein synthesis by binding reversibly to the P site on the 50S subunit of bacterial ribosomes. They interfere with aminoacyl translocation, preventing the transfer of tRNA from the A site to the P site of the rRNA complex. Without this translocation, the A site remains occupied and thus preventing amino acids from attaching (Gibreel & Taylor, 2006; Gibreel *et al.*, 2005; Payot *et al.*, 2006).

The main mechanisms of resistance to macrolides in *Campylobacter* are: (1) target modification and (2) efflux pumps (Iovine, 2013; Wieczorek & Osek, 2013). Macrolide resistance in *Campylobacter* is strongly associated with point mutations in the peptidyl encoding region in the domain V of the 23S rRNA gene at positions 2074 and 2075, and confer high-level macrolide resistance with the 2075 substitution being more common (Hao *et al.*, 2013; Perez-Boto *et al.*, 2010; Vacher *et al.*, 2005). In addition to target specific mutation, the efflux pump CmeABC also contributes to macrolide resistance by increasing transport of macrolides and could act synergistically with 23S rRNA mutations resulting in high-level macrolide resistance (Cagliero *et al.*, 2007; Gibreel *et al.*, 2007; Mamelli *et al.*, 2006; Perez-

Boto *et al.*, 2010). The CmeG efflux pump may also contribute to macrolide resistance, since insertional mutagenesis of *cmeG* causes an 8-fold reduction in erythromycin resistance (Iovine, 2013). A third mechanism of macrolide resistance involves altered membrane permeability mediated by expression of the major outer membrane porin (MOMP), chromosomally encoded by *porA* (Iovine, 2013).

Aminoglycosides resistance

This class, including gentamicin, amikacin, neomycin, tobramycin, kanamycin and streptomycin, has traditionally been used in therapeutic treatments, especially streptomycin and neomycin which were commonly used in veterinary medicine (Iovine, 2013). Aminoglycosides are bacterial protein synthesis inhibitors which act by binding to the 30S ribosome. Aminoglycosides contain amino-modified sugars that are positively charged and water-soluble. The initial binding of aminoglycosides to negatively charged bacterial membranes is electrostatic and relatively slow and the second phase is a rapid but reversible binding to the 30S segment of the ribosome (Iovine, 2013; Wieczorek & Osek, 2013). It is believed that aminoglycosides can interfere with the translocation of the nascent peptide chain from the ribosomal A site to the P site leading to premature termination, and interfere with proof-reading, leading to incorporation of incorrect amino acids and dysfunctional protein (Iovine, 2013).

The mechanisms of resistance in *Campylobacter* have not been systematically clarified but the main mechanism is described to be via aminoglycoside modifying enzymes (Iovine, 2013; Qin *et al.*, 2012; Wieczorek & Osek, 2013). Aminoglycoside resistance was first detected in *C. coli* and was mediated by a 3'-aminoglycoside phosphotransferase (encoded by *aphA-3*) that had been previously described as conferring kanamycin resistance in *Streptococcus* and *Staphylococcus*. This *aphA-3* gene is the most common source of aminoglycoside resistance in *Campylobacter*. Streptomycin resistance is encoded by *aadE*, a 6′-adenyl transferase and streptothricin resistance is encoded by *sat*, an acetyl transferase. These resistance genes are often located on plasmids or as recently shown, on a genomic island and are probable transferred from enterococci to *Campylobacter*. Other genes which confer kanamycin resistance were also detected on plasmids in *C. jejuni* and include *aphA-1* and *aphA-7*. The *aphA-7* gene has a similar G-C content to *C. jejuni* chromosomal DNA, suggesting it is intrinsic in *Campylobacter*. Also, integrons seem to have a role in aminoglycoside resistance (Chen *et al.*, 2013; Iovine, 2013; Qin *et al.*, 2012; Wieczorek & Osek, 2013). Furthermore, the efflux mechanisms may have a role to aminoglycoside resistance, but the mechanism is less clear. It was described that the presence of the efflux pump inhibitor phenylalanine-arginine-ß-naphthylamide decreased the resistance to gentamicin in *Campylobacter* strains (Duarte *et al.*, 2014). In addition, polymorphisms in the 5 bp immediately upstream of an intact IR of the *cmeABC* locus were also detected in gentamicin resistant strains (Duarte *et al.*, 2014).

Therefore, the contribution of efflux to aminoglycoside resistance in *Campylobacter* is not completely elucidated but is likely to be less important than the plasmid-borne drug-modifying enzymes described previously (Iovine, 2013).

Tetracyclines resistance

Tetracyclines were discovered in the 1940s and act against both Gram-negative and Gram-positive organisms. Tetracyclines were widely used in the past for both human and veterinary medicine. Nowadays, the widespread resistance and development of other antimicrobials has limited their use. Tetracycline, doxycycline and minocycline are the most commonly used members of this class. The tetracyclines are lipophilic protein synthesis inhibitors that likely use a combination of the hydrophobic pathway described for macrolides as well as outer membrane porins to gain access to the bacterial ribosome. Once inside the bacteria cytoplasm, tetracyclines reversibly bind to the 30S subunit of ribosomes and inhibit protein synthesis by preventing the attachment of charged aminoacyl-tRNA to the ribosomal A site (Iovine, 2013; Wieczorek & Osek, 2013).

Known mechanisms for tetracycline resistance in *Campylobacter* include (1) alteration of tetracycline ribosomal target and (2) interference with the efflux pump CmeABC (Iovine, 2013). The major mechanism of tetracycline resistance is protection of an unoccupied A site by binding of the bacterial protein TetO to that site. The TetO protein binds to the same ribosomal site as tetracycline and inhibits binding of tetracycline. The *tet(O)* gene in most cases is located on a plasmid but can also be located in different positions on the chromosome. The original hosts of the *tet(O)* gene are intestinal enterococci which have the capability to transfer it to *Campylobacter* (Iovine, 2013; Wieczorek & Osek, 2013). The contribution of efflux to tetracycline resistance has also been demonstrated. The inactivation of the CmeABC efflux pump by disruption of *cmeB* led to a decrease in the tetracycline resistance (Gibreel *et al.*, 2007; Lin *et al.*, 2002). It has also been suggested that CmeABC and TetO can act synergistically in increasing resistance to tetracyclines (Gibreel *et al.*, 2007; Iovine, 2013; Lin *et al.*, 2002).

ß-Lactams resistance

ß-Lactams have been the most widely available antimicrobials on the market. The ß-lactam antibiotics group includes penicillins, oxacillin, amoxicillin, ampicillin, carbenicillin, cephalosporins, carbapenems and monobactams and all of them contain the ß-lactam ring required for the antimicrobial activity. These antibiotics are used extensively in veterinary medicine, however emerging resistance has compromised their use. ß-Lactam antibiotics inhibit cell wall biosynthesis by binding to the bacterial peptidoglycan transpeptidases (also called as penicillin-binding proteins) required to catalyze the final cross linking step and

thereby inactivating them. The resulting bacterial cell walls lack structural integrity and are subject to osmotic swelling and consequently lysis (Iovine, 2013; Wieczorek & Osek, 2013).

There are two main mechanisms described to mediate ß-lactam resistance in *Campylobacter*: (1) enzymatic inactivation by ß-lactamases and (2) efflux (Iovine, 2013). Several resistance mechanisms exist but the most commonly studied is ß-lactamase production. The expression of a penicillinase-type of ß-lactamase in *Campylobacter* is described to confer resistance to amoxicillin, ampicillin and ticarcillin (Alfredson & Korolik, 2007). In addition, a ß-lactamase gene *OXA-61* associated with ampicillin resistance was identified in *Campylobacter* (Alfredson & Korolik, 2007) with a mutation in the promoter region being required to activate ß-lactamase production (Alfredson & Korolik, 2007; Wieczorek & Osek, 2013). Two genes encoding a metallo-ß-lactamase type of enzyme have also been described, although it is not yet clear if expression actually leads to ß-lactam resistance (Iovine, 2013). In addition to the target-specific mechanism, the CmeABC efflux pump has also been associated with the ampicillin resistance in *Campylobacter*. Insertional mutagenesis of *cmeB* led to an increase in ampicillin susceptibility. Similarly to macrolides, the reduced uptake due to alterations in outer membrane porins is also described as a possible mechanism of resistance, however the underlying mechanism is not yet clearly described (Iovine, 2013; Wieczorek & Osek, 2013).

2.8. Control of *Campylobacter* by natural compounds

The growing resistance of *Campylobacter* to antibiotics and its ability to form biofilms has created the need to find and develop new antimicrobial agents to control this foodborne pathogen. In addition to bacteria, oxidation processes are also a cause of food deterioration and loss of quality. Oxidative stress occurs due to the irregular generation of free radicals reactive oxygen species which triggers the oxidative stress and damaging the lipid and protein fractions of food products (Falowo *et al.*, 2014). Therefore, natural compounds have been explored as a source for new antimicrobials, as well as antioxidants. Besides having antimicrobial and antioxidant properties, natural products also receive the preference of consumers, thus making them an increasingly popular subject of research (Falowo *et al.*, 2014; Sanches-Silva *et al.*, 2014; Szczepanski & Lipski, 2014; Taylor, 2013; Tiwari *et al.*, 2009).

In recent years, natural compounds have received great attention as potential antibacterial agents, which results into a large number of scientific papers exploring and describing their antimicrobial activity, antioxidant, anti-biofilm and anti-QS (Dorman & Deans, 2000; Duarte *et al.*, 2012, 2013a; Ferreira *et al.*, 2012, 2014b; Luís *et al.*, 2014a, b; Nazzaro *et al.*, 2013; Silva *et al.*, 2011a, b; Vattem *et al.*, 2007).

2.8.1. Resveratrol

Natural polyphenols are molecules with recognized chemopreventive and therapeutic efficacy. Among them, resveratrol (3,5,4'-trihydroxystilbene) is commonly encountered in foods and drinks, such as red wine, grapes, berries, peanuts, pistachios and chocolate (Counet *et al.*, 2006; Kiselev, 2011). Resveratrol has been associated with several biological activities and with different targets and mechanism. Resveratrol has shown anti-cancer, anti-inflammatory, antioxidant and antimicrobial properties, in addition to preventing many aging processes and can help or to protect against ischemia-reperfusion, cardiovascular disease, neurodegenerative processes and metabolic diseases (Cottart *et al.*, 2014; Delmas *et al.*, 2011; Paulo *et al.*, 2011a; Sadruddin & Arora, 2009). However, resveratrol is characterized by limited aqueous solubility, poor bioavailability, weak absorption after oral administration and rapid metabolization *in vivo*. Many studies have shown that the main biological activities are exerted by *trans*-resveratrol rather than *cis*-resveratrol (Anisimova *et al.*, 2011; Bhat KPL *et al.*, 2001; Cottart *et al.*, 2014; Delmas *et al.*, 2011). However, *trans*-resveratrol is converted into its *cis* isomer, a less active form, upon light exposure (Figure 5).

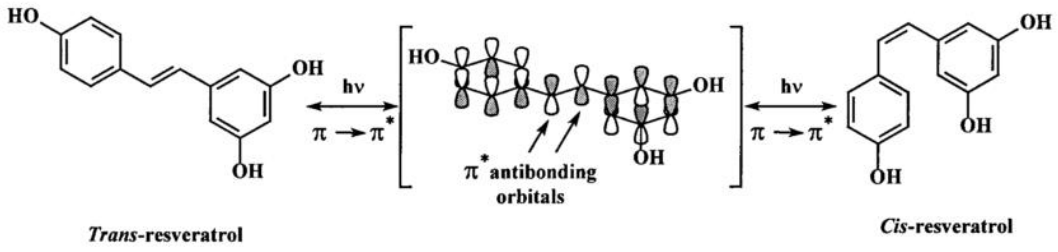

Figure 5. Mechanism for light-induced isomerisation of resveratrol. Resveratrol exists dominantly as the *trans* isomer, but exposure to light cause excitation of the electrons in the alkene to suffer a π to π* transition and induce isomerization through a perpendicular transition state to *cis*-resveratrol (Bhat KPL *et al.*, 2001).

In addition to several biological activities, resveratrol has also been studied for its antimicrobial properties. This compound has the ability to inhibit the growth of some pathogenic microorganisms such as Gram-positive and Gram-negative bacteria and fungi (Jung *et al.*, 2007; Lee *et al.*, 2014; Paulo *et al.*, 2010, 2011a, b). The antimicrobial activity of resveratrol against *Arcobacter* species which shares phenotypic similarities with *Campylobacter* has already been described (Ferreira *et al.*, 2014b). A bactericidal or bacteriostatic activity was observed depending on growth phase and resveratrol concentration against *A. butzleri* and *A. cryaerophilus*. Moreover, resveratrol caused a significant decrease on *Arcobacter* metabolic activity and a reduction in the intracellular DNA content, while being able to inhibit the efflux pump. This study suggested that the resveratrol antibacterial activity may result from the action of the compound in various targets, leading to the induction of cell death due to impairment of cellular functions (Ferreira *et al.*, 2014b).

Resveratrol also has the ability to inhibit several virulence factors of pathogenic bacteria, such as biofilms (Augustine *et al.*, 2014; Lee *et al.*, 2014; Paulo *et al.*, 2011b; Wang *et al.*, 2006). The anti-QS properties of resveratrol against *Proteus mirabilis*, by inhibiting the swarming and virulence expression was also described (Wang *et al.*, 2006), as well as the ability to inhibit the violacein production by the biosensor strain *Chromobacterium violaceum* (Alvarez *et al.*, 2012).

Although resveratrol has displayed good properties to be used as an antimicrobial agent in the control of many pathogens, its application is still limited due to its physicochemical properties and low bioavailability. In order to overcome these problems, many formulations based on cyclodextrins inclusion complex, microencapsulation, liposomes and nanoparticles have been developed (Augustin *et al.*, 2013; Bonechi *et al.*, 2012; Lu *et al.*, 2009; Santos *et al.*, 2011).

Inclusion complexes

Based on the number of published papers, cyclodextrins (CDs) are the most common host molecules used to form inclusion complexes in the food industry (Del Valle, 2004). CDs are a group of naturally occurring cyclic oligosaccharides composed of glucopyranose derived from starch, which are constituted by variable glucose residues linked by α-(1,4) glycosidic bonds (Figure 6).

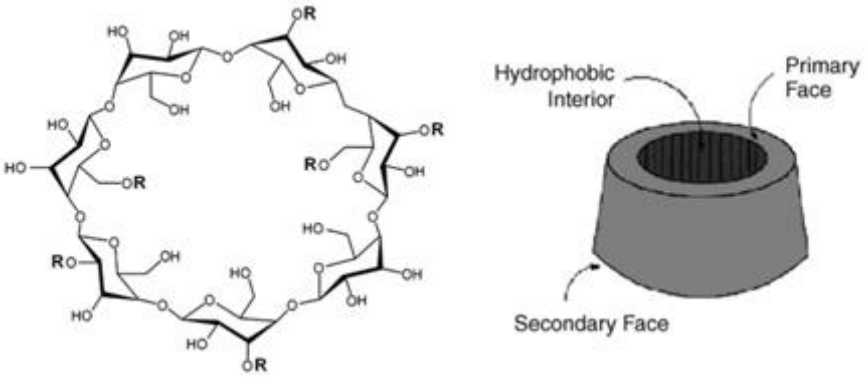

Figure 6. Chemical structure of a cyclodextrin (Del Valle, 2004).

The steric arrangement of glucose units in the CD results in a hollow truncated cone with a hydrophilic outside surface, which makes CDs water soluble, and a hydrophobic internal cavity, which enables CDs to form inclusion complexes with various hydrophobic guest molecules (Figure 6) (Stella & He, 2008; Del Valle, 2004; Zhang & Ma, 2013). CDs are divided into two groups: naturals, obtained with high yield, namely α-, ß-, and γ-CD consisting of six, seven, or eight glucose units, respectively; and chemically modified CDs. Many modified CDs have been synthesised, usually produced by aminations, esterifications or etherifications of primary and secondary hydroxyl groups of the CDs. Depending on the substituent, the

solubility of the modified CD is usually different from that of their parent CD. All derivatives have a changed hydrophobic cavity volume and these modifications can also improve solubility, stability against light or oxygen and help control the chemical activity of guest molecules (Kurkov & Loftsson, 2013; Del Valle, 2004). CDs possess negligible toxicity and many of them are generally recognized as safe (GRAS) by the United States Food Drug Administration, being currently used in food, pharmaceuticals, cosmetics, environment protection, packing and the textile industry (Kurkov & Loftsson, 2013; Loftsson & Duchêne, 2007; López-Nicolás *et al.*, 2014; Del Valle, 2004).

The formation of resveratrol/CDs inclusion complex has been widely described mainly in order to overcome resveratrol low solubility and to increase its stability and bioavailability (Davidov-Pardo & McClements, 2014; Duarte *et al.*, 2015a, b; Kumpugdee-Vollrath *et al.*, 2012; Lu *et al.*, 2009; Santos *et al.*, 2011; Silva *et al.*, 2014). These inclusion complex are known to have antioxidant (Duarte *et al.*, 2015a; Lu *et al.*, 2009, 2012), anti-cancer (Lu *et al.*, 2012; Venuti *et al.*, 2014), antibacterial (Duarte *et al.*, 2015b) and anti-biofilm activity (Duarte *et al.*, 2015b).

2.8.2. Coriander essential oil

Essential oils (EOs) are volatile, natural, complex compounds characterized by a strong odour and are formed as secondary metabolites by aromatic plants. EOs are usually obtained by steam or hydro-distillation from various aromatic plants. In nature, essential oils play an important role in the protection of the plants functioning as antibacterials, antivirals, antifungals, insecticides and also against herbivores by reducing their appetite for such plants. They also may attract some insects to favour the dispersion of pollens and seeds, or repel undesirable others (Bakkali *et al.*, 2008). They are known for their antiseptic, medicinal properties and their fragrance and they are used in packaging, preservation of foods and as antimicrobial, analgesic, sedative, anti-inflammatory, spasmolytic and locally anesthesic remedies (Bakkali *et al.*, 2008; Calo *et al.*, 2015; Jayasena & Jo, 2013). Nowadays, EOs are widely studied due to their antimicrobial and antioxidant properties (Bakkali *et al.*, 2008; Burt, 2004; Calo *et al.*, 2015; Falowo *et al.*, 2014; Jayasena & Jo, 2013; Ozogul *et al.*, 2015; Sanches-Silva *et al.*, 2014; Silva & Domingues, 2015). EOs are very complex mixtures which can contain between 20 to more than 60 components at different concentrations and are characterized by two or three major components at fairly high concentrations (20–70%) when compared to others components. For example, carvacrol (30%) and thymol (27%) are the major components of the *Origanum compactum* EO, while linalool (≈68%) is in majority in *Coriandrum sativum* EO (Bakkali *et al.*, 2008; Silva *et al.*, 2011a).

Coriander EO is known for its antioxidant activity, being considered a source of natural antioxidants and may be used in the food industry as a substitute of synthetic antioxidants

(Laribi *et al.*, 2015; Samojlik *et al.*, 2010; Silva & Domingues, 2015; Singh *et al.*, 2002). Moreover, its bactericidal effect and the anti-biofilm potential of coriander EO against several pathogens has also been described, as well as its mechanism of action (Lo Cantore *et al.*, 2004; Duarte *et al.*, 2013a; Silva & Domingues, 2015; Silva *et al.*, 2011b). However, concerning *Campylobacter,* there is only one study, describing its antimicrobial potential against *C. jejuni* in food (Rattanachaikunsopon & Phumkhachorn, 2010). The potential anti-QS of natural compounds has been extensively described, however, for coriander EO there are no QS studies (Ahmad *et al.*, 2015; Alvarez *et al.*, 2012; Castillo *et al.*, 2014; Nazzaro *et al.*, 2013).

Overall, due to the antibacterial, anti-biofilm, anti-QS and antioxidant potential of natural compounds, they could be used in the food industry to enhance shelf life of food products and increase food safety by helping to control the foodborne pathogen *Campylobacter*.

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Chapter 2 – Aims of the Thesis

Chapter 2 – Aims of the Thesis

The overall objective of this work was to study the epidemiology of *Campylobacter* species in Portugal and to explore the potential of natural compounds, like resveratrol and coriander essential oil, as new antibacterials to control this foodborne pathogen, and to provide strong evidences for a future development of a practical application aiming at reducing contamination in the food chain and the burden of disease.

The specific goals of this work were:

1. Characterization of *Campylobacter* isolates from different sources (human, food and animal samples), by evaluating their genetic profiles and susceptibility to antibiotics, as well as the mechanism underlying the antibiotics resistance.

2. Preparation and characterization of resveratrol-cyclodextrins inclusion complex and evaluation of the biological properties.

3. Evaluation of the antimicrobial activity of resveratrol and coriander essential oil, by the inhibition of planktonic cells and biofilms of *Campylobacter* species, as well as the capacity of these compounds to inhibit the quorum sensing system, and evaluation of their potential as antioxidants.

Chapter 3 - Paper I

A infeção humana por *Campylobacter* em Portugal – alguns dados epidemiológicos

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Chapter overview:

As described in the Chapter 1, the infection by *Campylobacter* species, mainly *C. jejuni* and *C. coli*, is considered the most common cause of acute gastroenteritis in humans. In Portugal, studies on the prevalence of this disease are scarce and with a very regional character, being the national status unclear. In 2009, a national working group was created to study the human campylobacteriosis, coordinated by the National Institute of Health Dr Ricardo Jorge (INSARJ). This network has the active participation of 17 laboratories, mostly hospital laboratories, and each laboratory is tasked with searching for *Campylobacter* in fecal samples and sending to the INSARJ the isolated strains, along with some epidemiological information.

In the current study, we present some data on the epidemiology of *Campylobacter* infection in Portugal. Between 2009 and 2012, 837 strains were analyzed, of which 84.5% were identified as *C. jejuni*, 14.8% as *C. coli*, 0.2% as *C. upsaliensis* and 0.1% as *C. concisus*. In addition, 0.2% of the samples were identified as *Arcobacter butzleri*. Concerning the distribution per age groups, we observed that 61.5% of the strains belonged to the group aged between 1 and 15 years and 25.2% of the strains were isolated from children aged between 26 days and 11 months. From all those strains, 125 strains (78 *C. jejuni* and 47 *C. coli*) were randomly chosen and analyzed for their resistance profiles to antibiotics. All the strains were resistant to nalidixic acid and 92.8% were resistant to ciprofloxacin. Moreover, 87.2% were classified as multidrug-resistant (MDR; resistant to at least 3 non-related antibiotics). Finally we proceeded to the genotyping of the strains by multi locus sequence typing (MLST) and we observed that *C. coli* strains were closely related, with all belonging to the clonal complex (CC) 828, while the *C. jejuni* strains were distributed in 15 different CCs.

In sum, this first study allowed for a national overview of the epidemiology of *Campylobacter* infection in humans. However, studies with strains of other sources, such as zoonotic, may be important to understand the whole process of transmission and infection of *Campylobacter*.

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_A infeção humana por *Campylobacter* em Portugal: alguns dados epidemiológicos

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_Introdução

A infeção por *Campylobacter* spp., sobretudo por *Campylobacter jejuni*, é considerada, nos países industrializados, a causa mais frequente de gastroenterite aguda nos humanos, excedendo mesmo as infeções causadas por espécies de *Salmonella, Shigella* ou *Escherichia coli* enteropatogénica. Outro dado importante é a tendência crescente desta infeção, verificada nos últimos anos na Europa, comparativamente com os casos de Salmonelose que têm vindo a decrescer, segundo um relatório que incluiu dados de 24 estados membros, no período entre 2006 e 2010 (1).

_O quadro clínico associado à infeção por *Campylobacter* traduz-se maioritariamente por diarreias inflamatórias acompanhadas de febre e dores abdominais. Apesar de na maioria dos casos ser uma infeção auto-limitada, podem surgir graves complicações como septicémias, infeções extraintestinais e sequelas neurológicas, como a síndrome de Guillain-Barré (2). Está bem estabelecido que a principal fonte de infeção é o consumo ou manipulação de alimentos contaminados, sobretudo carne de aves (3).

_Em Portugal, os estudos sobre a prevalência desta doença são escassos e de carácter muito regional, sendo o panorama nacional desconhecido. A partir de 2009 foi constituído um grupo de trabalho nacional (*), coordenado pelo Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA), para o estudo da Campylobacteriose humana,que conta atualmente com a participação ativa de 17 laboratórios, maioritariamente laboratórios hospitalares, com uma considerável cobertura geográfica. Cada laboratório, que realiza sistematicamente a pesquisa de *Campylobacter* em amostras fecais, envia para o INSA parte das estirpes isoladas, juntamente com alguma informação epidemiológica, permitindo assim fazer a caracterização das mesmas, incluindo vigilância da resistência aos antibióticos.

_Neste trabalho, são divulgados alguns dados referentes à epidemiologia da infeção por *Campylobacter* em Portugal, tendo como base as estirpes clínicas obtidas através deste grupo de trabalho.

_Métodos

As estirpes clínicas foram identificadas ao nível da espécie por PCR em Tempo-real com sondas de hibridação. Nos casos onde não foi possível obter identificação, foi realizado um PCR para o gene que codifica o *16S rRNA*, seguido de sequenciação.

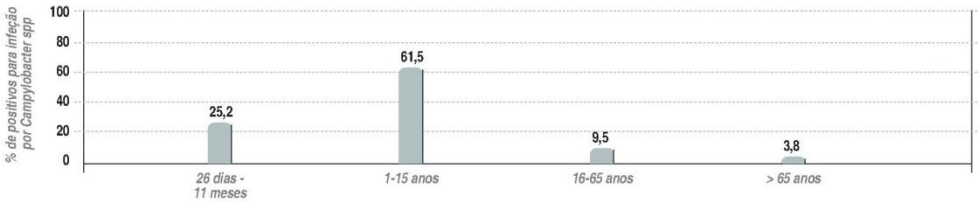
_A concentração mínima inibitória (CMI) de sete antibióticos (ácido nalidíxico, amoxicilina, ciprofloxacina, doxiciclina, eritromicina, gentamicina e tetraciclina) foi determinada pelo método de diluição em agar, segundo as recomendações padrão. A suscetibilidade antimicrobiana foi interpretada de acordo com os valores epidemiológicos estabelecidos pelo *European Commitee on Antimicrobial Susceptibility Testing* (EUCAST, 2012). As estirpes foram genotipadas por *Multi Locus Sequencing Typing* (MLST), por amplificação por PCR de sete genes *housekeeping (aspA, glnA, gltA, glyA, pgm, tkt e uncA)* (4). Os produtos de PCR foram purificados e sequenciados e os tipos de sequências e complexos clonais foram identificados utilizando o banco de MLST para *Campylobacter* (http://pubmlst.org/campylobacter).

_Resultados

Entre 2009 e 2012, foram analisadas 837 estirpes, das quais 84,5% foram identificadas como *C. jejuni*, 14,8% *C. coli*, 0,2% *C. upsaliensis*, 0,1% *C. concisus*, e em 0,2% das amostras foi identificado *Arcobacter butzleri*.

_A distribuição das estirpes por grupo etário foi a seguinte: idade entre 26 dias e 11 meses, 208 (25,2%); entre 1 e 15 anos, 507 (61,5%); entre 16-65 anos, 78 (9,5%); idade>65 anos, 31 (3.8%), sendo a infeção mais frequente no género masculino (59,3%) *(Gráfico 1)*.

Gráfico 1: *Distribuição das estirpes de Campylobacter spp por grupo etário.*



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_Em relação à resistência aos antibióticos e tipagem molecular, foram estudadas um total de 125 estipes, 78 *C. jejuni* e 47 *C. coli*. No geral, foi observada uma elevada taxa de estirpes resistentes *(Tabela 1)*, sendo as estirpes de *C. coli* mais resistentes do que as estirpes de *C. jejuni*. Todas as estirpes foram resistentes ao ácido nalidíxico, 92,8% foram resistentes às fluoroquinolonas e 76% resistentes à tetraciclina/doxiciclina. De realçar ainda a elevada prevalência de estirpes multirresistentes (resistentes a pelo menos 3 antibióticos estruturalmente não relacionados), que foi de 87,2%, bem como o aparecimento de 2 (1,6%) estirpes *C. coli* resistentes à gentamicina.

_Comparando o período do presente estudo (2009-12) e o período entre 1984-89, para os quais existe informação disponível para *Campylobacter* de origem humana em Portugal, observa-se um considerável aumento de estirpes resistentes aos antibióticos [5] *(Gráfico 2)*.

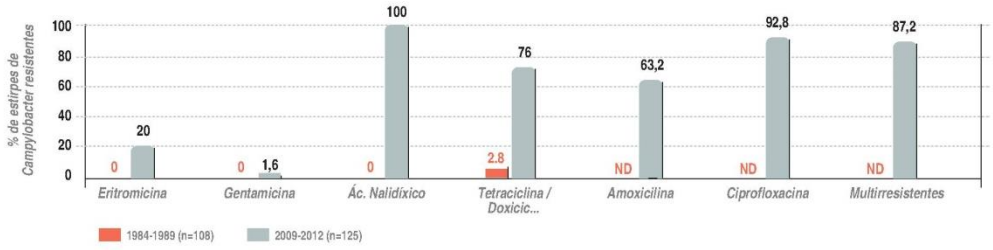
_Em relação à genotipagem por MLST, cada estirpe é caracterizada por uma sequência tipo, que resulta do conjunto dos alelos obtidos para os sete genes *housekeeping*. As estirpes que partilham pelo menos quatro de sete alelos idênticos são consideradas mais próximas, e como pertencendo ao mesmo complexo clonal (CC). No geral, observou-se que as estirpes de *C. coli* são mais conservadas, pertencendo todas ao CC-828, enquanto os isolados *C. jejuni* foram distribuídos em 15 CCs diferentes. Entre as estirpes estudadas, foram identificadas 19 sequências tipo, ainda sem designação na base internacional de MLST para *Campylobacter*. Aparentemente, não foi observada nenhuma relação entre o fenótipo de resistência e o genótipo das estirpes.

Tabela 1: *Resistência a diversos antibióticos de estirpes de Campylobacter jejuni e Campylobacter coli de origem humana, isoladas em Portugal, entre 2009-2012.*

Antibióticos	Intervalo CMI [a]	*Campylobacter jejuni (n=78)*			*Campylobacter coli (n=47)*		
		CMI_{50} [b]	CMI_{90} [c]	*Resistência % (n)*	CMI_{50} [b]	CMI_{90} [c]	*Resistência % (n)*
Ác. Nalidixico	2 – 32	>32	>32	**100 (78)**	>32	>32	**100 (47)**
Amoxicilina	2 – 32	32	>32	**57,7 (45)**	32	>32	**72,3 (34)**
Ciprofloxacina	0,25 – 4	>4	>4	**91 (71)**	>4	>4	**95,7 (45)**
Doxiciclina/Tetraciclina	0,125 – 2	2	>2	**65,4 (51)**	>2	>2	**93,6 (44)**
Eritromicina	1 – 16	<ou=1	4	**9 (7)**	4	>16	**38,3 (18)**
Gentamicina	0,25 – 4	<ou=0,25	0,5	0 (0)	<ou=0,25	0,5	**4,3 (2)**

CMI – concentração mínima inibitória
[a] Intervalo de concentrações testadas (µg/mL)
[b,c] CMI_{50} e CMI_{90} indica a concentração (µg/mL) que inibe o crescimento de 50% e 90% dos isolados.

Gráfico 2: *Taxas de resistência a diversos antibióticos de estirpes de Campylobacter jejuni e Campylobacter coli de origem humana, isoladas em Portugal, em dois períodos de tempo distintos, 1984-1989 [5] e 2009-2012.*



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_Discussão

A análise das características epidemiológicas da infeção por *Campylobacter* em Portugal mostra que a principal espécie que infeta o homem é *C. jejuni*, tal como descrito noutros estudos, apesar de a prevalência de *C. coli* observada ser superior ao que é reportado para os países ocidentais [6]. Como era esperado, o grupo etário de maior risco foram as crianças com idade>1 ano, sendo no entanto de sublinhar a elevada taxa de infeção mesmo nos recém-nascidos e bebés (25,2%).

_Em relação às antibioresistências, foram muito altas, incluindo aos macrólidos e fluoroquinolonas, que são os antibióticos mais frequentemente utilizados para tratar a infeção por *Campylobacter* mais prolongada ou severa. Além disso, observa-se uma tendência crescente de estirpes resistentes, p.ex. para a ciprofloxacina, se comparadas com as taxas descritas noutros trabalhos realizados anteriormente a 2009 [5,7], e a emergência de estirpes resistentes a outros grupos de antibióticos, como os aminoglicosídeos. As taxas de antibioresistência encontradas estão em consonância com as descritas para estirpes de *Campylobacter* de origem animal, refletindo a utilização destes antibióticos na produção animal que se destina ao consumo humano [8].

_Em conclusão, estes dados mostram a importância da infeção por *Campylobacter* em Portugal e reforçam a necessidade de se manter uma vigilância epidemiológica desta infeção, já que a emergência de estirpes multirresistentes sugere um aumento do seu potencial zoonótico.

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Chapter 4 - Paper II

Human, Food and Animal *Campylobacter* spp. isolated in Portugal: high genetic diversity and antibiotic resistance rate

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International Journal of Antimicrobial Agents. 2014; 44:306-313.

Chapter overview:

The previous study (Chapter 3) allowed establishing an overview of the epidemiology of the human infection with *Campylobacter*. In the present study, in addition to the 125 human strains previously studied (Chapter 3), we also studied the genetic and resistance profiles of *Campylobacter* strains isolated from food (39 strains) and animals (32 strains). The strains were genetically analysed by MLST as previously described (Chapter 3) and in order to distinguish closely related strains we also used the *flaA* short variable region typing. Similar to what was observed in Chapter 2, in this study the *C. coli* isolates were genetically more conserved than *C. jejuni* with the CC-828 being the predominant CC among *C. coli*. MLST data also showed that, within each species, genetically related isolates were recovered from different sources. The *flaA* typing results showed a huge variability for both species, and some *flaA* types comprised both *C. jejuni* and *C. coli* isolates. After this, the antibiotic susceptibility profiles were studied as previously described (Chapter 3), and we observed once more that all the isolates were resistant to nalidixic acid, while a MDR phenotype was observed in 84.7% of the isolates. In addition high rates of resistance to fluoroquinolones (91.3%) and tetracycline (79.6%) were also observed. The gentamicin resistance in *Campylobacter* species is rare, although in this study 3 *C. coli* isolates (2 from humans and 1 from food) were resistant to this antibiotic. Given the high antibiotic resistance rates observed, we decided to study the mechanism underlying the resistance phenotypes. To study quinolone resistance, we analyzed the QRDR of *gyrA* gene to locate point mutations. As expected, all the isolates resistant to ciprofloxacin carried the Thr-86-Ile mutation. In order to analyze erythromycin resistance-associated mutations, we tested the isolates by a real-time FRET (Fluorescence resonance energy transfer) assay; the A2075G mutation in the 23S rRNA gene was detected in all of the resistant strains. Since the resistance to gentamicin is not common in *Campylobacter* species, we decided to screen the presence of aminoglycoside-modifying enzymes coding genes (*aphA-3*, *aacA4*, *aadE* and *sat*) described as a possible mechanism in the aminoglycosides resistance (Chapter 1). We observed that the three gentamicin-resistant isolates carried the *aphA-3* gene and one of the isolates had a mutation in this gene, resulting in one amino acid substitution in the AphA3 deduced protein. Finally,

we analyzed the regulatory region of the cmeABC efflux pump, since efflux pumps are described as possible mechanisms conferring resistance to several antibiotics (Chapter 1). Five of the seven MDR strains presented polymorphisms in this region. In addition, when we used an efflux pump inhibitor, we observed that the MIC to gentamicin of three strains was reduced two- to four-fold, indicating the possible role of efflux pumps in the aminoglycosides resistance.

Overall, this study has contributed to understand the epidemiology of *Campylobacter* infection in Portugal, and also describes worrying antibiotic resistance rates. It also contributed to clarify some possible mechanism underlying the resistance to antibiotics by *Campylobacter* strains.

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Human, food and animal *Campylobacter* spp. isolated in Portugal: High genetic diversity and antibiotic resistance rates

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ABSTRACT

Infections by *Campylobacter jejuni* and *Campylobacter coli* are considered the major cause of bacterial gastroenteritis in humans, with food being the main source of infection. In this study, a total of 196 *Campylobacter* strains (125 isolates from humans, 39 from retail food and 32 from food animal sources) isolated in Portugal between 2009 and 2012 were characterised by multilocus sequence typing (MLST) and *flaA* short variable region (SVR) typing. Susceptibility to six antibiotics as well as the mechanisms underlying antibiotic resistance phenotypes was also studied. Based on MLST typing, *C. coli* strains were genetically more conserved, with a predominant clonal complex (CC828), than *C. jejuni* strains. In contrast, *C. coli* isolates were genetically more variable than *C. jejuni* with regard to *flaA*-SVR typing. A high rate of resistance was observed for quinolones (100% to nalidixic acid, >90% to ciprofloxacin) and, in general, resistance was more common among *C. coli*, especially for erythromycin (40.2% vs. 6.7%). In addition, most isolates (86%) were resistant to multiple antimicrobial families. Besides the expected point mutations associated with antibiotic resistance, detected polymorphisms in the *cmeABC* locus likely play a role in the multiresistant phenotype. This study provides for the first time an overview of the genetic diversity of *Campylobacter* strains from Portugal. It also shows a worrying antibiotic multiresistance rate and the emergence of *Campylobacter* strains resistant to antibiotics of human use.

1. Introduction

Campylobacter spp., mainly *Campylobacter jejuni* and *Campylobacter coli*, are well-known foodborne pathogens, reported as the major cause of bacterial gastroenteritis in humans worldwide [1,2]. They are commensal organisms found in the gastrointestinal tract of a variety of wild and domestic birds and mammals [1]. Poultry are considered important reservoirs of these micro-organisms, rarely showing signs of disease [1]. The main transmission route to humans is through the handling, preparation and consumption of contaminated food [1].

The main symptoms of campylobacteriosis are watery or bloody diarrhoea, nausea, abdominal pain and fever [3]. Extraintestinal manifestations of the infection may also occur, with special emphasis on neurological disorders such as Guillain–Barré syndrome and its variant Miller–Fisher syndrome [3].

Molecular typing of strains represents an important tool to study the genetic diversity of *Campylobacter* spp. and to trace individual strains that cause human infections [4].

Multilocus sequence typing (MLST), considered the gold-standard typing method for *Campylobacter*, exploits the relative conservation in the sequence of seven housekeeping genes for which variations are more likely to be selectively neutral [5]. Similarly, it was demonstrated that direct sequencing of PCR-amplified short variable regions (SVRs) of the *flaA* gene is a useful tool for *Campylobacter* genotyping, allowing similar or higher discriminatory power than MLST [4].

Use of antibiotics is not usually indicated for the treatment of uncomplicated campylobacteriosis cases, however severe systemic or chronic infection necessitates antibiotic therapy [1]. Therefore, the increase in antibiotic resistance of *Campylobacter* isolates is an issue of concern, particularly resistance to macrolides,

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fluoroquinolones and gentamicin, which are the drugs of choice for serious campylobacteriosis. Resistance to fluoroquinolones is mainly due to a mutation (Thr-86-Ile) in the quinolone resistance-determining region (QRDR) of the *gyrA* gene [6]. As in other bacteria, in *Campylobacter* spp., point mutations in the peptidyl-encoding region in domain V of the 23S rRNA gene at the base positions 2074, 2075 or both, have been associated with a high level of macrolide resistance [7]. The main mechanism of aminoglycoside resistance in *Campylobacter* spp. is via aminoglycoside-modifying enzymes (AphA, AadE, Sat); furthermore, the efflux systems may have a role, but the mechanism is not clear [6,8]. The CmeABC multidrug efflux system also contributes significantly both to intrinsic and acquired resistance of *Campylobacter* spp. to several structurally unrelated antimicrobials [9]. The *cmeABC* operon is transcriptionally repressed by the *cmeR* gene encoding a repressor, which binds to an inverted repeat (IR) region between *cmeR* and a periplasmic protein-encoding gene *cmeA*. It was recently demonstrated that mutations in the repressing site lead to an overexpression of the efflux pump and consequently to improved resistance to several antimicrobials [9].

In Portugal, studies regarding *Campylobacter* are very scarce. The first study was published in 1992 addressing epidemiological features of human isolates; the following few studies were mainly focused on antimicrobial susceptibility and occurrence of *Campylobacter* isolates either from food animals, food or humans [10–12].

The aim of the present study was to address the genetic diversity, by MLST and *flaA*-SVR typing, antibiotic susceptibility and mechanisms of resistance in *Campylobacter* isolates from Portugal comprising the three sources of isolation (human, food and animals).

2. Materials and methods

2.1. Campylobacter isolates

A total of 196 *Campylobacter* isolates (Table 1), all collected in Portugal between 2009 and 2012, were analysed, including 125 isolates from faecal samples of patients presenting with acute gastroenteritis, randomly selected from the collection of *Campylobacter* strains of the Department of Infectious Diseases of the National Institute of Health Dr Ricardo Jorge (INSARJ) (Lisbon, Portugal), 39 strains from retail food, belonging to the collection of enteric food pathogens of the Department of Nutrition of INSARJ, and 32 isolates from food animal sources, belonging to a collection of *Campylobacter* strains from the Faculty of Veterinary Medicine of Lisbon University (Lisbon, Portugal). All of the isolates were identified at the species level by a real-time PCR assay that differentiates between *C. jejuni*, *C. coli* and *C. fetus* [13].

2.2. Multilocus sequence typing

MLST was carried out as described by Dingle et al. [5,14]. Briefly, internal fragments of seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt* and *uncA*) were PCR amplified using the primers described in Table 2. Amplification products were purified using an ExoSAP-IT Kit (GE Healthcare, Uppsala, Sweden) and were subjected to sequencing on both strands by the dye termination method using a BigDye® Terminator v.1.1 Sequencing Standard Kit (PE Applied Biosystems Chemistry, Foster City, CA) and an ABI PRISM® 3130 xl Automated Sequencer Genetic Analyser (PE Applied Biosystems). Sequence analysis was performed using DNASTAR Lasergene® v.5.0. Allele numbers, sequence types (STs) and clonal complexes (CCs) were assigned by submitting the DNA sequence to the *Campylobacter* MLST database website (http://pubmlst.org/campylobacter).

2.3. flaA short variable region typing

flaA-SVR typing was performed according to Meinersmann et al. [16]. Purification and sequencing of PCR products were performed as described above. Nucleotide sequences were analysed with DNASTAR Lasergene® and were compared with the FlaA database (http://pubmlst.org/campylobacter), and SVR allele numbers were assigned accordingly.

2.4. Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) for six antimicrobial agents [amoxicillin, ciprofloxacin, erythromycin, gentamicin, nalidixic acid (all from Sigma–Aldrich Co., St Louis, MO) and tetracycline (USB, Santa Clara, CA)] were determined by the agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) recommendations [17]. Briefly, bacterial suspensions (2×10^4 CFU/spot) were inoculated on solid Mueller–Hinton agar (MHA) (LiofilChem, Milan, Italy) plates supplemented with 5% defibrinated horse blood (Fisher Scientific, Lisbon, Portugal), with and without the antimicrobials, and plates were incubated at 37 °C for 48 h under a microaerobic hydrogen-enriched atmosphere (Anoxomat™; MART Microbiology BV, Drachten, The Netherlands). *C. jejuni* ATCC 33560 and *C. coli* ATCC 33559 were used as control strains. Multidrug resistance was defined as resistance to three or more non-related antimicrobials.

2.5. Mechanisms of antibiotic resistance

Resistance mechanisms were evaluated for ciprofloxacin, erythromycin and gentamicin. Total DNA was extracted from a 24-h-old culture using a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. For each antibiotic, the genomic targets and primer sets used are listed in Table 2.

Briefly, for quinolone resistance, the QRDR of *gyrA* was amplified according to Zirnstein et al. [18] and was subjected to sequencing to locate point mutations. The point mutations A2074C and A2075G associated with macrolide resistance were determined by a real-time fluorescence resonance energy transfer (FRET) PCR assay using a melting curve analysis as described by Vacher et al. [7].

Regarding gentamicin resistance, isolates were screened for the presence of *aphA-3* and *aacA4* genes by PCR. Presence of the *aadE–sat4–aphA-3* region, which comprises the resistance markers encoding 6′-adenylyltransferase (*aadE*), streptothricin acetyltransferase (*sat*) and 3′-aminoglycoside phosphotransferase type III (*aphA-3*), was also evaluated by PCR. The inferred amino acid sequences of aminoglycoside-modifying enzymes detected were compared with those deposited in GenBank. Class 1 and 2 integrons were screened in the gentamicin-resistant isolates as previously described [19]. Positive and negative controls were used in all PCR reactions. PCR products were then purified and sequenced.

Polymorphisms in the regulatory region of *cmeABC* were evaluated by PCR and sequencing of the intergenic region between *cmeR* and *cmeA* genes. For *C. coli* isolates the primers described by Pérez-Boto et al. [9] were used, and for *C. jejuni* new primers were designed (Table 2; CmejejuniF and CmejejuniR) on the basis of previously sequenced bacteria (GenBank accession nos. AB592864-72).

2.6. Nucleotide sequence accession number

The sequence of the novel *aphA-3* variant was submitted to GenBank under accession no. JQ655275.

Table 1
Campylobacter spp. isolates distributed by source of isolation.

Source		No. of isolates		
		Total	*Campylobacter jejuni*	*Campylobacter coli*
Human				
Females	3–11 months	14	5	9
	1–15 years	32	24	8
	16–65 years	6	1	5
	>65 years	3	3	0
Males	1–11 months	16	8	8
	1–15 years	43	31	12
	16–65 years	8	5	3
	>65 years	3	2	1
Total		125	79	46
Food				
Poultry	Fresh meat	16	4	12
	Processed meat	15	1	14
Pig	Fresh meat	6	0	6
Bovine	Fresh meat	2	0	2
Total		39	5	34
Animal				
Poultry		22	5	17
Pig		10	0	10
Total		32	5	27
Total		196	89	107

2.7. Evaluation of an efflux pump inhibitor

To investigate the effect of the efflux pump inhibitor (EPI) phenylalanine-arginine β-naphthylamide (PAβN) on ciprofloxacin, erythromycin and gentamicin resistance, the MIC for each of these antibiotics was determined for three multidrug-resistant (MDR) and two reference strains in the presence and absence of PAβN (Sigma–Aldrich Co.). The compound was incorporated into MHA at a final concentration of 20 mg/L and the assay was repeated in three independent experiments as previously described [20].

2.8. Statistical analyses

Antibiotic resistance rates between groups were compared with the Mann–Whitney test. Proportions were compared with the χ^2 test. A *P*-value of <0.05 was considered significant.

3. Results

3.1. Genetic characterisation of Campylobacter isolates

A summary of the typing results (MLST and *flaA*-SVR) from all of the isolates is shown in Table 3. A total of 93 different STs and 19 CCs were found among the 196 isolates. At the time of writing, 26 STs not previously assigned in the MLST database were identified, and 11 STs remain unassigned. Eleven of the new STs were from human origin only, nine from food, five from animals and one was detected in both human and food isolates. A total of 19 STs remained unassigned to any existing CCs, however for all of them except one, the most likely CC could be assigned based on the sharing of four or more alleles.

For *C. jejuni* isolates, the following CCs were the most frequently observed: CC607 ($n = 10$); CC353 ($n = 9$); CC464 ($n = 7$); and CC257 ($n = 7$). The predominant CC607 included five different STs, two of which were new. The *C. coli* isolates were genetically more conserved than *C. jejuni*, with two predominant CCs: CC828 ($n = 85$), which included 35 STs, 11 of which were new; and CC1150 ($n = 6$), with two new STs. MLST data showed that, within each species, genetically related isolates are present from different sources, e.g. ST904 was assigned to four *C. jejuni* human isolates and one food isolate. Regarding *C. coli*, four STs (ST828, ST829, ST855 and ST2177) were assigned to isolates from the three studied sources.

flaA-SVR typing was successful in 192 of the 196 isolates. In total, 47 different *flaA*-SVR nucleotide alleles were identified, associated with 23 peptide alleles, and 18 of the 47 *flaA* nucleotide types were detected only once. Thirteen alleles were not found in the database. Unlike the results observed for the MLST data, *C. coli* isolates were genetically more variable than *C. jejuni* regarding *flaA*-SVR typing, being distributed by 26 different *flaA*-SVR nucleotide alleles compared with 25 different *flaA*-SVR nucleotide alleles for the *C. jejuni* isolates. Four *flaA* types (32, 49, 22 and 301) were found in both species. *flaA* type 66 was the predominant within *C. coli*, with 14 isolates from the three sources of isolation, followed by *flaA* type 17 comprising 10 isolates. Regarding *C. jejuni*, type 34 was the most frequently detected, comprising 10 isolates.

3.2. Antibiotic susceptibility

Results of susceptibility testing for the two *Campylobacter* species according to source are summarised in Table 4. Overall, a high rate of resistant strains was observed and, except for ciprofloxacin, the rate of resistant isolates was higher in *C. coli* than in *C. jejuni*, especially for erythromycin (40.2% vs. 6.7%; $P < 0.001$). The resistance rate to erythromycin for both species was higher in animal isolates both for *C. jejuni* (5.1% in human isolates, 0% in food isolates and 40.0% in animal isolates; $P = 0.009$) and *C. coli* (37.0% among human isolates, 29.4% among food and 59.3% among animals; $P = 0.052$).

All isolates were resistant to nalidixic acid, all *C. jejuni* isolates were susceptible to gentamicin, and only 3 (3.1%) of 97 *C. coli* isolates were resistant to this antimicrobial (2 human and 1 from food isolates). It is noteworthy that all except 7 *C. jejuni* isolates (82/89; 92.1%) were resistant to ciprofloxacin, and among *C. coli* 97 of 107 (90.7%) isolates presented this phenotype.

A MDR phenotype was observed for 166 (84.7%) of the 196 isolates, 83% among *C. jejuni* and 86% among *C. coli*. Resistance to all six antimicrobials was observed for two *C. coli* isolates of human origin, whilst susceptibility to all antimicrobials, excluding nalidixic acid, was observed in only five isolates (three *C. jejuni* of human origin and two *C. coli* from human and food origin). Multiple resistances to clinically relevant drugs (ciprofloxacin, erythromycin,

Table 2
Primer sequences and target genes used for multilocus sequence typing (MLST), *flaA* short variable region (SVR) typing and for the detection of the mechanisms of antibiotic resistance.

Typing method or antibiotic	Target	Primer	Sequence (5′→3′)	PCR product (bp)	$T_{annealing}$ (°C)	Reference
MLST	*aspA*	asp.jej.A9	AGTACTAATGATGCTTATCC	477	55	[5]
		asp.jej.A10	ATTTCATCAATTTGTTCTTTGC			
		asp.coli.S1	CAACTTCAAGATGCAGTACC	477	50	[14]
		asp.coli.S2	ATCTGCTAAAGTATGCATTGC			
	glnA	gln.jej.A1	TAGGAACTTGGCATCATATTACC	477	60	[5]
		gln.jej.A2	TTGGACGAGCTTCTACTGGC			
		gln.coli.S1	TTCATGGATGGCAACCTATTG	477	50	[14]
		gln.coli.S2	GCTTTGGCATAAAAGTTGCAG			
	gltA	glt.jej.A1	GGGCTTGACTTCTACAGCTACTTG	402	55	[5]
		glt.jej.A2	CCAAATAAAGTTGTCTTGGACGG			
		glt.coli.S1	GATGTAGTGCATCTTTTACTC	402	50	[14]
		glt.coli.S2	AAGCGCTCCAATACCTGCTG			
	glyA	gly.jej.A1	GAGTTAGAGCGTCAATGTGAAGG	507	55	[5]
		gly.jej.A2	AAACCTCTGGCAGTAAGGGC			
		gly.coli.S1	TCAAGGCGTTTATGCTGCAC	507	50	[14]
		gly.coli.S2	CCATCACTTACAAGCTTATAC			
	pgm	pgm.jej.A7	TACTAATAATATCTTAGTAGG	498	55	[5]
		pgm.jej.A8	CACAACATTTTTCATTTCTTTTTC			
		pgm.coli.S1	TTATAAGGTAGCTCCGACTG	498	50	[14]
		pgm.coli.S2	GTTCCGAATAGCGAAATAACAC			
	tkt	tkt.jej.A3	GCAAACTCAGGACACCCAGG	459	55	[5]
		tkt.jej.A6	AAAGCATTGTTAATGGCTGC			
		tkt.coli.S1	AGGCTTGTGTTTTCAGGCGG	459	50	[14]
		tkt.coli.S2	TGACTTCCTTCAAGCTCTCC			
	uncA	unc.jej.A7	ATGGACTTAAGAATATTATGGC	489	50	[5]
		unc.jej.A8	ATAAATTCCATCTTCAAATTCC			
		unc.coli.S1	AAGCACAGTGGCTCAAGTTG	489	50	[14]
		unc.coli.S2	CTACTTGCCTCATCCAATCAC			
flaA-SVR	*flaA*	fla242FU	CTATGGATGAGCAATT(AT)AAAAT	402	52	[16]
		fla625.RU	CAAG(AT)CCTGTTCC(AT)ACTGAAG			
Ciprofloxacin	*gyrA* (QRDR region)	GZgyrA5	ATTTTTAGCAAAGATTCTGAT	673	58	[18]
		GZgyrA6	CCATAAATTATTCCACCTGT			
Erythromycin	23S rRNA	Camp-A	CCTCCCACCTATCCTGCAC	263	60	[7]
		Camp-S	AGCATTAGCGAAGCTCTTGAT			
Gentamicin	*aphA-3*	aphA-3F	ATGGCTAAAATGAGAATATC	795	48	This study
		aphA-3R	CTAAAACAATTCATCCAG			
	aadEsat4–aphA-3	aadE-F	ATGAGATCAGAAAAGGAAG	2129	48	This study
		aphA-3R	CTAAAACAATTCATCCAG			
	aacA4	aac(5)	TTGCGATGCTCTATGAGTGGCTA	482	55	[15]
		aac(6)	CTCGAATGCCTGGCGTGTTT			
Integrons	*intI1*	intI1-F	TCTCGGGTAACATCAAGG	243	49	[19]
		intI1-R	AGGAGATCCGAAGACCTC			
	intI2	intI2-F	CTTACCTGCACTGGATTAAG	289	53	[19]
		intI2-R	TTGCGAGTATCCATAACCTG			
Multidrug resistance	Cme efflux pump	CmecoliF3	AATGTTTTAGCCGATACT	428	58	[9]
		CmecoliR4	AACACCGCTTACTTGAGG			
		CmejejuniF	TTGCCAATTGGATAGAAAATAATC	770	58	This study
		CmejejuniR	TCGTATTCCTTTTGAGAGATTGC			

gentamicin and tetracycline) were observed for three *C. coli* isolates (two human and one food isolate).

3.3. Determinants of antibiotic resistance

For ciprofloxacin resistance-associated mutations, primers based on *C. jejuni gyrA* [18] were used for amplification and sequencing both for *C. jejuni* and *C. coli* resistant isolates. Under our conditions, a positive PCR fragment as well as reliable DNA sequences was obtained for all 179 ciprofloxacin-resistant isolates. The Thr-86-Ile (C257T) *gyrA* mutation was the single polymorphism detected in all cases. None of the 17 isolates (7 *C. jejuni* and 10 *C. coli*) that was resistant to nalidixic acid but susceptible to ciprofloxacin presented mutations in the *gyrA* QRDR.

For the erythromycin resistance-associated mutations, 10 susceptible (4 *C. coli* and 6 *C. jejuni*) and all 49 resistant (43 *C. coli* and 6 *C. jejuni*) isolates were tested by the real-time FRET assay. The A2075G mutation was detected in all of the resistant strains, whilst the A2074C mutation or the double genotype A2074C/A2075G were not observed. All of the susceptible isolates presented the wild-type genotype.

The three gentamicin-resistant isolates (*C. coli* 226199, *C. coli* 223 and *C. coli* 873) presented MICs of 16, 64 and 128 mg/L, respectively, and all three carried the *aphA-3* gene, with identical nucleotide sequences to the *aphA-3* gene from *C. coli* strain SX81, which harboured the *aadE–sat4–aphA-3* cluster [8]. However, none of the *Campylobacter* strains harboured this cluster. Owing to a C→T transition at nucleotide 503, the AphA3 protein produced by *C. coli* 226199 (food origin) differed by one amino acid substitution at position 168 (GenBank accession no. JQ655275) (Table 5). The PCR for *aacA4* and class 1 and 2 integrons was negative.

Seven *C. jejuni* and seven *C. coli* strains with different patterns of resistance were studied (Table 5) for polymorphisms in the repressing site of *cmeABC*. Five of the seven MDR strains presented polymorphisms: three strains (one *C. coli* and two *C. jejuni*) had a modified IR, either with single or dinucleotide deletions, or with a G→A transition; and two *C. coli* strains presented polymorphisms in the 5 bp immediately upstream of an intact IR.

Table 3
Distribution of clonal complexes (CCs), sequence types (STs) and *flaA* short variable region (SVR) types of the 196 *Campylobacter* spp. isolates according to source.

Campylobacter jejuni isolates						*Campylobacter coli* isolates					
CC	ST[a]	flaA-SVR nucleotide allele[b]	No. of isolates from:			CC	ST[a]	flaA-SVR nucleotide allele[b]	No. of isolates from:		
			Human	Animals	Food				Human	Animals	Food
21	31	36	1	0	0	828	828	30; 325; 866; NF	2	1	1
	262	37	1	0	0		829	28; 1390; NF	1	1	1
	883	8	2	0	0		830	17; NF	0	0	2
	3769	260	1	0	0		854	500	0	0	1
	760	36	0	0	1		855	**66**; NF	5	1	4
22	22	NF	1	0	0		890	310	0	0	1
45	25	22	1	0	0		901	301	0	0	1
	583	NF	1	0	0		902	66	0	0	2
48	475	105	1	0	0		1058	NF	0	2	0
49	49	11	2	0	0		1067	222	1	0	0
53	52	57	1	0	0		1153	13	0	1	0
	775	57	1	0	0		1436	660	0	0	1
206	122	49	1	0	0		1586	NF	1	0	0
	227	49	1	0	0		1628	13; **301**	4	0	1
	572	32	1	0	0		1750	**726**; 736	0	8	1
	6815	NF	1	0	0		1769	23	1	0	1
257	584	301	1	0	0		2177	32; 66; **NF**	2	2	3
	824	34	4	0	0		2631	30; 1596	2	0	0
	990	16	2	0	0		2715	310	0	1	0
353	5	97	7	0	0		2820	NF	1	0	0
	2036	287	0	0	1		3017	23	1	0	0
	6812	453	1	0	0		5111	13; 17	1	0	1
354	354	18	3	1	0		5659	**30**; 49	2	0	2
362	2123	NF	0	1	0		6233	**17**; 1348	4	3	0
443	51	21; 67	2	0	0		**6819**	NF	0	1	0
	2807	21	2	0	0		**6824**	652	0	1	0
	6522	NF	1	0	0		**6825**	NF	0	0	1
446	**6832**	222	0	0	1		**6826**	343	0	0	2
464	464	34; 222; **260**	7	0	0		**6828**	17	1	0	0
574	305	57	1	0	0		**6830**	23	0	0	1
607	607	49; NF	2	0	0		**6833**	310	0	0	1
	904	**34**; 49; 260	4	0	1		**6834**	NF	0	0	1
	1707	14	1	0	0		**6835**	NF	0	0	1
	6814	34	1	0	0		**6857**	1348	2	0	0
	6827	34	0	1	0		**6858**	NF	0	2	0
658	523	NF	3	0	0	1150	**6817**	NF	3	0	0
	1044	5	1	0	0		**6820**	23; 1297; NF	3	0	0
	2180	402	2	0	0	828[c]	1009	17	1	0	0
49[c]	466	NF	1	0	0		1591	900	0	0	1
206[c]	2258	402	1	0	0		4394	66	1	0	0
353[c]	2065	14	4	0	0		4988	NF	3	0	0
	6813	NF	1	0	0		**6816**	**321**; 599	1	0	2
573[c]	3766	1386	1	0	0		**6823**	500	0	1	0
	6831	NF	0	0	1		**6828**	321	0	0	1
1034[c]	5760	NF	1	0	0	1150[c]	**6821**	NF	1	0	0
UA	882	NF	1	0	0		**6822**	1312	1	0	0
UA	UA	14; **45**; 97	6	0	0		**6829**	1312	1	0	0
UA	UA	NF	1	2	0	UA	UA	NF	0	2	0

UA, unassigned STs; NF, allele not found in database; UA, unassigned to any CC.
[a] STs in boldface indicate new assigned STs.
[b] Alleles in boldface are predominant.
[c] Most likely CC sharing ≥4 alleles found in database.

Table 4
Resistance profile to six antibiotics of *Campylobacter jejuni* and *Campylobacter coli* strains of human, food and animal origin.

Antibiotic	MIC range (mg/L)	% Resistance (*n*) in *Campylobacter jejuni* strains[a]				MIC range (mg/L)	% Resistance (*n*) in *Campylobacter coli* strains[a]			
		Total (*n* = 89)	Humans (*n* = 79)	Food (*n* = 5)	Animals (*n* = 5)		Total (*n* = 107)	Humans (*n* = 46)	Food (*n* = 34)	Animals (*n* = 27)
Amoxicillin	≤2 to >32	58.3 (49/84)	57.0 (45)	80.0 (4)	N/D	≤2 to >32	66.7 (44/66)	73.9 (34)	50.0 (10/20)	N/D
Ciprofloxacin	≤0.25 to >4	92.1 (82)	91.1 (72)	100 (5)	100 (5)	≤0.25 to >4	90.7 (97)	95.7 (44)	94.1 (32)	77.8 (21)
Erythromycin	≤1 to >16	6.7 (6)	5.1 (4)	0	40.0 (2)	≤1 to >16	40.2 (43)	37.0 (17)	29.4 (10)	59.3 (16)
Gentamicin	≤0.25–2	0	0	0	0	≤0.25–128	3.1 (3/97)	4.3 (2)	2.9 (1)	0/17
Nalidixic acid	>32	100 (89)	100 (79)	100 (5)	100 (5)	>32	100 (107)	100 (46)	100 (34)	100 (27)
Tetracycline	0.5 to >4	66.3 (59)	65.8 (52)	60.0 (3)	80.0 (4)	0.25 to >4	90.7 (97)	93.5 (43)	88.2 (30)	88.9 (24)

MIC, minimum inhibitory concentration; N/D, not determined.
Resistance breakpoints established by European Committee on Antimicrobial Susceptibility Testing (EUCAST) [21] and Clinical and Laboratory Standards Institute (CLSI) [17] for *C. jejuni*/*C. coli*, respectively, were used: amoxicillin, >16/>8 mg/L; ciprofloxacin, >0.5 mg/L; erythromycin, >4/>8 mg/L; gentamicin, >2 mg/L; nalidixic acid, >16 mg/L; and tetracycline, >2 mg/L.
Data including the MICs of all isolates for all antimicrobials studied are given in Supplementary Tables S1 and S2.

Table 5
Antimicrobial phenotype and sequence analysis of the quinolone resistance-determining region (QRDR) of the *gyrA* gene, 23S rRNA and inverted repeat at *cmeABC* locus of 12 *Campylobacter* isolates and 2 reference strains.

Isolate	Origin	Antimicrobial phenotype						Fluoroquinolone resistance	Macrolide resistance	Aminoglycosides resistance	Polymorphisms in the IR at *cmeABC* locus
		AMX	CIP	ERY	GEN	NAL	TET	*gyrA*, Thr-86-Ile mutation	23S rRNA, A2075G mutation	*aphA3*, mutations	
C. jejuni ATCC 33560	Collection	S	S	S	S	R	S	No	No	N/A	No mutations
C. jejuni 123	Human	R	R	R	S	R	R	Yes	Yes	N/A	No mutations
C. jejuni 203	Human	S	S	S	S	R	S	No	No	N/A	No mutations
C. jejuni 727	Human	S	S	S	S	R	S	No	No	N/A	No mutations
C. jejuni 1035	Human	R	R	S	S	R	R	Yes	No	N/A	G→A transition in the first half-site of the IR
C. jejuni 227190	Food	R	R	S	S	R	R	Yes	No	N/A	No mutations
C. jejuni 227202	Food	S	R	S	S	R	R	Yes	No	N/A	Nucleotide A deletion in the second half-site of the IR
C. coli ATCC 33559	Collection	S	S	S	S	R	R	No	No	N/A	No mutations
C. coli 111	Human	S	S	S	S	R	S	No	No	N/A	No mutations
C. coli 129	Human	S	R	S	S	R	S	Yes	No	N/A	No mutations
C. coli 223	Human	R	R	R	R	R	R	Yes	Yes	Yes, no mutation	Polymorphisms in the 5-bp immediately upstream of the IR
C. coli 873	Human	R	R	R	R	R	R	Yes	Yes	Yes, no mutation	Polymorphisms in the 5-bp immediately upstream of the IR
C. coli 225837	Food	S	R	S	S	R	S	Yes	No	N/A	No mutations
C. coli 226199	Food	S	R	R	R	R	R	Yes	Yes	Yes, point mutation C503T	Dinucleotide CA deletion in the second half-site of the IR

AMX, amoxicillin; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; NAL, nalidixic acid; TET, tetracycline; IR, inverted repeat; S, susceptible; R, resistant; N/A, not applicable.

3.4. Effect of an efflux pump inhibitor on antibiotic resistance

The role of the EPI PAβN on susceptibility to ciprofloxacin, erythromycin and gentamicin was evaluated. MICs to ciprofloxacin and erythromycin were not altered in the presence of PAβN for the three MDR *C. coli* strains tested (Table 6). In contrast, the presence of the EPI reduced the MIC to gentamicin of all the three strains by two- to four-fold (Table 6).

4. Discussion

This study provides for the first time an overview of the genetic diversity of *Campylobacter* strains from several sources of isolation in Portugal. MLST analysis showed 93 different STs and 19 CCs, with *C. jejuni* and *C. coli* isolates showing distinct MLST profiles as previously described [14]. For *C. jejuni*, CC607 and CC353 were associated with poultry, also being found in human isolates. Data from the MLST database show that strains from both of these STs are found in human and chicken isolates mainly from European countries, but also from other countries such as Canada, Thailand and Uruguay, suggesting a wide distribution of these types.

C. coli strains belonged predominantly to CC828, and within this complex to ST855, comprising both human and poultry isolates, and ST1750 containing poultry isolates only as previously reported [22]. Regarding ST1750, no studies reported this type as predominant, and from the online database only 10 isolates from human and poultry origins, from different European countries, were recorded as ST1750.

Among the 46 different STs assigned to *C. jejuni* isolates, 44 comprised exclusively isolates from one source of isolation, whilst only two comprised isolates from two different origins. Nevertheless, only ten non-human strains were studied, likely contributing to this finding. In contrast, among the 47 STs identified for *C. coli*, 11 of them comprised isolates from different sources, namely, human, animals (mostly poultry) and food (mostly poultry meat) isolates. These results confirm that *Campylobacter* infections in humans arise from different sources, with poultry being the main source [23].

The usefulness of *flaA*-SVR typing in combination with MLST was assessed and a total of 47 *flaA* types were identified, with types 66, 17 and 34 being most frequently represented. The *flaA* types comprised both *C. jejuni* and *C. coli* isolates, likely due to the frequent interspecies recombination, which explains the unsuitability of this typing as a standalone marker for species identification [4]. In addition, *flaA* types were assessed to the same ST, and the same *flaA* type was found in different STs, showing that *flaA*-SVR typing provides limited correlation with the data obtained by MLST, likely due to the hypervariable nature of this locus [4].

Use of antimicrobial agents, particularly fluoroquinolones, in veterinary production is associated with the spread of resistant *Campylobacter* strains, with potential effects on food safety and human health [1]. In the current study, a high prevalence of resistance to several antibiotics was observed: nalidixic acid (100%); ciprofloxacin (91.3%); and tetracycline (79.6%). Moreover, most isolates (86%) were resistant to three or more antimicrobial families. In addition, despite the fact that the rate of resistance to each antimicrobial alone was higher for *C. coli* than for *C. jejuni* (except for ciprofloxacin), the ability of both species to exhibit resistance to multiple antimicrobials, including fluoroquinolones and macrolides, increases the interest from a public health perspective [11].

In this study, the rate of resistance to ciprofloxacin was remarkably high, irrespective of the source of isolation, as previously reported in other Portuguese studies: 98% for broiler isolates and 80% for human isolates [10,11]. These rates are comparable with those reported in other countries such as Spain, where 100%

Table 6
Minimum inhibitory concentrations (MICs) of ciprofloxacin, erythromycin and gentamicin in the absence and presence of the efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN) for three multidrug-resistant *Campylobacter coli* isolates.

Strain	MIC (mg/L)					
	Ciprofloxacin		Erythromycin		Gentamicin	
	−PaβN	+PaβN	−PaβN	+PaβN	−PaβN	+PaβN
C. coli 223	32	32	16	16	64	32
C. coli 873	32	32	>16	>16	128	32
C. coli 226199	32	32	16	16	16	8

resistance to ciprofloxacin among *C. coli* isolates from pigs and broilers was found [24], but higher than those found in isolates from Germany, France or the UK [25,26]. Regarding nalidixic acid, all of the isolates studied presented resistance to this antimicrobial, irrespective of the species and source of isolation, similarly to that reported in Spain [24], but once again higher than that reported in other European countries [24,25]. Regarding the human isolates in particular, >90% of the isolates from both species were resistant to ciprofloxacin, which is much higher than that described in other countries [27].

The overall prevalence of resistance to erythromycin (25%) was higher in the current study than that reported for other countries for each source (human or animal) [27]. Also according to the literature, erythromycin resistance was more common among *C. coli* than *C. jejuni* [25,26].

The high resistance rates observed in this study are likely related to the widespread use of antibiotics in food-producing animals in Portugal, one of the countries with the highest consumption of fluoroquinolones and macrolides for this purpose among the EU member states [28].

Gentamicin resistance in *Campylobacter* spp. is a rare event all over European countries [11,25]. In the present study, only three *C. coli* presented this phenotype, although with high MICs. Once again, the appearance of gentamicin-resistant strains in food animals and in humans likely reflects the use of aminoglycosides for veterinary treatment [1].

Knowledge of the mechanisms underlying resistance phenotypes may provide a basis for controlling the emergence and spread of antibiotic resistance. All of the studied *Campylobacter* spp. isolates resistant to ciprofloxacin carried the Thr-86-Ile amino acid substitution in the QRDR of the *gyrA* gene, which, however, did not confer resistance to nalidixic acid only, according to previously reported data [29]. Indeed, for the 17 isolates that were resistant to nalidixic acid and susceptible to ciprofloxacin, no detectable *gyrA* QRDR mutations was found, including the Thr-86-Ala mutation reported to be associated with nalidixic acid resistance only in a few *C. jejuni* strains isolated from poultry [29]. No other genetic determinant has been associated with this phenotype in *Campylobacter* spp. There is growing evidence that the CmeABC efflux system may play a role in fluoroquinolone resistance in *Campylobacter* [30]. In the current study, polymorphisms in the repressing site of the CmeABC multidrug efflux system were observed for some ciprofloxacin-resistant isolates, supporting the hypothesis that different mechanisms of fluoroquinolone resistance may co-exist and act synergistically. None the less, other resistant isolates lack polymorphisms at this locus. Furthermore, the use of an EPI had no effect on the susceptibility to ciprofloxacin, even on strains displaying polymorphisms in the repressing site. A more extensive study will be useful to clarify the role of efflux pumps and *gyrA* mutations on quinolone and fluoroquinolone resistance.

The transitional mutation A2075G was associated with erythromycin resistance according to previous studies [30,31]. Similarly to ciprofloxacin resistance, efflux pump activity did not contribute to erythromycin resistance in the isolates in the current study, as also reported by Bolton et al. [20], but contrasting with other studies demonstrating a putative role of the efflux pumps in *Campylobacter* erythromycin resistance [30,31].

Gentamicin resistance in *Campylobacter* occurs mostly via aminoglycoside phosphotransferases, which are usually plasmid-borne and are typically organised as a cluster [6]. In the current study, all the three gentamicin-resistant *C. coli* strains harboured the *aphA-3* aminoglycoside resistance marker at the chromosome level, but not as part of the *aadE–sat4–aphA-3* resistance cluster. The food origin strain with the lowest MIC for gentamicin (16 mg/L) displayed a point mutation leading to an amino acid substitution in AphA3, but likely this mutation is not contributing to this phenotype. Based on the MLST and *flaA*-SVR typing, these strains were not clonal, suggesting that a rapid spread of this phenotype is, for the moment, unlikely.

Following the same trend of these isolates, no associations between antibiotic resistance patterns and MLST or *flaA*-SVR profiles were found, with resistance phenotypes being dispersed throughout the different genotypes (data not shown). The contribution of efflux systems to aminoglycoside resistance remains unclear [6]. In the current study, a decrease in gentamicin MIC in the presence of the EPI was observed, suggesting a putative role in gentamicin resistance of *Campylobacter* as well. However, more molecular studies will be required in order to clarify this mechanism, since no studies have reported the effect of efflux pumps on *Campylobacter* gentamicin resistance before. Mutations in the repressing site lead to overexpression of the efflux pump and to enhanced resistance to several antimicrobials [9,30]. In the current study, all three MDR *C. coli* and two of the four MDR *C. jejuni* isolates studied presented polymorphisms at the *cmeABC* locus, strongly suggesting the role of this efflux pump in the MDR phenotype, in addition to the previously described point mutations in specific molecular targets.

In conclusion, this study is a contribution to the understanding of the epidemiology of *Campylobacter* infection, providing for the first time an overview of the genetic diversity of *Campylobacter* isolates from several sources in Portugal. We also emphasise a worrying antibiotic multiresistance rate and the emergence of strains resistant to antibiotics of human use, drawing attention to the need to monitor the trends of antibiotic resistance in this foodborne pathogen.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijantimicag.2014.06.012.

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Supplementary Table S1

Minimum inhibitory concentrations (MICs) of all *Campylobacter jejuni* isolates for the six antimicrobials studied.

Source	Isolate	MIC (mg/L)					
		AMX	CIP	ERY	GEN	NAL	TET
Human							
	5	≤2	**>4**	≤1	≤0.25	**>32**	**>4**
	16	4	**>4**	≤1	1	**>32**	**>4**
	21	**32**	**>4**	2	≤0.25	**>32**	1
	25	**>32**	**>4**	2	≤0.25	**>32**	1
	54	8	**1**	4	0.5	**>32**	**>4**
	55	**>32**	**>4**	4	≤0.25	**>32**	**>4**
	57	4	**>4**	≤1	≤0.25	**>32**	**>4**
	58	**>32**	**>4**	4	≤0.25	**>32**	**>4**
	67	**>32**	**>4**	≤1	≤0.25	**>32**	2
	112	4	**4**	≤1	≤0.25	**>32**	**>4**
	115	8	**>4**	≤1	≤0.25	**>32**	1
	117	4	**>4**	4	0.5	**>32**	**4**
	118	**>32**	**>4**	4	0.5	**>32**	**>4**
	120	**>32**	**>4**	4	≤0.25	**>32**	**>4**
	123	**>32**	**>4**	**16**	1	**>32**	**>4**
	126	**>32**	**4**	**>16**	≤0.25	**>32**	**>4**
	136	16	**2**	**8**	≤0.25	**>32**	**>4**
	137	16	0.5	4	≤0.25	**>32**	**>4**
	141	≤2	**2**	≤1	≤0.25	**>32**	**>4**
	149	≤2	**>4**	≤1	≤0.25	**>32**	**>4**
	166	**>32**	**>4**	≤1	≤0.25	**>32**	2
	168	4	**>4**	≤1	≤0.25	**>32**	**>4**
	171	**>32**	≤0.25	≤1	≤0.25	**>32**	0.5
	175	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	192	**>32**	**>4**	≤1	≤0.25	**>32**	1
	203	8	≤0.25	≤1	≤0.25	**>32**	0.5
	218	**>32**	**>4**	≤1	≤0.25	**>32**	2
	220	8	**>4**	≤1	≤0.25	**>32**	2
	233	**>32**	**>4**	≤1	1	**>32**	**>4**
	239	≤2	**4**	≤1	≤0.25	**>32**	**>4**
	251	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	282	**32**	**>4**	≤1	≤0.25	**>32**	**>4**
	285	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	349	≤2	**2**	≤1	≤0.25	**>32**	**>4**
	357	**>32**	**>4**	≤1	≤0.25	**>32**	2
	421	**>32**	**>4**	4	≤0.25	**>32**	0.5
	446	**32**	**>4**	2	≤0.25	**>32**	**>4**
	460	**>32**	≤0.25	≤1	≤0.25	**>32**	**>4**
	463	16	**>4**	**>16**	≤0.25	**>32**	**>4**
	517	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	527	4	**>4**	≤1	≤0.25	**>32**	0.5
	543	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**

1

Table 1 (cont.).

Source	Isolate	MIC (mg/L)					
		AMX	CIP	ERY	GEN	NAL	TET
Human							
	582	4	**4**	≤1	≤0.25	**>32**	**>4**
	587	≤2	**4**	≤1	≤0.25	**>32**	**>4**
	607	≤2	**1**	≤1	≤0.25	**>32**	**>4**
	614	≤2	**4**	≤1	≤0.25	**>32**	**>4**
	626	**>32**	**>4**	≤1	≤0.25	**>32**	0.5
	630	**32**	**>4**	≤1	≤0.25	**>32**	1
	640	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	645	**>32**	**>4**	4	≤0.25	**>32**	**>4**
	649	**32**	**2**	4	≤0.25	**>32**	**>4**
	655	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	690	≤2	**4**	≤1	≤0.25	**>32**	**>4**
	727	8	≤0.25	2	≤0.25	**>32**	0.5
	734	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	736	**>32**	**>4**	2	≤0.25	**>32**	2
	753	**>32**	**>4**	4	≤0.25	**>32**	**>4**
	838	**>32**	**>4**	≤1	0.5	**>32**	**>4**
	865	16	**>4**	2	0.5	**>32**	**>4**
	872	**>32**	**4**	≤1	0.5	**>32**	**>4**
	874	**>32**	**>4**	2	0.5	**>32**	**>4**
	878	**>32**	**>4**	≤1	0.5	**>32**	2
	879	4	≤0.25	≤1	≤0.25	**>32**	0.5
	892	**>32**	**>4**	≤1	0.5	**>32**	2
	896	**>32**	**>4**	2	0.5	**>32**	2
	930	**>32**	**>4**	2	0.5	**>32**	**>4**
	932	16	**>4**	4	≤0.25	**>32**	**>4**
	935	16	**>4**	≤1	≤0.25	**>32**	2
	936	16	**>4**	≤1	0.5	**>32**	**>4**
	963	4	**4**	≤1	0.5	**>32**	0.5
	964	8	**4**	≤1	≤0.25	**>32**	**>4**
	969	**>32**	**>4**	≤1	≤0.25	**>32**	0.5
	971	8	**4**	2	≤0.25	**>32**	**>4**
	975	8	**4**	≤1	≤0.25	**>32**	0.5
	999	16	**>4**	2	1	**>32**	1
	1001	**32**	0.5	2	≤0.25	**>32**	1
	1006	**32**	**>4**	4	≤0.25	**>32**	**>4**
	1013	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	1035	**32**	**>4**	4	2	**>32**	**>4**
Food							
	224937	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	225421	**32**	**>4**	≤1	≤0.25	**>32**	1
	225859	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	227190	**>32**	**>4**	4	≤0.25	**>32**	**>4**
	227202	4	**>4**	≤1	≤0.25	**>32**	1

2

Table 1 (cont.).

Source	Isolate	Minimal inhibitory concentration (mg/L)					
		AMX	CIP	ERY	GEN	NAL	TET
Animal							
	122	ND	**>4**	**16**	0.5	**>32**	**>4**
	641	ND	**>4**	≤1	≤0.25	**>32**	**>4**
	649	ND	**4**	≤1	≤0.25	**>32**	**>4**
	682	ND	**>4**	**>16**	0.5	**>32**	**>4**
	683	ND	**>4**	2	0.5	**>32**	0.5

ND, not determined. Bold means resistance.

Resistance breakpoints established by EUCAST and CLSI to *C. jejuni* were used: amoxicillin (AMX), >16 mg/L; ciprofloxacin (CIP), >0.5 mg/L; erythromycin (ERY), >4 mg/L; gentamicin (GEN), >2 mg/L; nalidixic acid (NAL), >16 mg/L; tetracycline (TET), >2 mg/L.

Supplementary Table S2

Minimum inhibitory concentrations (MICs) of all *Campylobacter coli* isolates for the six antimicrobials studied.

Source	Isolate	Minimal inhibitory concentration (mg/L)					
		AMX	CIP	ERY	GEN	NAL	TET
Human							
	20	**>32**	**>4**	2	0.5	**>32**	**>4**
	29	**32**	**>4**	8	≤0.25	**>32**	**>4**
	53	**>32**	**>4**	**>16**	0.5	**>32**	**>4**
	59	**16**	**>4**	≤1	≤0.25	**>32**	**>4**
	60	**>32**	**>4**	**16**	≤0.25	**>32**	**>4**
	65	4	≤0.25	≤1	≤0.25	**>32**	**>4**
	69	**>32**	**>4**	**>16**	≤0.25	**>32**	**>4**
	72	**>32**	**>4**	4	≤0.25	**>32**	**>4**
	83	**16**	**4**	**>16**	≤0.25	**>32**	**>4**
	84	**>32**	**>4**	**16**	≤0.25	**>32**	**>4**
	87	**>32**	**4**	**>16**	0.5	**>32**	**>4**
	111	4	≤0.25	≤1	≤0.25	**>32**	1
	129	8	**4**	≤1	≤0.25	**>32**	0.5
	223	**32**	**>4**	**16**	**64**	**>32**	**>4**
	243	8	**>4**	4	≤0.25	**>32**	**>4**
	297	4	**>4**	≤1	≤0.25	**>32**	**>4**
	344	**>32**	**>4**	8	≤0.25	**>32**	**>4**
	355	≤2	**>4**	8	≤0.25	**>32**	**>4**
	365	**>32**	**>4**	**16**	≤0.25	**>32**	**>4**
	447	**16**	**4**	≤1	0.5	**>32**	**>4**
	473	**16**	**4**	2	0.5	**>32**	**>4**
	483	8	**>4**	**>16**	≤0.25	**>32**	**>4**
	486	**>32**	**4**	≤1	≤0.25	**>32**	**>4**
	512	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	526	**>32**	**>4**	**>16**	≤0.25	**>32**	**>4**
	539	**>32**	**4**	**>16**	≤0.25	**>32**	**>4**
	549	**>32**	**4**	**>16**	≤0.25	**>32**	**>4**
	586	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	619	**32**	**>4**	8	≤0.25	**>32**	**>4**
	623	**>32**	**4**	2	≤0.25	**>32**	**>4**
	672	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	706	**16**	**4**	≤1	0.5	**>32**	0.5
	723	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	740	**>32**	**>4**	**>16**	≤0.25	**>32**	**>4**
	799	**16**	**>4**	**>16**	0.5	**>32**	**>4**
	831	**>32**	**>4**	8	1	**>32**	**>4**
	835	8	**2**	≤1	0.5	**>32**	**>4**
	841	8	**>4**	4	≤0.25	**>32**	**>4**
	866	8	**>4**	≤1	0.5	**>32**	**>4**
	873	**>32**	**>4**	**>16**	**128**	**>32**	**>4**
	883	**>32**	**>4**	**>16**	≤0.25	**>32**	**>4**
	887	**>32**	**>4**	**>16**	≤0.25	**>32**	**>4**

1

Table 2 (cont.).

Source	Isolate	MIC (mg/L)					
		AMX	CIP	ERY	GEN	NAL	TET
Human							
	960	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	988	**32**	**>4**	2	≤0.25	**>32**	**>4**
	1002	≤2	**4**	≤1	≤0.25	**>32**	**>4**
	1015	8	**>4**	2	≤0.25	**>32**	**>4**
Food							
	215613	4	**>4**	**16**	≤0.25	**>32**	**>4**
	219418	≤2	**>4**	2	0.5	**>32**	**>4**
	219517	**32**	**4**	**16**	≤0.25	**>32**	**>4**
	219530	**32**	**4**	**16**	≤0.25	**>32**	**>4**
	219872	4	**>4**	≤1	0.5	**>32**	**>4**
	219897	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	224703	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	224704	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	225423	4	**>4**	2	≤0.25	**>32**	**>4**
	225837	≤2	**4**	≤1	≤0.25	**>32**	0.25
	225861	4	**>4**	2	≤0.25	**>32**	1
	225868	**>32**	**>4**	2	≤0.25	**>32**	**>4**
	226188	4	**>4**	4	≤0.25	**>32**	**>4**
	226199	4	**>4**	**16**	**16**	**>32**	**>4**
	227193	**>32**	**>4**	**16**	≤0.25	**>32**	**>4**
	227246	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	227262	8	**>4**	4	≤0.25	**>32**	**>4**
	227306	**>32**	**>4**	≤1	≤0.25	**>32**	**>4**
	227686	4	**>4**	≤1	≤0.25	**>32**	0.25
	228997	**>32**	**>4**	**16**	≤0.25	**>32**	**>4**
	427	ND	**>4**	2	≤0.25	**>32**	**>4**
	431	ND	**>4**	≤1	≤0.25	**>32**	**>4**
	437	ND	**>4**	1	≤0.25	**>32**	**>4**
	440	ND	**>4**	4	≤0.25	**>32**	**>4**
	442	ND	**>4**	1	≤0.25	**>32**	**>4**
	443	ND	**4**	**>16**	0.5	**>32**	**>4**
	450	ND	**>4**	1	1	**>32**	**>4**
	509	ND	**>4**	**16**	≤0.25	**>32**	**>4**
	543	ND	**>4**	**16**	≤0.25	**>32**	**>4**
	544	ND	**>4**	**16**	0.5	**>32**	**>4**
	545	ND	≤0.25	2	1	**>32**	**>4**
	546	ND	**>4**	4	1	**>32**	**>4**
	561	ND	**>4**	2	0.5	**>32**	**>4**
	563	ND	0.25	1	0.5	**>32**	0.5

2

Table 2 (cont.).

Source	Isolate	MIC (mg/L)					
		AMX	CIP	ERY	GEN	NAL	TET
Animal							
	648	ND	**>4**	**16**	1	**>32**	**>4**
	650	ND	**>4**	**16**	0.5	**>32**	**>4**
	660	ND	**>4**	**>16**	0.5	**>32**	**>4**
	670	ND	**>4**	**>16**	1	**>32**	**>4**
	671	ND	**>4**	**16**	1	**>32**	**>4**
	672	ND	**>4**	**16**	0.5	**>32**	**>4**
	673	ND	0.5	4	0.5	**>32**	**>4**
	678	ND	**>4**	4	0.5	**>32**	**>4**
	680	ND	**4**	**>16**	≤0.25	**>32**	**>4**
	684	ND	**>4**	**>16**	≤0.25	**>32**	**>4**
	685	ND	**>4**	**>16**	≤0.25	**>32**	**>4**
	686	ND	**>4**	**>16**	≤0.25	**>32**	**>4**
	689	ND	**>4**	**16**	≤0.25	**>32**	**>4**
	697	ND	**>4**	4	≤0.25	**>32**	**>4**
	700	ND	**>4**	1	≤0.25	**>32**	**>4**
	702	ND	**>4**	**16**	≤0.25	**>32**	**>4**
	725	ND	**>4**	1	≤0.25	**>32**	0.25
	2	ND	**>4**	2	≤0.25	**>32**	**>4**
	3	ND	≤0.25	≤1	1	**>32**	**>4**
	5	ND	0.5	2	1	**>32**	**>4**
	6	ND	**>4**	**16**	0.5	**>32**	**>4**
	7	ND	**>4**	**>16**	0.5	**>32**	**>4**
	41	ND	**>4**	**>16**	0.5	**>32**	**>4**
	42	ND	**4**	2	1	**>32**	**>4**
	43	ND	0.25	≤1	≤0.25	**>32**	**>4**
	49	ND	0.25	≤1	≤0.25	**>32**	1
	57	ND	≤0.25	**16**	≤0.25	**>32**	0.5

ND, not determined. Bold means resistance.

Resistance breakpoints established by EUCAST and CLSI to *C. coli* were used: amoxicillin (AMX), >8 mg/L; ciprofloxacin (CIP), >0.5 mg/L; erythromycin (ERY), >8 mg/L; gentamicin (GEN), >2 mg/L; nalidixic acid (NAL), >16 mg/L; tetracycline (TET), >2 mg/L.

Chapter 5 - Paper III

Chapter 5 – Paper III

Resveratrol encapsulation with methyl-ß-cyclodextrin for antibacterial and antioxidant delivery applications

Andreia Duarte, Ana Martinho, Ângelo Luís, Ana Figueiras, Mónica Oleastro, Fernanda C. Domingues, Filomena Silva

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Chapter overview:

The results previously obtained and described in Chapters 3 and 4 showed that *Campylobacter* isolates isolated in Portugal, from different sources have high antibiotic resistance rates, even to those antibiotics used in the treatment of severe *Campylobacter* human infections. Given this, we aimed to study if resveratrol, a natural compound described to have several biological activities, could help to overcome antibiotic resistance and control *Campylobacter*. However, the *in vivo* efficacy of resveratrol is limited due to its low aqueous solubility and light sensibility. So, in order to improve the solubility of resveratrol, we proceed to its complexation with a methylated-ß-cyclodextrin.

In this study, we successfully accomplished the resveratrol-cyclodextrin complexation and the inclusion complex was characterized by several techniques. We showed that the complexation allowed a 400 fold increase in the resveratrol aqueous solubility and the resveratrol dissolution in a food stimulant medium was also increased. Concerning the resveratrol biological properties, it is known to possess antioxidant and anti-cancer activity (Chapter 1). So, we showed that the inclusion complex was able to maintain the very strong antioxidant activity of resveratrol and had the ability to reduce the cell viability of Caco-2 cells. Since this inclusion complex, in addition to increasing resveratrol solubility, was able to maintain resveratrol biological properties, we have also evaluated its cytotoxicity in human erythrocytes, having observed no hemolytic activity. Finally, since the results encouraged the application of resveratrol and its inclusion complex, we tested their antimicrobial activity against *Campylobacter* strains randomly chosen from the ones studied in Chapter 4. We demonstrated that both resveratrol and inclusion complex were able to inhibit the growth of all the tested strains.

In conclusion, we showed that besides improving resveratrol solubility, the inclusion complex also preserved the biological properties of resveratrol. This, together with their anti-*Campylobacter* activity encourages the application of this natural compound to control this foodborne pathogen.

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Resveratrol encapsulation with methyl-β-cyclodextrin for antibacterial and antioxidant delivery applications

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ABSTRACT

Resveratrol has been described to possesses several biological activities such as antioxidant, antimicrobial, anti-inflammatory and anticarcinogenic. However, its *in vivo* efficacy is still limited owing to its low aqueous solubility. The main goal of this study is to increase resveratrol aqueous solubility using for this purpose randomly methylated-β-cyclodextrin. Inclusion complex (IC) characterization and dissolution studies were performed as well as the evaluation of the IC antioxidant, cytotoxicity and anti-*Campylobacter* activities. Resveratrol complexation caused an effective 400 fold improvement in its aqueous dissolution. Both resveratrol and IC showed good antibacterial activity against *Campylobacter* spp. with minimum inhibitory concentration values ranging from 50 to 100 μg/mL for pure resveratrol and from 64 to 512 μg/mL for the IC, while also exhibiting a very good antioxidant activity and no haemolytic activity. Resveratrol complexation with methylated-β-cyclodextrin increased its solubility while maintaining resveratrol's biological properties.

1. Introduction

Despite the advances in food safety, foodborne diseases remain a major health issue with Campylobacteriosis being the most commonly reported zoonosis in European Union (EFSA & ECDC, 2015), with the consumption and handling of contaminated food products being the major source of this zoonosis (Duarte et al., 2014). So, it is important to find alternative control strategies that meet food industries' requests of safer and more naturally preserved foods. The development of controlled-release active packaging systems to control and/or eradicate foodborne pathogens has gained relevance in recent years (Sung et al., 2013; Tao, Hill, Peng, & Gomes, 2014). Many of these packages incorporate natural products as antimicrobial agents since they are perceived as being safer and thus, preferred by consumers (Sung et al., 2013).

Resveratrol is a stilbene phytoallexin that possesses several biological activities (Ferreira, Silva, Queiroz, Oleastro, & Domingues, 2014; Paulo, Ferreira, Gallardo, Queiroz, & Domingues, 2010) which have paved the way for the development of resveratrol-containing products by the food and pharmaceutical industries (Jeandet et al., 2012). However, the *in vivo* efficacy of resveratrol products is still limited owing to its low bioavailability, a consequence of its low aqueous solubility and rapid clearance from the circulation (Cottart, Nivet-Antoine, Laguillier-Morizot, & Beaudeux, 2010). In an attempt to overcome these hurdles, many formulations based on microencapsulation, cyclodextrins, liposomes and nanoparticles have been described (Santos, Veiga, & Ribeiro, 2011).

Cyclodextrins (CDs) are cyclic oligosaccharides derived from starch, that, due to this specific structure, CDs can interact with molecules to form ICs with various organic and inorganic substances both in solution and solid state (Figueiras, Carvalho, Ribeiro, Torres-Labandeira, & Veiga, 2007; Silva, Figueiras, Gallardo, Nerín, & Domingues, 2014). To overcome the limited aqueous solubility of native CDs, new modified CDs have been synthesized through the substitution of the hydroxyl groups by several functional groups

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such as methyl moieties, as in the case of randomly methylated-β-cyclodextrin. Apart from the improvement in terms of solubility, modified CDs also exhibit lower toxicity when compared to their native counterparts (Stella & He, 2008). Given the fact that these oligosaccharides possess negligible toxicity and pharmacologically inactive excipients for both pharmaceutical and food industry (E459), CDs are currently used in food formulations for flavour protection or delivery and, also, in the development of controlled-release active packaging systems (Del Valle, 2004; Higueras, López-Carballo, Gavara, & Hernández-Muñoz, 2015).

In this study, we focused on increasing resveratrol aqueous solubility using methylated-β-cyclodextrin. Aiming the development of food-based applications for resveratrol such as a biodegradable antimicrobial food packaging for meat preservation and control *Campylobacter*, the antimicrobial activity against *Campylobacter* spp. was assessed and its antioxidant activity was also evaluated, since meat oxidation generally results in the loss of product sensorial properties and decreased shelf-life (Fraqueza & Barreto, 2009). Due to compound release from the package and consequent ingestion, its dissolution profile in a food simulant as well as haemolytic activity were assessed. The possible cytotoxicity of resveratrol and IC in the intestinal epithelium, the main resveratrol absorption site, was also studied in human intestinal Caco-2 cells.

2. Materials and methods

2.1. Formation and characterization of the inclusion complex

2.1.1. Chemicals

trans-Resveratrol ($M_w = 228.24$ g/mol) was purchased from TCI Europe N.V. (Zwijndrecht, Belgium). Randomly methylated beta-cyclodextrin (RM-β-CD; CRYSMEB®, $M_w = 1191$ Da) was kindly provided by Roquette Freres S.A. (Lestrem, France).

2.1.2. Phase solubility studies

Phase solubility studies were carried out as previously described by Silva et al. (2014) with 500 μL methylated-β-cyclodextrin solutions at concentrations ranging from 0 to 0.02 mol/L being added to an excess amount of resveratrol (2 mg). The apparent stability constants (K_s) were calculated according to the equations established by Higuchi and Connors (Higuchi & Connors, 1965). Under the conditions of the phase-solubility studies, an A_L-type phase-solubility diagram with a slope less than unity would be observed, meaning that the complexes formed had a 1:1 stoichiometry. Resveratrol loading capacity into methylated-β-CD in the dried, lyophilized inclusion complex (IC) was also evaluated.

2.1.3. Inclusion complex and physical mixture preparation

IC was prepared as described by Silva et al. (2014). An excess amount of resveratrol (20 mg) was added to a 0.15 mol/L methylated-β-cyclodextrin aqueous solution (1 mL) in a glass tube and the suspension was incubated protected from light in an orbital shake at 250 rpm at 25 °C, for 24 h. Afterwards, the IC was frozen at −80 °C, lyophilized and stored in a desiccator prior to analysis.

Resveratrol and methylated-β-cyclodextrin physical mixture (PM) was prepared by blending uniformly, in a ceramic mortar, equimolar (1:1) amounts of resveratrol and CD, previously sieved through a 200 μm mesh.

2.1.4. Characterization of inclusion complex in solid state

FTIR-ATR spectra were obtained using a Thermo Scientific Nicolet iS10 FT-IR spectrometer associated with a Smart iTR* ATR horizontal reflexion accessory as described by Silva et al. (2014).

Differential scanning calorimetry (DSC) curves were carried out using a Netzsch DSC 204 Phoenix differential scanning calorimeter (Netzsch GmbH, Selg, Germany) calibrated with indium (99.98%, mp 156.65 °C, Netzsch GmbH, Selg, Germany). The thermal behaviour was studied by heating the samples in a sealed aluminium pan from 50 to 300 °C, at a rate of 10 °C/min and under a nitrogen flow of 20 mL/min, using an empty pan sealed as reference.

X-ray powder diffraction (XRD) patterns were obtained with a Rigaku, model DMAX III/C diffractometer system equipped with copper (Cu) as anode material and a graphite monochromator using a voltage of 30 kV and a current of 35 mA. The diffractograms were recorded in the 2θ angle range between 3 and 60° at a scan step size of 2θ/min. Crystallinity was determined as described previously (Figueiras et al., 2007) using the three peaks with the highest intensities.

Scanning electron microscopy (SEM) analysis was performed using an Hitachi S-2700 microscope (Tokyo, Japan), operated at an accelerating voltage of 20 kV at variable magnifications. The samples were fixed on a brass stub using double-sided tape and then made electrically conductive by coating with gold using an Emitech K550 sputter coater (London, England).

2.2. Antioxidant activity – DPPH scavenging assay

The antioxidant activity of resveratrol and IC was determined by the radical scavenging activity method using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as previously described by Luís, Domingues, and Duarte (2012). The radical scavenging activity was calculated as follows: $I\,\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 is the absorbance of the control and Abs_1 is the absorbance of the sample. The IC_{50} (concentration providing 50% of inhibition) was calculated graphically by the use of a calibration curve in the linear range of compound concentration *vs.* scavenging effect. The antioxidant activity was expressed as the Antioxidant Activity Index (AAI), calculated as follows: AAI = final concentration of DPPH in the control sample/IC_{50}. Therefore, the AAI was calculated considering the mass of DPPH and the mass of sample in the reaction, resulting in a constant for each sample tested. The AAI allowed the following classification: poor ($AAI \leq 0.5$), moderate ($0.5 < AAI \leq 1.0$), strong ($1.0 < AAI < 2.0$) or very strong antioxidant activity ($AAI \geq 2.0$) (Luís et al., 2012; Scherer & Godoy, 2009). All assays were carried out in duplicate and all DPPH solutions were prepared daily.

2.3. In vitro dissolution studies

The dissolution profiles of resveratrol and IC were determined in accordance to the standard method used by Figueiras et al. (2007) with slight modifications. Dissolution studies were performed using ethanol 100 mL/L as dissolution medium and food simulant for fresh meat products as described in the European Commission regulation No 10/2011. The assays were performed at 4 and 20 °C, in the dark, under constant magnetic agitation at 75 rpm. 700 μg of resveratrol or its equivalent quantity in the IC were dispersed over 20 mL of the dissolution medium. After defined time intervals, 1 mL of each sample was withdrawn from the dissolution medium and replaced with the same volume in order to assure sink conditions. The withdrawn samples were filtered and resveratrol concentration was determined using the previously described HPLC-DAD method (Silva et al., 2014). The experiment was carried out in triplicate.

2.4. Evaluation of the cytotoxicity

2.4.1. Haemolytic studies

Haemolysis of human erythrocytes by resveratrol, methylated-β-cyclodextrin and IC was estimated according to the method described by Ishiguro et al. (2011) with minor modifications. Fresh

blood was collected and erythrocytes were separated by centrifugation, washed twice with phosphate-buffered saline and resuspended to give a haematocrit of 100 mL/L. Erythrocyte's suspension (0.1 mL) was added to different concentrations of resveratrol, methylated-β-cyclodextrin and IC (0.9 mL) and the mixtures were gently agitated during 30 min at 37 °C. After centrifugation, the absorbance of the supernatant was measured at 543 nm. Results were expressed as a mean percent of total haemolysis, which was obtained when erythrocytes were incubated in pure deionised water.

2.4.2. MTT assay

Caco-2 cells (American Type Culture Collection, Manassas, VA, USA), a heterogeneous human epithelial colorectal adenocarcinoma cell line, were used to evaluate potential cytotoxicity induced by the studied compounds. Cells were cultured in RPMI 1640 supplemented with 100 mL/L foetal bovine serum, 2 mmol/L L-glutamine and 100 units/L penicillin, 0.10 mg/L streptomycin and 0.25 μg/L amphotericin B at 37 °C in a humidified incubator under an atmosphere of 5% CO_2/95% air. Three to four days before the experiment, cells were seeded in 96-well cell culture plates (15,000 cells per well) and 24 h before incubation, the medium was substituted for RPMI 1640 containing the antibiotics listed above without foetal bovine serum. Incubations included five concentrations of resveratrol, methylated-β-cyclodextrin or IC prepared in RPMI 1640 with antibiotics. After 24 h of incubation, MTT assay was performed. Each concentration from each compound was made in triplicate and the experiment was repeated three times, independently.

Cellular viability of Caco-2 cells was measured using the MTT assay. Each replicate of the experiments was incubated with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; Sigma–Aldrich, Inc., St. Louis, MO, USA) in Krebs solution (132 mmol/L NaCl, 4 mmol/L KCl, 1.4 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 6 mmol/L glucose and 10 mmol/L HEPES, pH 7.4) for 90–120 min at 37 °C. Viable cells converted MTT to a water-insoluble precipitate that was solubilized with the addition of 40 mmol/L HCl in isopropanol followed by incubation in the dark with gentle agitation. Afterwards, the absorbance at 570 nm was recorded using a microplate spectrophotometric reader.

2.5. Antibacterial activity

2.5.1. Bacterial strains

In this study, two reference strains (*Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* ATCC 33559) and four *Campylobacter* isolates were used. *C. coli* 53 and *C. coli* 873 were isolated from faecal samples of patients with acute gastroenteritis and *C. jejuni* 225421 and *C. coli* 219872 were isolated from poultry fresh meat. The strains were stored in Brain Heart Infusion broth with 200 mL/L glycerol at −80 °C and prior to susceptibility testing each strain was inoculated on Brucella agar plates supplemented with 50 mL/L defibrinated horse blood to ensure optimal growth.

2.5.2. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) for resveratrol and IC were determined by the microdilution method (McDermott, Bodeis-Jones, Fritsche, Jones, & Walker, 2005). Briefly, serial two-fold dilutions of resveratrol (from 400 to 3.125 μg/mL) and IC (from 2048 to 16 μg/mL) were prepared in 96-well plate in Mueller Hinton broth (MHB). A maximum of 15 mL/L of dimethyl sulfoxide concentration was used to increase the solubility of pure resveratrol. Bacterial suspensions were prepared in MHB, until a turbidity of 0.5 McFarland was reached, diluted and added to each well, to yield a final concentration of about 5×10^5 colony-forming units (CFU)/mL per well and the plates were incubated at 37 °C for 48 h under microaerobic conditions. After the incubation the growth was visually assessed. At least three independent assays were performed and the modal MIC values were selected.

2.6. Statistics

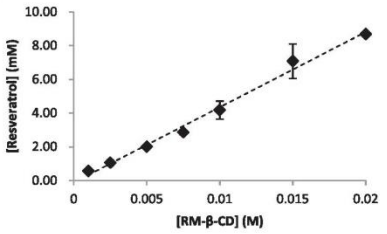
Statistical analysis was performed for antioxidant activity and MTT data. Groups were compared using one-way ANOVA followed by Tukey's *post hoc* test. A p value of <0.05 was used to indicate statistically significant differences.

3. Results and discussion

Although resveratrol presents an immense potential for the food industry, its low aqueous solubility still needs to be circumvented. As CDs are known to increase the solubility and stability of compounds and its use is allowed as food additive, in this work these compounds were chosen to incorporate resveratrol, assessing the possible solubility enhancement. Phase-solubility studies revealed that methylated-β-cyclodextrin forms IC with resveratrol in a 1:1 stoichiometry (Fig. 1b), exhibiting a A_L type curve (Fig. 1a) which is in agreement with the results described by other authors (Bertacche, Lorenzi, Nava, Pini, & Sinico, 2006). The K_S for resveratrol indicated that the complex formed is moderately stable (Bertacche et al., 2006; Das, Lin, Ho, & Ng, 2008). Resveratrol inclusion in methylated-β-cyclodextrin allowed resveratrol solubilisation up to a maximum amount of 16 mg/mL, which yields a 400 fold increase in resveratrol solubility (Das et al., 2008). Regarding resveratrol loading capacity into the cyclodextrin, 80% of resveratrol was successfully loaded into the complex, yielding 0.8 mg of resveratrol in 1 mg of the lyophilized IC.

IC formation was further confirmed by means of FTIR, DSC, XRD and SEM analysis. Fig. 2a shows the multiple FTIR spectra obtained for the resveratrol, methylated-β-cyclodextrin, PM and IC. The FTIR spectrum for resveratrol, from 1700 to 700 cm^{-1}, showed the typical four intense bands of trans-resveratrol at 1605, 1583, 1381 cm^{-1} and 964 cm^{-1} (Shi et al., 2008). Methylated-β-cyclodextrin, PM and IC FTIR spectra exhibited a strong band at 1004 cm^{-1}, characteristic of methylated-β-cyclodextrin (Ribeiro, Figueiras, Santos, & Veiga, 2008). The major differences were observed between the FTIR spectra of IC and PM. All four typical

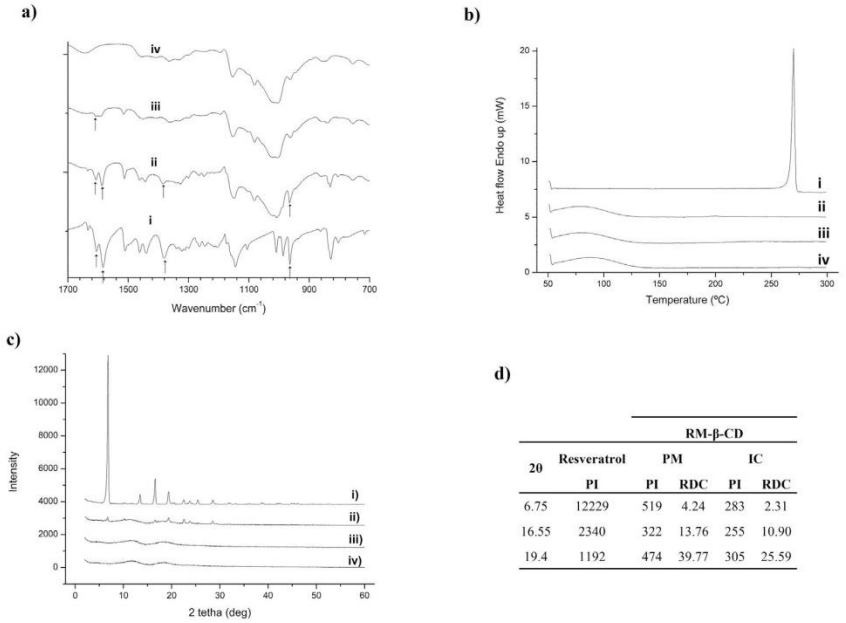
a)



b)

CD	Maximum solubility (mg/mL)	f_s	K_S (M^{-1})	Stoichiometry	R^2
RM-β-CD	16.9 ± 1.3	444.4	1482.9 ± 13.7	1:1	0.996

Fig. 1. Phase solubility studies between resveratrol and randomly methylated-β-cyclodextrin: initial straight line portion of the phase solubility diagrams (a); maximum solubility achieved with the complexation, f_s (solubility factor; ratio between resveratrol solubility in the presence and in the absence of cyclodextrin), K_S value, stoichiometry and correlation coefficient (b) (n = 2).



d)

2θ	Resveratrol	RM-β-CD PM		RM-β-CD IC	
	PI	PI	RDC	PI	RDC
6.75	12229	519	4.24	283	2.31
16.55	2340	322	13.76	255	10.90
19.4	1192	474	39.77	305	25.59

Fig. 2. Characterization of complex formation between resveratrol and randomly methylated-β-cyclodextrin: FTIR-ATR spectra (a) with arrows indicating the characteristic FTIR bands of resveratrol, DSC thermograms (b), X-ray diffractograms (c) of resveratrol (i), resveratrol – methylated-β-cyclodextrin physical mixture (ii), resveratrol – methylated-β-cyclodextrin inclusion complex (iii) and methylated-β-cyclodextrin (iv); and peak intensities (PI) and relative degree of crystallinity (RDC) values (d) for resveratrol – methylated–β-cyclodextrin physical mixtures (PM) and inclusion complexes (IC).

resveratrol bands can be seen almost as clear in the pure compound as in the PM; whereas, in the IC, three of them disappeared and only the band corresponding to C–C aromatic double bond stretching ($\approx$1605 cm^{-1}) could be identified, confirming IC formation (Figueiras et al., 2007).

Concerning DSC results (Fig. 2b), resveratrol thermogram is typical of an anhydrous crystalline substance with a sharp melting endothermic peak at 270 °C, which is in the melting point range provided by other authors (Bertacche et al., 2006). Methylated-β-cyclodextrin yielded a broad endothermic peak around 90 °C associated with loss of water molecules from the CD cavity (Fernandes, Vieira, & Veiga, 2002; Ribeiro et al., 2008). In the thermograms obtained for the complex, the absence of the melting endotermic peak at 270 °C can be noticed, providing some evidences of complexation, as the melting point of a molecule tends to shift or even disappear when it is embedded inside the CD cavity (Karoyo, Sidhu, Wilson, & Hazendonk, 2013). Contrary to the expected, the DSC thermograms of IC and PM were very similar. Since no overlapping between resveratrol melting peak and CD dehydration peak was seen, this fact might indicate that a partial complexation could have occurred during the mixing of the compounds in the PM preparation (Bertacche et al., 2006).

The XRD diffractograms of pure compounds, PM and IC are displayed in Fig. 2c and the relative degree of crystallinity (RDC) is presented in Fig. 2d. The hollow pattern obtained for methylated-β-cyclodextrin diffractogram proved its amorphous state (Figueiras et al., 2007; Silva et al., 2014). For the PM, the diffractogram obtained is an overlap of resveratrol and CD single patterns, although there was a visible decrease in resveratrol peak intensities, as a probable consequence of the CD's amorphous character (Ribeiro et al., 2008). The X-ray pattern obtained for the complex indicates that an IC is formed, as it is distinct from the ones of pure compounds. Also, the RDC data seems to corroborate this result, lower RDCs values were obtained for the IC at several diffraction angles.

SEM was used to allow the microscopic visualization of morphologic alterations in pure compounds (CD and resveratrol) and systems (PM and IC) structures (Fig. 3). Resveratrol microphotograph (Fig. 3a) showed parallelogram-shaped crystals, indicative of its crystalline structure (Bertacche et al., 2006). Methylated-β-cyclodextrin (Fig. 3b) is composed by spherical particles with an amorphous character (Figueiras et al., 2007). The PM microphotograph (Fig. 3c), allowed to observe the typical methylated-β-cyclodextrin structure with some resveratrol agglomerates adhered at its surface. In contrast, the IC (Fig. 3d) shows irregular-shaped particles and none of the pure compounds can be distinguished. Notwithstanding the fact that SEM could not undoubtedly confirm the complex formation, this drastic change of the particle size and shape might be indicative of a new solid phase/component, as a result of the inclusion of resveratrol within the CD cavity (Bertacche et al., 2006; Naidu et al., 2004).

DPPH is a stable free radical that has been extensively applied to the study of the antioxidant activity of natural compounds (Villaño, Fernández-Pachón, Moyá, Troncoso, & García-Parrilla, 2007). The results of the DPPH assay are presented in Table 1. It was observed that resveratrol has a very strong antioxidant activity, as well as the IC, which is in agreement with the data previously described by Lu, Cheng, Hu, Zhang, and Zou (2009). The differences in scavenging capacity between free and complexed resveratrol are small, and no significant statistical differences ($p < 0.05$) were observed, which suggests that the inclusion process had little influence on the antioxidant activity and that the IC was able to maintain resveratrol's antioxidant activity. In sum, the IC formed maintained

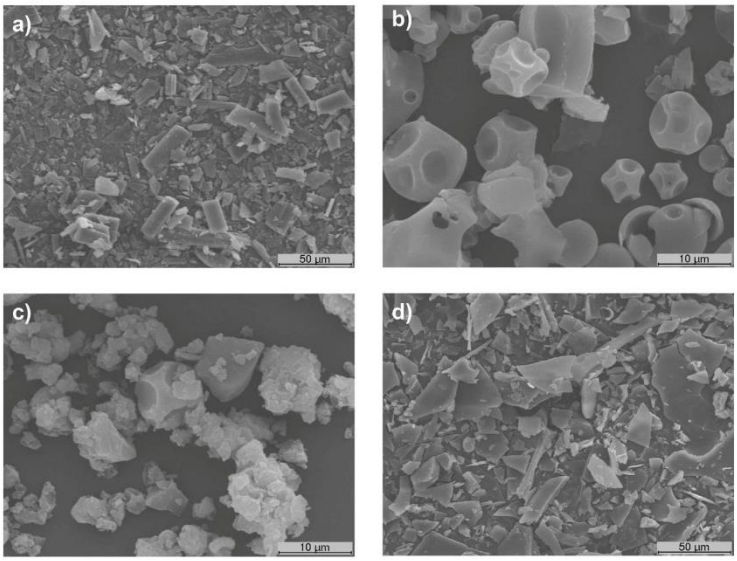


Fig. 3. Scanning electron microscopy photographs of resveratrol (a), methylated-β-cyclodextrin (b), resveratrol – methylated-β-cyclodextrin physical mixture (c) and resveratrol – methylated-β-cyclodextrin inclusion complex (d).

resveratrol's antioxidant activity, meaning that it could, potentially, be used by the food industry to improve product shelf-life.

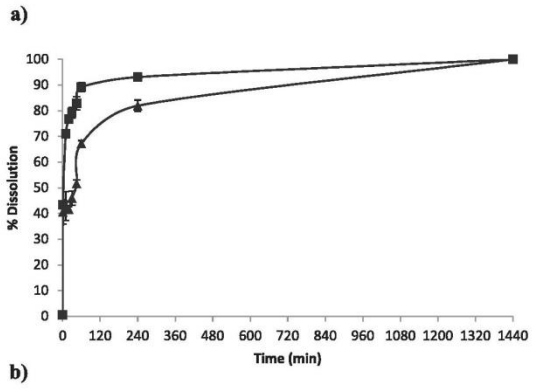
The dissolution behaviour of resveratrol and its IC at 4 °C are given in Fig. 4a and the dissolution percentage obtained after 60 min for both samples is compiled in Fig. 4b. In the dissolution medium used, significant differences between the dissolved percentage of free resveratrol and IC were observed. After 10 min, the percentage of resveratrol dissolved was nearly 43% in the case of resveratrol, whereas, when complexed with methylated-β-cyclodextrin, this value increased to 70%. After 60 min of incubation, the percentage of dissolution increased to 67% and 89% for resveratrol and IC respectively. For the assays performed at 20 °C, resveratrol dissolution profile was similar to the profile at 4 °C, with 95% resveratrol dissolution for the IC obtained only after 10 min (data not shown). Resveratrol dissolution is enhanced when this compound is complexed with methylated-β-cyclodextrin. This effect can be attributed to the surfactant-like properties of the CD molecule that is able to reduce the interfacial tension between resveratrol and the dissolution medium (Figueiras et al., 2007; Priya, Sivakamavalli, Vaseeharan, & Stalin, 2013). So, it is important to assess resveratrol toxicity as it could effectively migrate from the food packaging to the meat product.

The haemolysis assay is used to investigate the cytotoxicity of compounds on erythrocytes' membrane through the haemoglobin released. Regarding haemolysis results, resveratrol showed haemolytic activity at concentrations higher than 100 µg/mL (2% of haemolysis) while the IC presented lower haemolytic activity with about 2% of haemolysis at a concentration of 2000 µg/mL and methylated-β-cyclodextrin exhibited the highest hemolytic activity, approximately 13% for the maximum concentration used (0.02 mol/L). These data support the fact that resveratrol and IC are compatible with erythrocytes, with the IC showing higher compatibility. Our results are in accordance with other data, since resveratrol exhibited no hemolytic effect on human erythrocytes up to 100 µg/mL of concentration (Jung, Seu, & Lee, 2007).

Table 1
Antioxidant properties of resveratrol and inclusion complex. Results are presented as mean ± standard deviation of three replicates.

	IC_{50} (µg/mL)	AAI	Antioxidant Activity
Resveratrol	13.5 ± 0.6	4.0 ± 0.4	Very Strong
Inclusion Complex	11.0 ± 2.7	4.6 ± 0.5	Very Strong

No significant difference ($p < 0.05$) was observed.



	RV dissolution at 60 min (%)
Resveratrol	67.2±1.1
Inclusion Complex	89.2±1.8

Fig. 4. Dissolution profiles (a) of resveratrol (▲) and inclusion complex (■). Test conditions: ethanol 100 mL/L, 4 °C in the dark. Percentage of *in vitro* dissolution profiles of resveratrol and inclusion complex after 60 min (b). Results are presented as mean ± standard deviation of three independent experiments.

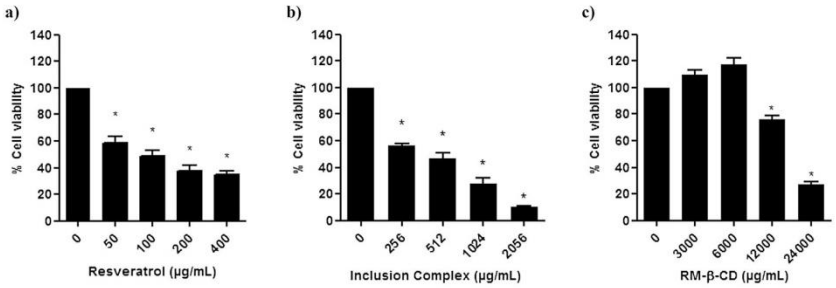


Fig. 5. Cytotoxicity of resveratrol (a), inclusion complex (b) and methylated-β-cyclodextrin (c) on Caco-2 cells. Cell viability was expressed as the percentage of untreated control and data are expressed as mean ± standard deviation of three independent assays. *Correspond to significant difference ($p < 0.05$).

Since the final goal of this work is to increase resveratrol aqueous solubility in order to a future application in an antimicrobial food packaging, it becomes important to investigate cytotoxicity of both resveratrol and IC in intestinal cells, since the intestinal epithelium is the main absorption site for this compound (Santos et al., 2011). Therefore, the effects of resveratrol, IC and methylated-β-cyclodextrin on Caco-2 cell viability were assessed using the MTT assay. As shown in Fig. 5, resveratrol treatment (50–400 µg/mL) significantly decreased ($p<0.05$) cell viability and the treatment with 100 µg/mL inhibited about 50% of Caco-2 cell viability. Concerning the IC, all tested concentrations also significantly decreased cell viability, with an inhibition of about 90% for the highest concentration. Methylated-β-cyclodextrin had no cytotoxic effect with concentrations between 0 and 6000 µg/mL, but at the highest concentration tested (24,000 µg/mL) a decrease of 73% in cell viability was observed. Our results demonstrate the potential of resveratrol and IC in decreasing the cell viability of Caco-2 cells. Zhang, Anderson, Kaushik, and Dwivedi (2009) reported similar results, since they showed that resveratrol treatment starting at 25 µM decreased cell viability of Caco-2 cells. Other reports demonstrated that resveratrol at lower concentrations inhibited Wnt signal throughput in colon-derived cells due to regulation of β-catenin localization (Hope et al., 2008). This activity may also contribute to the potential of resveratrol in the cancer prevention. Taking in account their potential applications, many studies have revealed the activity of CDs in drug complexation and their effects on drugs pharmacokinetic. However, limited data is available regarding their toxicity. The toxicological effects of β-CDs' are thought to be associated mainly to inclusion complexation with the cholesterol present in cellular membranes; in fact, the high affinity of methylated-β-CDs towards cholesterol became so evident that, currently, these CDs are widely applied as cholesterol sequestering agents (Kiss et al., 2010). Nevertheless, in our study, only with the highest methylated-β-cyclodextrin concentration, a significant reduction in cell viability was observed, indicating a low toxicity of this CD against Caco-2 cells. This could be due to the low number of methyl groups (3–4) on this CD molecule, as an increase on the number of methyl groups on the CD molecule enhanced its toxic effect (Kiss et al., 2010). Concerning resveratrol – methylated-β-CD IC, the information is limited and no cell viability studies were found. Nevertheless, the potential of resveratrol in decreasing the cell viability of Caco-2 cells is maintained in the IC form, meaning that the IC is able to maintain resveratrol biological properties.

The very strong antioxidant activity and low cytotoxicity of the resveratrol and IC encourage its future application in food industry. Thus, the antimicrobial activity of the resveratrol and IC was evaluated against *Campylobacter* spp. The MIC values obtained for *C. coli* and *C. jejuni* are presented in Table 2. Pure resveratrol and IC were able to inhibit all the tested strains, with exception of the IC against to the *C. coli* reference strain. The tested compounds were able to inhibit the growth of the four *Campylobacter* isolates that exhibit a multidrug resistance profile to several antibiotics (Duarte et al., 2014). The MIC values ranged from 50 to 100 µg/mL for pure resveratrol and from 64 to 512 µg/mL for the IC. The MICs for resveratrol are in accordance with the existing data regarding resveratrol action against other food pathogens (Ferreira et al., 2014; Paulo, Oleastro, Gallardo, Queiroz, & Domingues, 2011). Also, the MIC values for pure resveratrol and IC were very similar in the case of the two *C.* coli isolates (*C. coli* 873 and *C. coli* 219872), while the MIC values concerning the IC against the other strains were two to five times higher than the obtained for resveratrol. Giving the proposed model for the use of inclusion complex for drug delivery to microbial cells, the dissociation of the IC needs to occur for the pure compound to exert its antibacterial activity inside the bacterial cell, which could indicate that the reduction of the antimicrobial activity of the compound by the complexation is positively correlated with the value of the IC K_S, as more stable complexes could imply a lower dissociation rate of the antimicrobial from the IC (Leclercq, Nardello-Rataj, Rauwel, & Aubry, 2010; Lu, Zhao, Wang, & Wang, 2011). In this way, resveratrol had to be free to interact with the bacterial membrane, since the antimicrobial activity of an IC could be explained by four steps: diffusion of the complex in the solution; contact with the bacterial membrane by collision; dissociation of the complex and interaction of the free molecule with the bacterial membrane (Lu et al., 2011). It has also been reported that the formation of ICs between CDs and biocides could reduce the antimicrobial activity of the biocides (Leclercq et al., 2010; Lu et al., 2011).

In conclusion, the results showed that resveratrol-methylated-β-cyclodextrin IC could be prepared at a 1:1 M ratio and that the complexation effectively enhanced the resveratrol's solubility and

Table 2
Minimal inhibitory concentrations (MIC) of resveratrol and inclusion complex against two *Campylobacter* clinical isolates (*C. coli* 53 and *C. coli* 873), two food isolates (*C. jejuni* 225421 and *C. coli* 219872) and two reference strains (*C. jejuni* ATCC 33560 and *C. coli* ATCC 33559). Results are presented as mean ± standard deviation of three replicates.

Campylobacter strains	MIC (µg/mL)	
	Resveratrol	Inclusion complex
C. coli 53	100	256
C. coli 873	50	64
C. jejuni 225421	100	512
C. coli 219872	50	64
C. jejuni ATCC 33560	50	128
C. coli ATCC 33559	100	>2048

dissolution in a food simulant for fresh meat products. Besides improving resveratrol solubility, the IC also preserved the potential of resveratrol in decreasing the cell viability of Caco-2 cells, as well as the very strong antioxidant activity of resveratrol. The non-cytotoxicity of resveratrol and IC against human erythrocytes was also demonstrated. This, together with the anti-*Campylobacter* activity exhibited by the IC encourages its application in the food industry to control this foodborne pathogen, namely in poultry products packaging, as they are known to be heavily contaminated with *Campylobacter* spp.

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Chapter 6 - Paper IV

Resveratrol inclusion complexes: antibacterial and anti-biofilm activity against *Campylobacter* spp. and *Arcobacter butzleri*

Andreia Duarte, Ana Alves, Susana Ferreira, Filomena Silva, Fernanda C. Domingues

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Chapter overview:

As described in the previous chapters, C. *jejuni* and *C. coli* are well known foodborne pathogens. In addition, *Arcobacter* species, in particular *A. butzleri*, are also known to be human and animal pathogens. In Chapter 5, we demonstrated some biological properties of resveratrol and its potential to control *Campylobacter*.

In this study, we evaluated the potential of resveratrol and its inclusion complex with hydroxypropyl-γ-cyclodextrin (previously characterized by our research group) to control planktonic cells and biofilms of *Campylobacter* species and *A. butzleri*. The assays with *A. butzleri* were performed by Ana Alves during her master thesis work, resulting in a contribution to the paper presented in this chapter. In this work, we first assessed if resveratrol and the inclusion complex had ability to inhibit the growth of planktonic cells of both microorganisms, with a bactericidal effect being observed. In addition we observed that the inclusion complex may act by depolarizing the membrane of the cells and through the reduction of the metabolic activity of the cells. Then, we demonstrated that both resveratrol and inclusion complex had the ability to reduce the biofilm formation by the tested strains and to diminish biofilms already formed. Finally, we showed the potential of resveratrol and inclusion complex in the inhibition of the quorum sensing (QS) mechanism by using a biosensor strain. Since bacterial biofilms are related with quorum sensing mechanism, the inhibitory action in the QS system could be related with the anti-biofilm action of resveratrol and inclusion complex.

So, in addition to the antibacterial activity of resveratrol against *Campylobacter* previously described in the chapter 5, in the present study we also showed the potential of this compound to control biofilms of *Campylobacter* and *Arcobacter*. In sum, in Chapters 5 and 6 we showed that the natural compound resveratrol possess anti-*Campylobacter* activity and the resveratrol inclusion complexes in addition to maintain resveratrol properties, increases its solubility and stability. So both resveratrol and inclusion complexes could be used as natural compounds to control the foodborne pathogen *Campylobacter*.

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Resveratrol inclusion complexes: Antibacterial and anti-biofilm activity against *Campylobacter* spp. and *Arcobacter butzleri*

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ABSTRACT

Worldwide, the consumption or handling of contaminated food has been described as one of the major causes of foodborne illness with campylobacteriosis being the most commonly reported zoonosis. *Campylobacter jejuni* and *Campylobacter coli* are considered the major cause of bacterial gastroenteritis, while *Arcobacter* spp. are also known to be human and animal pathogens. Furthermore, these bacteria are able to form biofilms which have become a relevant issue in a wide range of food industries since they are more resistant to disinfectants and so, more difficult to eliminate. This question gives rise to the research on the use of alternative substances that can effectively prevent biofilm formation or eradicate the biofilm already formed. Given this, the aim of this study was to evaluate the antimicrobial and anti-biofilm activity of resveratrol-hydroxypropyl-γ-cyclodextrin inclusion complexes (IC) against *C. jejuni*, *C. coli* and *A. butzleri* as well as their quorum sensing (QS) inhibition activity. Besides improving resveratrol solubility, the ICs showed anti-*Campylobacter* and anti-*Arcobacter* activity, inhibited biofilm formation and promoted the biofilm dispersion even at sub-MIC concentrations for both genera. It was also demonstrated the anti-QS activity of the ICs through the inhibition of violacein production by *Chromobacterium violaceum*. In conclusion, since the use of natural compounds can improve the safety and security of foods, our results showed that this IC could be developed as a new anti-biofilm agent and QS inhibitor to enhance the shelf-life and safety of foods.

1. Introduction

Despite the recent improvements in food safety, foodborne illness remains frequent in Europe and the United States (EFSA, (European Food Safety Authority), & ECDC, (European Centre for Disease Prevention & Control), 2015; Painter et al., 2013). It is estimated that, each year, approximately 300,000 persons in the European Union suffer from foodborne illness caused by a major pathogen (EFSA, (European Food Safety Authority), & ECDC, (European Centre for Disease Prevention & Control), 2015). According to the latest EFSA report, campylobacteriosis was the most commonly reported zoonosis in 2013, being *Campylobacter jejuni* and *Campylobacter coli* the major cause of bacterial gastroenteritis in humans worldwide (Duarte et al., 2014; EFSA, (European Food Safety Authority), & ECDC, (European Centre for Disease Prevention & Control), 2015). One of the major causes of foodborne illness continues to be the consumption of raw or undercooked chicken or the cross-contamination of other foods during raw chicken handling; which is mostly due to the high microbial load of fresh poultry products (Painter et al., 2013). This elevated microbial load present in chickens and other avian species is mainly a consequence of the fact that these animals serve as natural reservoir hosts for *Campylobacter* spp. which are able to colonize their intestinal tract (Silva et al., 2011b). Other pathogens also commonly found in poultry meat and products are *Arcobacter* spp., although the source of poultry contamination is still not clear (Ferreira, Fraqueza, Queiroz, Domingues, & Oleastro, 2013; Wesley & Miller, 2010). Within the *Arcobacter* genus, the species *A. butzleri*, *A. cryaerophilus* and *A. skirrowii* are known to be human and animal pathogens, with *A. butzleri* being included in the list of microbes considered to be a serious hazard to human health by the International Commission on Microbiological Specifications for Foods (Collado & Figueras, 2011). Since poultry meat is a highly perishable and very popular food commodity, with a growing increased consumption world (Daniel, Cross, Koebnick, & Sinha, 2011), the microbiological safety of poultry products is of extreme importance. Therefore, the development of new antimicrobial strategies to control and/or eliminate *Campylobacter* and *Arcobacter* contamination in poultry and poultry products are in demand, not only to deal with the economic burden that represents bacterial foodborne illness but also to extend the shelf-life of these products. These newly developed strategies must also be effective in controlling and/or eliminating bacterial biofilms since it has been described that *Campylobacter* and *Arcobacter* could develop biofilms (Ferreira et al., 2013; Gunther & Chen, 2009) that are more resistant to disinfectants

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and become a major issue within the food industry, mainly the ones dedicated to poultry meat processing (Srey, Jahid, & Ha, 2013). Bacterial biofilm formation and other physiological activities, such as antibiotic resistance and motility can be related to the intercellular communication mechanisms like quorum sensing (QS) (Deep, Chaudhary, & Gupta, 2011) which has also been implied in bacterial proliferation in foods and food spoilage.

Keeping this into consideration, the inhibition of cell-to-cell communication underlying the QS could be considered a viable strategy to ensure food safety and quality (Alvarez et al., 2014; Zhang et al., 2014).

Resveratrol (3,5,4′-trihydroxystilbene) is a stilbene naturally present in foodstuffs with described antimicrobial activity against several pathogens (Ferreira, Silva, Queiroz, Oleastro, & Domingues, 2014b; Paulo, Ferreira, Gallardo, Queiroz, & Domingues, 2010; Paulo, Oleastro, Gallardo, Queiroz, & Domingues, 2011). However, this compound's bioactivity still remains an issue due to its low bioavailability stemming from its poor stability and solubility in aqueous media (Chen et al., 2007; Delmas et al., 2011). To overcome these challenges, in recent years, several encapsulation strategies have been proposed such as resveratrol inclusion in liposomes/niosomes, yeast cells, biopolymer particles such as proteins, chitosan and cyclodextrins (Augustin, Sanguansri, & Lockett, 2013; Davidov-Pardo & McClements, 2014). Cyclodextrins are the most commonly used encapsulating agents to form inclusion complexes (IC) in the food industry and are generally recognized as safe by the United States Food Drug Administration (Davidov-Pardo & McClements, 2014). Results have shown that, besides being able to significantly increase resveratrol aqueous solubility, cyclodextrins also improved or maintained the antioxidant activity of this compound (Das, Lin, Ho, & Ng, 2008; Davidov-Pardo & McClements, 2014; Lu, Cheng, Hu, Zhang, & Zou, 2009), while being capable of protecting resveratrol from the external environmental factors, such as temperature, light and pH, by entrapping it inside their cavities (Pinho, Grootveld, Soares, & Henriques, 2014a).

Envisioning the application of resveratrol IC in the food industry, the goal of this study was to evaluate the antimicrobial activity of resveratrol-hydroxypropyl-γ-cyclodextrin ICs against *C. jejuni*, *C. coli* and *A. butzleri* while unveiling its mode of action in these bacterial cells. The ability of these IC in the inhibition of biofilm formation and in the dispersion of established biofilms was also evaluated, as well as its QS inhibition activity.

2. Material and methods

2.1. Inclusion complex formation

trans-Resveratrol was obtained from TCI Europe N.V. and hydroxypropyl-γ-cyclodextrin (HP-γ-CD) from Sigma-Aldrich. The inclusion complex (IC) of resveratrol with HP-γ-CD was prepared as described by Silva, Figueiras, Gallardo, Nerín, and Domingues (2014) and resveratrol concentration in the IC was quantified by HPLC–DAD (Silva et al., 2014).

2.2. Bacterial strains

In this study, two *Campylobacter* spp. and two *Arcobacter butzleri* isolates were used. *C. coli* 873 was isolated from a faecal sample of a patient with acute gastroenteritis and *C. jejuni* 225421 was isolated from fresh poultry meat (Duarte et al., 2014). *A. butzleri* AB36/11 was isolated from poultry caecum and the strain INSA776 was isolated from a faecal sample of a patient with diarrhoea and abdominal pain (Ferreira, Queiroz, Oleastro, & Domingues, 2014a). All the strains were stored in Brain Heart Infusion (BHI) broth with 20% (v/v) glycerol at −80 °C and prior to antimicrobial susceptibility assays, each strain was inoculated on blood agar plates supplemented with 5% defibrinated horse blood (Oxoid, England) to ensure optimal growth.

2.3. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MICs) for the IC and resveratrol were determined by the microdilution method according to the previously described methods to *Campylobacter* and *Arcobacter* spp. (Ferreira et al., 2014b; McDermott, Bodeis-Jones, Fritsche, Jones, & Walker, 2005). Briefly, serial two-fold dilutions of IC, ranging from 2048 to 16 μg/mL, and resveratrol from 400 to 3.125 μg/mL were prepared in a 96-well plate (50 μL per well) in Mueller Hinton broth (MHB, LiofilChem, Italy). A maximum of 1.5% of dimethyl sulfoxide (DMSO) was used to increase resveratrol aqueous solubility. The bacterial suspensions, with a turbidity of 0.5 McFarland, were prepared from overnight cultures on blood agar plates and then diluted and added to each well to yield a final concentration of about 10^6 colony-forming units (CFU)/mL. The plates were incubated at 37 °C for 48 h under microaerobic conditions and, after incubation, growth was visually assessed and confirmed spectrophotometrically at 590 nm. At least three independent assays were performed and the modal MIC values were selected.

2.4. Time–kill curves

Bacterial suspensions from exponentially-growing cultures obtained after 6 h of incubation in MHB at 37 °C and 100 rpm under microaerobic conditions were exposed to the IC and resveratrol at final concentrations of 1×, 2× and 4 × MIC and a final cell concentration of about 10^6 CFU/mL. HP-γ-CD, DMSO and culture medium were used as growth controls. Tubes were incubated at 37 °C under microaerobic (*Campylobacter* strains) or aerobic (*A. butzleri*) conditions and after 0, 2, 4, 6, 8, 10 and 24 h of incubation a sample of 20 μL was removed to assess cell concentration (CFU/mL) by the drop plate method (Chen, Nace, & Irwin, 2003). Data was obtained from three independent experiments. Bactericidal activity was defined as a reduction of 99.9% of the total number of CFU/mL and bacteriostatic activity as the maintenance or reduction of less than 99.9% of the original inoculum.

2.5. Flow cytometry assay

2.5.1. Exposure of bacteria to the inclusion complex

Bacterial suspensions for flow cytometry assays were prepared as described for time–kill curves. After 6 h of incubation, samples were centrifuged at 5000 rpm for 10 min and washed with PBS buffer. The resulting suspensions were used for fluorescent staining.

2.5.2. Staining procedure

To evaluate membrane depolarization, the previously prepared bacterial suspensions were incubated with 2.5 μg/mL of bis-(1,3-dibutylbarbituric acid) trimethine oxonol (BOX; Molecular Probes®, Carlsbad, CA) in PBS buffer supplemented with 4 mM EDTA (pH 7.4) for 15 min in the dark at room temperature. In order to assess respiratory activity, the suspensions were incubated with 5 mM of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Inc., Warrington, PA) in BHI broth for 2 h at 37 °C and 100 rpm under microaerobic conditions. After the incubation with BOX or CTC, cells suspensions were centrifuged at 10,000 rpm, for 5 min and resuspended in PBS buffer with 4 mM EDTA for total cell staining with 10 μM of SYTO® 40 (Molecular Probes®, Carlsbad, CA). SYTO® 40 has the capacity to penetrate the cell, binding to nucleic acids, allowing to distinguish cells from possible residues or cellular remains. The final suspensions were incubated in the dark for 15 min at room temperature, washed, resuspended in PBS and analysed in the flow cytometer.

2.5.3. Flow cytometry acquisition data

Samples were acquired on a CyAn ADP (Beckman Coulter, USA) flow cytometer and analysis was performed using Summit 4.3 (Beckman Coulter, USA) software. Fluorescence signals were collected by FL1

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(BOX), FL4 (CTC) and FL6 (SYTO® 40) bandpass filters and a total of 5000 events in the gated region were collected for each sample. Fluorescence and light scatter signals were acquired logarithmically. All the assays were performed at least twice.

2.6. Anti-biofilm activity

Biofilms were grown in 96-well flat-bottom polystyrene microtiter plates using a previously described method (Reeser, Medler, Billington, Jost, & Joens, 2007) with some modifications. Briefly, bacterial isolates were grown overnight in MHB at 37 °C under microaerobic conditions and the turbidity of the suspension was adjusted to 0.1 OD_{620}. Serial IC and resveratrol two-fold dilutions were prepared in MHB in a 96-well plate (50 μL per well) with final concentrations of IC ranging between 0.125 and 4 times the MIC value for each strain, and for resveratrol between 0.125 and 2 times the MIC value, due to its lower aqueous solubility. Then, 50 μL of the abovementioned bacterial suspension was added to each well. The plates were incubated for 48 h at 37 °C under microaerobic conditions. To study the IC and resveratrol effect on preformed biofilms, biofilms were prepared as mentioned above by inoculating 50 μL of the bacterial suspension into the wells of a polystyrene microtitre plate containing 50 μL of MHB. Following incubation at 37 °C for 48 h, the medium was removed and 100 μL of each IC and resveratrol concentration was added to the biofilm in the wells. The plates were further incubated at 37 °C for 48 h. For positive control, 100 μL of culture medium was added instead of IC, whereas for negative control only the culture medium was used. After incubation, biofilm biomass was evaluated by the crystal violet (CV) staining method described by Reeser et al. (2007) with some slight alterations. The loosely attached cells were removed from each well by washing twice with distilled water, the wells were allowed to dry for 10 min and stained with 0.1% (wt/vol) CV for 30 min. After CV removal, the wells were washed twice with distilled water, and allowed to dry. The remaining CV was dissolved in 95% ethanol and the absorbance at 570 nm was determined using a microplate reader.

2.7. Quorum-sensing inhibition (QSI) assay

To detect the QSI activity of the IC and resveratrol, a standard disc diffusion assay was performed with the biosensor strain *Chromobacterium violaceum* ATCC 12472 (Borges et al., 2014). Briefly, Luria–Bertani (LB) agar plates were inoculated with an overnight culture of *C. violaceum* ATCC 12472 and sterile paper discs (6 mm in diameter) loaded with 20 μL of the IC, resveratrol, HP-γ-CD and DMSO were placed over the plates. After incubation for 24 h at 30 °C, both bacterial growth and pigment inhibition were measured as total diameter 1 (d_1) in mm, while bacterial growth inhibition was measured as diameter 2 (d_2). Therefore, QSI, assessed by pigment inhibition, was determined by subtracting the diameter of bacterial growth inhibition (d_2) from the total diameter (d_1) ($QSI = d_1 - d_2$).

3. Results

3.1. Antibacterial activity

The inhibitory effect of the IC and resveratrol against *C. jejuni*, *C. coli* and *A. butzleri* was demonstrated and the MIC values are listed in Table 1. For the IC, it was observed a difference in the MIC of the *A. butzleri* clinical isolate (MIC = 64 μg/mL) and the MIC of the animal isolate (MIC = 256 μg/mL), with the first one being almost 4 times more susceptible than the latter (Table 1). For resveratrol, this difference was not observed with a MIC value of 100 μg/mL for both strains. Similarly, for *Campylobacter* it was observed that the clinical isolate (MIC = 64 μg/mL) was more susceptible to the IC than the food isolate (MIC = 256 μg/mL) and the same trend was observed for resveratrol (Table 1). Time–kill profiles of IC and resveratrol on exponential-growing cells were also evaluated and are displayed in Fig. 1. For *C. jejuni*, after a 6 h incubation with the IC, it was observed a reduction in the initial inoculum of about 1.4 $\log_{10}$ CFU/mL for all tested concentrations, showing that the effect was independent of the concentration used and a similar effect was observed for resveratrol (Fig. 1A). Nonetheless, for *C. coli* and *A. butzleri*, the time–kill profiles obtained showed a growth inhibition dependent on the concentration used, with both *A. butzleri* strains being more susceptible to the IC and resveratrol action than *Campylobacter* spp. (Fig. 1). For *Campylobacter* strains, a bactericidal effect of the IC and resveratrol was only observed after 24 h of incubation for all tested concentrations (Fig. 1A, B), with exception to the effect of 4 × MIC of resveratrol against *C. coli* which caused a bactericidal effect after 10 h of incubation. However, after 6 h of incubation with the maximum IC concentration, a bactericidal effect on the two *A. butzleri* isolates was observed (Fig. 1C, D), revealing that the killing kinetics for *A. butzleri* was faster than for *Campylobacter* spp. In the case of resveratrol, after 6 h of incubation, a bactericidal effect was observed for all tested concentrations. Concerning the cyclodextrin and DMSO effect on bacterial growth, it was found that the highest concentrations tested did not inhibit the growth of all tested (Fig. 1).

Table 1
Minimal inhibitory concentrations (MIC) of resveratrol and inclusion complex against two *Campylobacter* spp. and two *Arcobacter butzleri* isolates.

Strains	MIC (μg/mL)	
	Inclusion complex	Resveratrol
C. jejuni 225421	256	100
C. coli 873	64	50
A. butzleri AB36/11	64	100
A. butzleri INSA776	256	100

3.2. Effect of the inclusion complex on membrane depolarization and metabolic activity

Flow cytometry was used to elucidate the mechanism of action of the IC against *Campylobacter* spp. and *A. butzleri* cells, through the use of a dual staining with SYTO® 40 and BOX or CTC. Depolarized cells allow the accumulation of BOX inside the cell and viable bacterial cells are able to reduce CTC resulting in the production of an insoluble fluorescent CTC-formazan product (Silva, Ferreira, Queiroz, & Domingues, 2011a). Overall, after 6 h of incubation with the IC, membrane depolarization was more pronounced for *A. butzleri* than for *Campylobacter* spp., with metabolic activity reduction being less marked for *C. coli* incubated with 4 × MIC of IC (Table 2). Regarding membrane depolarization, the IC had a more noticeable effect on *C. jejuni* cells, as with 1 × and 2 × MIC a depolarization of approximately 60% of *C. jejuni* cells was obtained, contrasting with only 21% of depolarized *C. coli* cells when using 2 × MIC value (Table 2). However, with 4 × MIC, membrane depolarization of the majority of the cells was observed for *C. coli* cells (86.57 ± 11.96%) in contrast with *C. jejuni* (67.87 ± 0.52%). For *A. butzleri* cells, the incubation with 1 × MIC of IC led to 74% and 55% of depolarization for *A. butzleri* AB36/11 and *A. butzleri* INSA776, respectively (Table 2). Moreover, with 2 × and 4 × MIC, it was observed an almost complete membrane depolarization for both strains, since the percentage of depolarization obtained in these assays was higher than 95% (Table 2). Concerning CTC-stained cells, after incubation with IC, the reduction on metabolic active cells was more pronounced for the *C. jejuni* than for *C. coli* and *A. butzleri*. *C. coli* CTC^+ stained cells dropped from about 89% to 69% after 6 h of incubation with 4 × MIC of IC, however the decrease in the viability of *C. jejuni* was more evident since there the percentage of CTC stained cells dropped to about 31% (Table 2). These results are in agreement with the killing kinetics observed for both *Campylobacter* strains in the time–kill assays (Fig. 1A, B). Concerning *A. butzleri* cells, metabolic activity reduction caused by the IC was similar for both strains under study, with a decrease from 95% to 36%, and from 92% to 47% for *A. butzleri* AB 36/11 and INSA776, respectively, when

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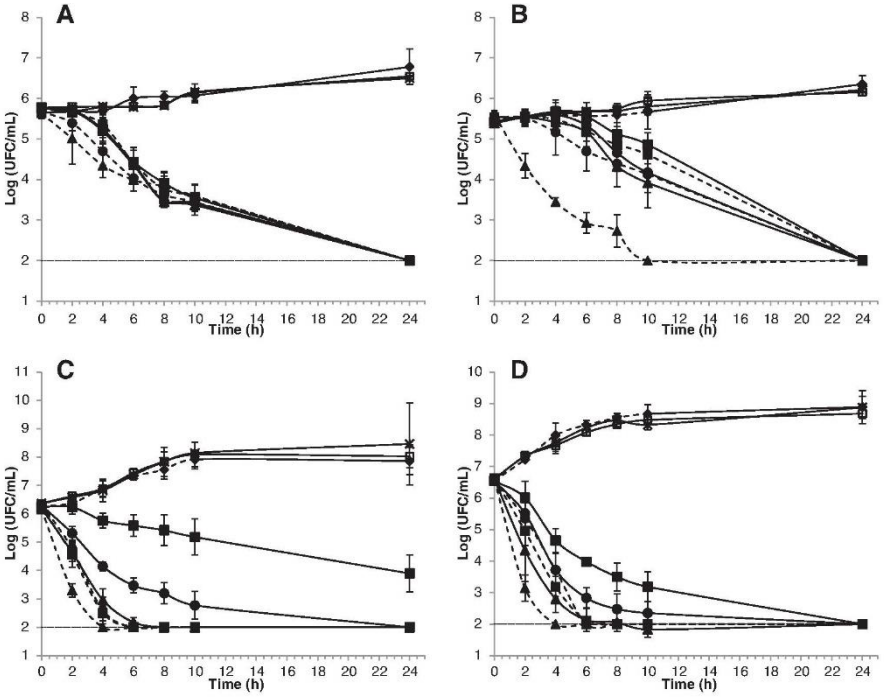

Fig. 1. Time–kill curves of the inclusion complex (full line) and resveratrol (dashed line) against *C. jejuni* 225421 (A), *C. coli* 873 (B), *A. butzleri* AB36/11 (C) and *A. butzleri* INSA 776 (D). Growth control (□); HP-γ-CD control (*); DMSO control (♦); 1× (■); 2× (●) and 4× (▲) the MIC, over a 24 h period.

incubated with the IC at 4 × MIC. When used alone, HP-γ-CD did not cause a significant change in both parameters assessed by flow cytometry, as can be seen by the identical percentages of stained cells for both growth (culture medium) and HP-γ-CD controls (Table 2).

3.3. Effect of the inclusion complex and resveratrol on biofilm formation and dispersion

Concerning the effect of the IC on biofilm formation (Fig. 2) by the four strains, when using a concentration of 4 × MIC (Fig. 2A) an inhibition ranging from 62% to 78% on the total biofilm biomass was achieved. For resveratrol, with the maximum concentration tested (2 × MIC) the biofilm inhibition ranged between 63% and 94% (Fig. 2B). Overall, when comparing concentrations of 1 × and 2 × MIC, resveratrol showed a higher inhibitory effect than the IC, showing similar effects for subinhibitory concentrations. Regarding the effect against established biofilms (Fig. 3), the highest IC concentration resulted in a 60% decrease in biofilm biomass for both *Campylobacter* strains; and by 75% and 80% for *A. butzleri* AB36/11 and INSA776, respectively (Fig. 3A). Concerning the effect of resveratrol on pre-established biofilms, concentrations equal to 2 × MIC caused a reduction on biofilm formation of about 80% for both *Campylobacter* strains and *A. butzleri* AB36/11; and a reduction of 64% for the *A. butzleri* INSA 776 (Fig. 3B). For all strains, even at subinhibitory IC and resveratrol concentrations, inhibited biofilm formation as well as diminished established biofilm.

3.4. Anti-QS activity of the inclusion complex

A disc diffusion assay using the biosensor strain *C. violaceum* ATCC 12472 was performed for the QS inhibition (QSI) screening. The IC and resveratrol produced halos around the discs which indicated *C. violaceum* growth inhibition with concomitant loss of violacein pigment (Table 3) with this pigment loss being indicative that the IC possessed QSI activity. Concerning the cyclodextrin and DMSO control, no halos were observed around the disc indicating that the antibacterial and anti-QS effect of the IC are due to resveratrol.

Table 2
Percentage of fluorochrome-stained cells assessed by flow cytometry of *Campylobacter* spp. and *A. butzleri* isolates treated with the inclusion complex (mean values ± SD; n = 2).

	BOX^+ stained cells (%)				CTC^+ stained cells (%)			
	C. jejuni 225421	*C. coli* 873	*A. butzleri* AB36/11	*A. butzleri* INSA776	*C. jejuni* 225421	*C. coli* 873	*A. butzleri* AB36/11	*A. butzleri* INSA776
Growth control	7.69 ± 0.81	4.79 ± 0.06	15.01 ± 1.88	14.49 ± 1.15	93.06 ± 1.95	89.26 ± 4.33	95.35 ± 4.65	92.35 ± 2.70
HP-γ-CD control	7.00 ± 0.59	3.50 ± 0.33	14.33 ± 3.1	11.59 ± 0.18	90.90 ± 3.85	92.73 ± 3.32	86.02 ± 0.08	91.12 ± 4.16
1 × MIC	32.68 ± 1.92	14.81 ± 0.47	74.05 ± 1.48	54.98 ± 0.14	66.42 ± 0.82	78.03 ± 4.91	85.60 ± 3.73	86.92 ± 1.44
2 × MIC	60.37 ± 5.76	21.32 ± 1.13	93.48 ± 1.02	96.50 ± 2.63	61.22 ± 1.61	71.54 ± 10.49	73.56 ± 0.28	79.77 ± 3.24
4 × MIC	67.87 ± 0.52	86.57 ± 11.96	96.57 ± 0.92	98.57 ± 1.80	31.27 ± 2.82	68.77 ± 8.27	36.69 ± 4.46	46.62 ± 2.88

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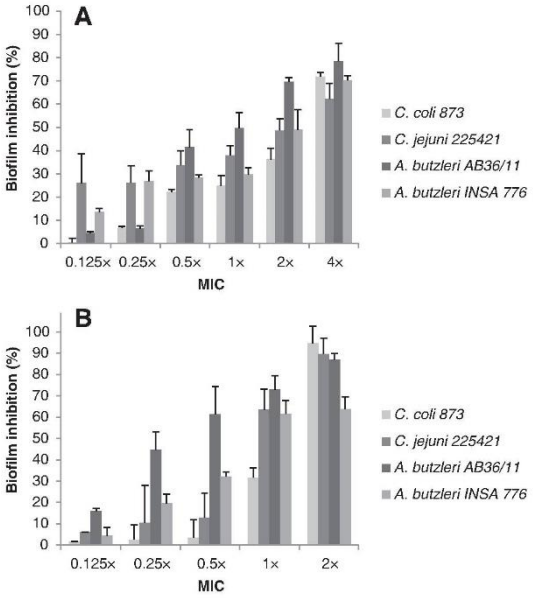

Fig. 2. Effect of different inclusion complex (A) and resveratrol (B) concentrations on biofilm formation by *Campylobacter* spp. and *Arcobacter butzleri*. Biofilm formation was evaluated by measuring total biomass and the results are expressed as percentage of biofilm inhibition.

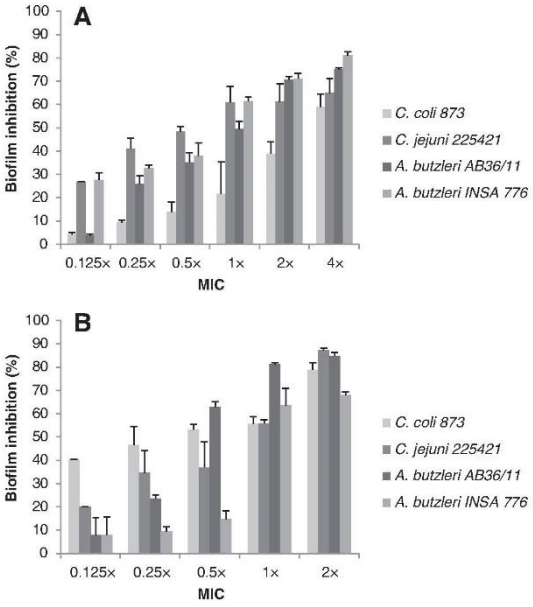

Fig. 3. Effect of inclusion complex (A) and resveratrol (B) concentrations in the elimination of pre-established biofilms of *Campylobacter* spp. and *Arcobacter butzleri*. Biofilm formation was evaluated by measuring total biomass and the results are expressed as percentage of biofilm inhibition.

4. Discussion

C. jejuni and *C. coli* are well-known foodborne pathogens and are considered the major cause of bacterial gastroenteritis in humans (Silva et al., 2011b) while *Arcobacter* species are emerging human pathogens, with *A. butzleri* being the most prevalent of this genus (Ferreira et al., 2014a). The consumption of contaminated food or water has been described as the most probable route of these microorganism transmissions to humans and/or animals (Collado & Figueras, 2011; Silva et al., 2011b), therefore there is a need to develop strategies to control these microorganisms. Thus, in this study it has been determined the antimicrobial activity of IC formed between HP-γ-CD and resveratrol on *C. jejuni*, *C. coli* and *A. butzleri*. When evaluating *Campylobacter* and *Arcobacter* susceptibility to the IC, it was observed an inhibitory effect of the IC with MIC values ranging from 64 to 256 μg/mL, whereas the MIC values concerning resveratrol ranged from 50 to 100 μg/mL. Additionally, the MIC values for IC and resveratrol were very similar in the case of *C. coli* 873 strain, while for the other strains the MIC values for the IC were about two times higher than the ones obtained for resveratrol. Previous studies reported that the antimicrobial activity of an IC is dependent of various factors, namely, the value of the apparent stability constants and complex stoichiometry and geometry (Leclercq, Nardello-Rataj, Rauwel, & Aubry, 2010). The reduction of the antimicrobial activity of a compound by its inclusion in a cyclodextrin has been previously reported, with this reduction being positively correlated with the apparent stability constant of the IC formed (Pinho, Soares, & Henriques, 2014b; Silva, Nerín, & Domingues, 2015).

Based on the results of time–kill curves, it was possible to conclude that the IC and resveratrol display a bacteriostatic or bactericidal effect, with this effect being time- and concentration-dependent and with a faster killing kinetics in the case of resveratrol in comparison to the IC. A bactericidal effect was observed with 4 × MIC of the IC for the two strains of *A. butzleri* after 6 h of incubation, with a bacteriostatic effect being observed for incubation of AB36/11 isolate with 1 × MIC of IC. Concerning *Campylobacter* strains, a bactericidal effect was observed with all the tested concentrations of IC after 24 h of incubation with *C. jejuni* being more susceptible than *C. coli*. These distinct susceptibility profiles have also been described for antibiotics in several studies, demonstrating a higher rate of resistance among *C. coli* strains than *C. jejuni* (Duarte et al., 2014; Pezzotti et al., 2003). The bactericidal effect of stilbene–cyclodextrin inclusion complexes, has been described, being the effect also observed after 24 h of incubation (Silva et al., 2015). The encapsulation of active compounds is one of the several approaches to achieve a more controlled diffusion from a packaging material and a prolonged duration (Lacoste, Schaich, Zumbrunnen, & Yam, 2005). So, the slower antibacterial effect of ICs when compared with the pure compound could mean a more controlled release and a more prolonged effect of the active compound when incorporated into a food package, making it a good candidate to be incorporated in controlled-release packaging systems.

The possible mechanism of action of the IC against *C. jejuni*, *C. coli* and *A. butzleri* cells was studied through flow cytometry assays, using SYTO® 40, BOX and CTC fluorochromes. According to the results, it is possible to verify, for all the studied strains, an increase of the percentage of BOX-positive cells along with the raise of IC concentration used. When applied the MIC concentration, the depolarization of the cell membrane is higher for the *A. butzleri* AB36/11 strain, however, the effect of this IC concentration in culturable cells was more noticeable on *A. butzleri* INSA776 cells (Fig. 1). Relatively to *Campylobacter* spp. the membrane depolarization is higher in *C. jejuni* cells when using the MIC concentration, nonetheless in the time–kill curves the reduction of culturable cells is not so evident and was similar with all the concentrations tested (Fig. 1). The cell membrane depolarization is a transition state which occurs before membrane permeabilization, and may be caused by several factors or antimicrobial agents, with the cells in this state having the capacity to regain culturability (Díaz, Herrero, García,

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Table 3
Anti-QS activity of hydroxypropyl-γ-cyclodextrin (HP-γ-CD), dimethyl sulfoxide (DMSO), inclusion complex and resveratrol by disc diffusion method using *C. violaceum* ATCC 12472 as indicator strain.

	Inhibition zones against *C. violaceum* ATCC 12472 (diameter in mm)		
	Growth + pigment inhibition (d_1)	Growth inhibition (d_2)	QS inhibition (d_1–d_2)
HP-γ-CD	ND	ND	ND
DMSO	ND	ND	ND
Inclusion complex	21.50 ± 0.71	9.50 ± 0.71	12.00 ± 0.00
Resveratrol	19.50 ± 0.71	11.00 ± 0.00	8.50 ± 0.71

Values of diameters are mean ± SD of three independent replicates. ND, inhibition not detected.

& Quirós, 2010). This ability may sustain the lack of direct correlation between the percentage of depolarized cells and the time–kill curves results. Considering that resveratrol was formerly described as having effect in *Arcobacter* metabolic activity (Ferreira et al., 2014b), this effect was also evaluated for the IC. From Table 2, it is suggested that the decrease in metabolic activity of the cells depends on the increase of concentrations of IC used. For *C. jejuni* the incubation with the IC led to a significant decrease in the CTC-positive cells with all the tested concentrations, however for *C. coli* no significant decrease of the CTC-positive cells was observed. In contrast, for *A. butzleri* AB36/11, the addition of the IC led to a significant reduction of CTC-positive cells when incubated with 2× and 4× MIC and for *A. butzleri* INSA776, this reduction was only significant with 4× MIC. Ferreira et al. (2014b) had previously obtained a significant reduction in *A. butzleri* metabolic activity by exposure to resveratrol, however with no correlation with the compound concentration. This trend was not observed here, which can be due to the encapsulation of resveratrol and the exposure of the cells to the active groups of the compound.

The ability of microorganisms to form biofilms is associated with the bacterial virulence, the ability to colonize and survive in the environment and antibiotic resistance (Candon, Allan, Fraley, & Gaynor, 2007); thus making it extremely relevant to test the IC effectiveness on the formation and dispersion of biofilms. The results obtained show an inhibition of the biofilm formation for both strains of *A. butzleri* and both species of *Campylobacter* in the presence of the IC, with this inhibition being influenced by the IC's concentration (Fig. 2A). Regarding biofilm dispersion, a destruction and/or disaggregation of the preformed biofilm was observed for all the tested strains (Fig. 3A), being these effects dependent of IC concentration, similarly to what was already observed for the biofilm formation inhibition assays. It should be highlighted that the IC was able to inhibit biofilm formation as well as to reduce biofilm previously established even at concentrations lower than the MIC value. Resveratrol was also able to inhibit biofilm formation and to reduce pre-formed biofilms (Figs. 2B; 3B); however, due to its low aqueous solubility, it was only possible to test concentrations up to 2× MIC values. The anti-biofilm effect of resveratrol was higher than the one obtained with the IC, since a lower resveratrol concentration (2× MIC) yielded a similar effect to the highest IC concentration used (4× MIC). This lower IC efficacy could be due to the lower dissociation rate of the resveratrol from the complex, resulting in a lower availability of free active compound and consequently a diminished anti-biofilm effect. In sum, this study demonstrates for the first time that resveratrol-HP-γ-CD complex exhibits anti-biofilm activity against *Campylobacter* spp. and *A. butzleri* in a dose-dependent manner. Nonetheless, the anti-biofilm activity of resveratrol against several pathogens has already been described, being suggested that this activity may be related to the regulation of biofilm-related genes such as adhesion, motility or even QS genes (Augustine, Goel, Sivakumar, Kumar, & Thomas, 2014; Lee, Kim, Ryu, Cho, & Lee, 2014a; Lee, Lee, Ryu, Cho, & Lee, 2014b). So, the anti-biofilm activity of the IC could be explained by the effect of the active compound, resveratrol, in genes associated with biofilms formation, and the effect on the pre-establish biofilm could be mainly due to the effect on QS, since this process has been documented to play a role in the formation and maintenance of *Campylobacter* biofilms (Gölz, Sharbati, Backert, & Alter, 2012; Reeser et al., 2007).

Strategies for combating bacterial biofilms and QS mechanism through the use of natural compounds can improve the safety and security of foods, also improving the human health (Nazzaro, Fratianni, & Coppola, 2013; Zhang et al., 2014). Some data also suggest that targeting QS could be a new strategy for fighting the virulence factors and biofilms of the foodborne pathogen *C. jejuni* (Castillo, Heredia, Arechiga-Carvajal, & García, 2014), which could mean a significant improvement in food safety. Notwithstanding that the anti-QS activity of resveratrol and other stilbenes had been already described against some foodborne pathogens (Lee et al., 2014b; Wang et al., 2006), the results of our study showed the potential of the IC as QSI, since an inhibition of violacein production by the biosensor strain *C. violaceum* was observed. The mechanism of QSI could be achieved by several factors, and for resveratrol it has been suggested that its anti-QS activity is due to its capacity to mimic the QS signals and disrupts the bacterial QS system (Wang et al., 2006).

Nowadays, the development of controlled-release packaging is emerging, since a controlled release allows a prolonged duration of active compound delivery, but also to the predictability and reproducibility of release rates, thus surpassing some of the hurdles nowadays faced in the development of new and effective food packaging systems (Martínez-Abad, Lagarón, & Ocio, 2014). In this sense, the IC slower antibacterial activity could indicate a slower release of the compound from the CD cavity, thus encouraging the application of this IC within the food industry, namely in the development of controlled release packaging to control the foodborne pathogens *Campylobacter* and *Arcobacter*.

5. Conclusions

In conclusion, the present study demonstrated for the first time the antibacterial, anti-biofilm and anti-QS potentials of the IC between resveratrol and HP-γ-CD. Besides improving resveratrol's solubility, the IC showed anti-*Campylobacter* and anti-*Arcobacter* activity, inhibiting in vitro biofilm formation and promoting biofilm dispersion even at sub-MIC concentrations. The IC also inhibited QS-dependent violacein production in *C. violaceum*. Our results strongly suggest that this IC could be considered by the food and packaging industries as a new anti-biofilm agent and/or QS inhibitor to enhance shelf life and increase food safety, meeting consumer expectations to have pathogen-free food without the use of chemical additives or preservatives.

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Chapter 7 - Paper V

Antioxidant properties of Coriander essential oil and Linalool and their potential to control *Campylobacter* spp.

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Chapter overview:

In Chapters 5 and 6 we have shown that resveratrol is a natural compound with antibacterial and anti-biofilm activity against C*ampylobacter*. Now, taking into account the antibacterial activity of coriander essential oil (EO) against several pathogens previously demonstrated by our research group, we decided to study the potential of this EO and its major compound linalool also in controlling *Campylobacter* species.

In this study, we started with assessing the potential of coriander EO and linalool to inhibit the growth of *C. coli* and *C. jejuni*, with the results showing a bactericidal action. Then the effect against *Campylobacter* biofilms was assessed, and we showed that even at sub-inhibitory concentrations both compounds were able to inhibit the biofilm formation as well as inhibiting the growth of already established biofilms. Since QS has been described to be related with biofilm formation and food spoilage, we also evaluated the potential of these compounds in inhibiting the QS mechanism. Coriander EO and linalool had the ability to reduce the violacein production of the biosensor strain used, which means that these compounds interfere with the QS system. So, we showed that coriander EO and linalool have the ability to inhibit planktonic cells and biofilms of *Campylobacter*. Since *Campylobacter* is frequently found in food products, being its consumption or handling the major source of human infection, coriander EO and linalool could be used in the food industry. Then, we evaluated the antioxidant potential of these compounds and we observed that both had an exceptional ability to inhibit lipid peroxidation.

In conclusion, our results suggest that coriander EO and linalool could be used in the food industry to control the foodborne pathogen *Campylobacter*, while enhancing the shelf life of food products without the use of chemical additives or preservatives.

Antioxidant properties of Coriander essential oil and Linalool and their potential to control *Campylobacter* spp.

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Abstract

Foodborne diseases remain common around the world with Campylobacteriosis being the most commonly reported zoonosis in the European Union in 2013. *Campylobacter jejuni* and *C. coli* are the main species associated with human illness. Furthermore, *Campylobacter* can develop biofilms which is becoming a major problem within the food industry. In addition to foodborne pathogens, oxidation is a non-microbial cause of deterioration of food causing loss of quality and safety. Thus, there is an urgent need in the food industry for new and effective strategies that can help prevent food contamination, spoilage and consequently, foodborne illnesses. Essential oils are known for their antimicrobial and antioxidant properties and are already widely used in the food industry. So, the aim of this work was to study the antimicrobial and anti-biofilm activity of coriander essential oil and its major compound linalool against *C. jejuni* and *C. coli* strains, as well as their effect in the quorum sensing (QS) system and their potential as antioxidants. Our results, demonstrated that both compounds have anti-*Campylobacter* activity, inhibited *in vitro* biofilm formation and promoted biofilm dispersion even at sub-MIC concentrations and interfered with the QS system through the inhibition of violacein production. Moreover, the essential oil and linalool were shown to have radical scavenging properties and lipid peroxidation inhibition ability which could make them potential alternatives to synthetic antioxidants. In sum, our results demonstrated the antibacterial, anti-biofilm, anti-QS and antioxidant potentials of the coriander essential oil and its major compound, linalool, suggesting that they could be used in the food industry to enhance shelf life of food products and increase food safety without requiring chemical additives or preservatives.

Keywords: Coriander essential oil; Linalool; anti-*Campylobacter*; anti-biofilm; Quorum-sensing; antioxidant.

1. Introduction

Despite advances in food safety, foodborne diseases remain common throughout the world and a growing threat to global public health. The ingestion and/or contact with contaminated food are the major cause of foodborne illness (CDC, 2015; EFSA & ECDC, 2015). *Salmonella*, *E. coli* and *Campylobacter* are the three major pathogens that cause foodborne illnesses in United States (CDC, 2015), while Campylobacteriosis was the most commonly reported zoonosis in the European Union in 2013 (EFSA & ECDC, 2015). Campylobacteriosis is usually associated to the consumption of undercooked meat, environmental contamination, and cross-contamination between raw and cooked food (Hardy, Lackey, Cannon, Price, & Silbergeld, 2011; Silva et al., 2011c). Poultry is considered to be the main source of food-related human campylobacteriosis (Corry & Atabay, 2001; EFSA & ECDC, 2015). The most common species of *Campylobacte*r associated with human illness are *C. jejuni* and *C. coli* (Fitzgerald, 2015). In addition, it has also been described that *Campylobacter* could develop biofilms that are more resistant to disinfectants and thus becoming a major problem within the food industry (Gunther & Chen, 2009; Srey, Jahid, & Ha, 2013). Quorum sensing (QS) communication is associated to the bacterial biofilm formation and antibiotic resistance, as well as to the bacterial proliferation in foods and food spoilage, therefore, QS inhibition could be a good strategy to control *Campylobacter* and to ensure food safety (Alvarez, Moreira, & Ponce, 2012; Gölz, Sharbati, Backert, & Alter, 2012; Nazzaro, Fratianni, & Coppola, 2013). It is thus extremely important to search for new and effective measures to reduce *Campylobacter* proliferation in food and thus reduce the incidence of *Campylobacter* infection in humans.

In addition to the foodborne pathogens, oxidation is a well-known non-microbial cause of deterioration of food and loss of quality and safety (Falowo, Fayemi, & Muchenje,

2014; Sanches-Silva et al., 2014). Oxidative stress occurs due to the irregular generation of free radicals reactive oxygen species which triggers the oxidative stress and damage to macromolecules such as the lipid and protein fractions of food products (Falowo et al., 2014). Since antioxidants are compounds that interact with free radicals and prevent the oxidation of other molecules, finding compounds that have both antimicrobial and antioxidant activities has a great potential for the application in food systems (Sanches-Silva et al., 2014). There has been a growing interest in the use of natural compounds for application in food products, mainly due to the preference of consumers for natural ingredients and the concerns about the toxic effects of synthetic compounds (Falowo et al., 2014).

Essential oils are known for their antimicrobial and antioxidant properties and their use in the food industry has been widely described (Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Calo, Crandall, O'Bryan, & Ricke, 2015; Falowo et al., 2014; Jayasena & Jo, 2013; Sanches-Silva et al., 2014; Silva & Domingues, 2015). In short, essential oils are very complex natural mixtures and are characterized by two or three major components at fairly high concentrations (20–70%), such as linalool (≈68%) that is the major component of the coriander essential oil (Bakkali et al., 2008; Silva, Ferreira, Duarte, Mendonça, & Domingues, 2011a). The antioxidant effects of coriander oil have been described, suggesting that this oil could be considered as a source of natural antioxidants and used as a potential substitute for synthetic antioxidants in the food industry (Laribi, Kouki, M'Hamdi, & Bettaieb, 2015; Samojlik, Lakić, Mimica-Dukić, Daković-Švajcer, & Božin, 2010; Silva & Domingues, 2015; Singh, Kapoor, Pandey, Singh, & Singh, 2002). The bactericidal effect and the anti-biofilm potential of coriander essential oil against several pathogens has also been described (Duarte, Ferreira, Oliveira, & Domingues, 2013; Lo Cantore, Iacobellis, De Marco, Capasso, &

Senatore, 2004; Silva & Domingues, 2015; Silva, Ferreira, Queiroz, & Domingues, 2011b). In addition, the potential of coriander oil to control *C. jejuni* in raw meat has already been described (Rattanachaikunsopon & Phumkhachorn, 2010). Nevertheless, the effect against *C. coli* has not yet been described, as well as the potential of this essential oil to prevent or eliminate biofilms formed by these foodborne pathogens. So, the aim of this work was to study the antimicrobial and anti-biofilm activity of coriander essential oil and its major compound linalool against *C. jejuni* and *C. coli* strains. Finally, the role of coriander oil and linalool in the QS system was also studied, as well as its potential as an antioxidant.

2. Materials and Methods

2.1. Antibacterial agents and Bacterial strains

Coriander essential oil and its major compound linalool (obtained from Sigma – Aldrich St. Louis, MO) were tested against two reference strains (*Campylobacter jejuni* ATCC 33560 and *Campylobacter coli* ATCC 33559) and two *Campylobacter* isolates (*C. jejuni* 225421 and *C. coli* 873) (Duarte et al., 2014). The strains were stored in Brain Heart Infusion broth with 20% glycerol at −80 °C and prior to susceptibility testing each strain was inoculated on Brucella agar plates (Oxoid, England) supplemented with 5% defibrinated horse blood to ensure optimal growth. The chemical composition of commercial coriander essential oil is reported in the literature, being linalool (64.38%), geranyl acetate (5.82%), camphor (4.88%), p-cymene (4.54%) and α-pinene (4.04%) the major compounds identified (Silva, et al., 2011a).

2.2. Antibacterial activity

121 **2.2.1. Disc diffusion test**

122 The susceptibility of the *Campylobacter* strains to the antimicrobials was evaluated by
123 the disc diffusion test, according to the standard M2-A8 from Clinical Laboratory
124 Standards Institute (CLSI, 2003). The pure compounds (10 µL/disc) and a 50% (v/v)
125 solution of the compound in dimethyl sulfoxide (DMSO) (10 µL/disc) were added to the
126 discs (6mm of diameter). DMSO was used as control. The inoculum was prepared in a
127 sterile solution of 0.85% sodium chloride and the optical density of the suspension was
128 adjusted to 0.5 McFarland (≈ 1×10^8 colony forming units (CFU)/mL). Then the
129 Müeller-Hinton Agar (MHA, LiofilChem, Italy) plates were inoculated, allowed to dry
130 and the discs previously prepared were placed over the agar. The plates were incubated
131 at 37 ºC for 48 h under microaerobic conditions and after incubation the diameter of the
132 inhibition zone was measured. This assay was performed in triplicate.

133 **2.2.2. Vapour-phase antimicrobial activity**

134 The effect of the vapour of coriander essential oil and linalool against *Campylobacter*
135 was evaluated as described by Fisher and Phillips (2006). Briefly, MHA plates
136 inoculated as previously described were exposed to vapours by placing one disc
137 impregnated with 10 µL of the antibacterial compounds onto the lid of the Petri dish
138 (approximately 8 mm from the bacteria). Then, the plates were incubated at 37 ºC for 48
139 h under microaerobic conditions and the zones of inhibition were measured (diameter in
140 mm). This assay was performed in triplicate.

141 **2.2.3. Determination of minimum inhibitory concentration (MIC) and**
142 **Minimum Bactericidal Concentration (MBC)**

143 The minimum inhibitory concentration (MIC) and minimum bactericidal concentration
144 (MBC) for coriander essential oil and linalool were determined by the microdilution
145 method as described by Duarte et al. (2015b). Briefly, serial two-fold dilutions of
146 coriander oil and linalool (from 32 to 0.25 µL/mL) were prepared in 96-well plates in
147 Müeller Hinton broth (MHB) and a maximum of 2% of DMSO was used to increase the
148 solubility. Bacterial suspensions were prepared with a turbidity of 0.5 McFarland,
149 diluted in MHB and added to each well to yield a final concentration of 5×10^5 CFU/mL
150 per well. DMSO and culture medium were used as growth controls. The plates were
151 incubated at 37 °C for 48 h under microaerobic conditions and after the incubation the
152 growth was visually assessed. The MIC was defined as the lowest concentration of
153 compound without visible growth. From the wells without visible growth, 10 µL were
154 plated and after incubation the number of colonies was counted. The MBC was defined
155 as the lowest compound concentration which caused the death of 99.9% of the bacterial
156 inoculum. At least three independent assays were performed.

157 **2.3. Anti-biofilm activity**

158 The effect of coriander essential oil and linalool on the ability of *Campylobacter* to
159 form biofilms was determined following the protocol described by Duarte, Alves,
160 Ferreira, Silva and Domingues (2015a). Briefly, the bacterial suspensions were prepared
161 from overnight cultures in MHB at 37 °C under microaerobic conditions and the
162 turbidity was adjusted to an optical density at 620nm (OD_{620nm}) of 0.1. Serial two-fold
163 dilutions of the compounds were prepared in culture medium (MHB) in a 96-well plate
164 (50 µL per well) with final concentrations of 0.5×, 1×, 2× and 4× MIC value for each
165 strain. Then, 50 µL of the bacterial suspensions were added to each well. The wells
166 without compound were used as biofilm growth control and the wells with medium
167 culture (MHB) were considered as negative control. The plates were incubated for 48 h

168 at 37 °C under microaerobic conditions. Then, the biofilm biomass was evaluated by the
169 crystal violet (CV) assay, as described below. The effect of the compounds on
170 preformed biofilms of *Campylobacter* was also evaluated. *Campylobacter* biofilms
171 were prepared as mentioned above by inoculating 50 µL of the bacterial suspension into
172 the wells of a 96-well plate containing 50 µL of MHB and incubated at 37 °C for 48 h
173 under microaerobic conditions. Following incubation, the medium was removed and
174 100 µL of each compound was added to the biofilm in the wells in order to obtain a
175 final concentration ranging from 0.5× to 4× MIC value. For positive and negative
176 controls, 100 µL of culture medium was added. The plates were incubated at 37 °C for
177 48 h. After incubation, biofilm biomass was evaluated by the CV assay. The content of
178 the wells was removed, washed twice with distilled water and allowed to dry for 10
179 min. Then the remaining biofilm in each well was stained with 0.1% (wt/v) CV for 30
180 min at room temperature. After CV removal, the wells were washed twice with distilled
181 water, and allowed to dry. The remaining CV was dissolved in 95% ethanol and the
182 absorbance at 570 nm was determined using a microplate reader. The assay was
183 repeated at least three times.

184 **2.4. Anti-QS activity**

185 The biosensor strain *Chromobacterium violaceum* ATCC 12472 was used to determine
186 the anti-QS effect of coriander essential oil and linalool. The bacteria suspension of *C.*
187 *violaceum* was grown aerobically in Luria-Bertani (LB) broth at 30 °C and 250 rpm
188 overnight.

189 **2.4.1. Disc diffusion assay**

190 The disc diffusion assay was used to study the anti-QS potential of coriander oil and
191 linalool by using the biosensor strain *Chromobacterium violaceum* ATCC 12472, as

described by Borges et al. (2014). In summary, *C. violaceum* inoculum was adjusted to an OD_{620nm} of 1 and the LB agar plates were inoculated. Sterile discs with 10 µL of the pure compounds and 10 µL of a 50% (v/v) solution of the compounds in DMSO were placed over the plates and incubated at 30 ºC for 24 h. Discs with DMSO were used as control and the assay was realized in triplicate. After incubation, the zone of growth inhibition and pigment inhibition were evaluated and the QS inhibition was defined as the inhibition of violacein production around the discs.

2.4.2. Quantification of pigment production

The QS inhibition caused by the coriander essential oil and linalool was also quantified by a broth assay. The violacein production by *C. violaceum* when exposed to the antibacterial agents was quantified according to Borges et al. (2014). The inoculum was adjusted to an OD_{620nm} of 0.1 and different concentrations of coriander oil and linalool were added to the inoculum. After 24 h of incubation at 30 °C and 150 rpm the violacein extraction was performed. Briefly, 1 mL of culture from each sample was centrifuged at 14549 g for 10 min. Then, the supernatant was discarded, and the pellet was solubilized in 1 mL of DMSO, vortexed for 30 s to solubilize the violacein and centrifuged at 14549 g for 10 min. Finally, 200 µL of the supernantant containing the violacein were transferred to a 96-well microtiter plate to measure the OD at 585 nm. The assay was performed in three independent experiments. The results were expressed as percentage of violacein inhibition.

2.5. Evaluation of the antioxidant activity

2.5.1. DPPH scavenging assay

The antioxidant activity of coriander essential oil, linalool and standards (gallic acid and quercetin) was determined by the radical scavenging activity method using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical as previously described by Luís et al. (2014) with few modifications. Briefly, 0.1 mL of methanolic solutions of the coriander oil, linalool or standards at different concentrations were added to 3.9 mL of a DPPH methanolic solution. Three DPPH methanolic solutions (0.2000, 0.1242 and 0.0800 mM) were tested due to the linearity range of DPPH solutions. A solution of methanol (0.1 mL) mixed with 3.9 mL of DPPH was used as control sample. After 90 min of incubation at room temperature in the dark, the absorbance was measured at 517 nm. The radical scavenging activity was calculated as follows: $I\ \% = [(Abs_0 - Abs_1)/Abs_0] \times 100$, where Abs_0 was the absorbance of the control and Abs_1 was the absorbance of the sample. The IC_{50} (concentration providing 50% of inhibition) was calculated graphically by using a calibration curve in the linear range of compound concentration *vs.* scavenging effect. The Antioxidant Activity Index (AAI) was used to express the antioxidant activity and was calculated as follows: AAI = final concentration of DPPH in the control sample / IC_{50}. The AAI allowed the following classification: poor ($AAI \leq 0.5$), moderate ($0.5 < AAI \leq 1.0$), strong ($1.0 < AAI < 2.0$) or very strong antioxidant activity ($AAI \geq 2.0$) (Luís et al., 2014; Scherer & Godoy, 2009). All assays were carried out in duplicate.

2.5.2. β-Carotene bleaching test

A solution of β-carotene (20 mg/mL in chloroform) was prepared and 20 µL were added to 40 µL of linoleic acid, 400 mg of Tween 40 and 1 mL of chloroform. The mixture was then evaporated at 45 ºC for 5 min in a rotary vacuum evaporator to remove chloroform and immediately diluted with 100 mL of oxygenated distilled water and vigorously agitated to form an emulsion. Then, 5 mL of the emulsion was transferred to

239 test tubes containing 300 µL of the compounds in methanol at different concentrations.
240 About 5 mL of the emulsion and 300 µL of methanol were used as control and butylated
241 hydroxytoluene (BHT) in methanol, at the same concentration as samples, was used as
242 standard. The tubes were then gently vortexed and placed in a water bath at 50 ºC for 2
243 h. The absorbances of the samples, standard and control were measured at 470 nm,
244 using a spectrophotometer, against a blank (an emulsion without β-carotene). The
245 measurements were performed at initial time (t=0 h) and at final time (t=2 h) of
246 incubation. The antioxidant activity was measured as percentage of inhibition of β-
247 carotene's oxidation: $I\ \% = (Abs^{t=2}_{sample} - Abs^{t=2}_{control})/(Abs^{t=0}_{control} - Abs^{t=2}_{control})$ (Luís
248 et al., 2014). The experiments were performed in triplicate.

249 **2.6. Statistical analysis**

250 Data was analyzed by one-way ANOVA test and differences between groups were
251 compared by Tukey's *post hoc* tests. A p value of <0.05 indicates statistically
252 significant differences.

253

254 **3. Results and Discussion**

255 **3.1. Antibacterial activity**

256 The antibacterial activity of coriander essential oil and its major compound, linalool,
257 against *C. jejuni* and *C. coli* was assessed by the disc diffusion and vapour-phase
258 methods and the results are shown in Table 1. For both compounds, when tested at the
259 pure state were able to inhibit the microbial growth of the strains all over the plate
260 (diameter of inhibition >85 mm) by the diffusion in the agar medium. So, in order to
261 compare the activity against the several strains, coriander oil and linalool were diluted
262 with DMSO to a final concentration of 50%. Concerning coriander oil, it was possible

263 to observe a similar effect against both *C. jejuni* strains, while for the *C. coli* strains a
264 higher diameter of inhibition was observed for the *C. coli* 873 ($p<0.05$) (Table 1). The
265 effect of linalool at 50% of concentration was similar to the effect observed with
266 coriander oil, however, a significant higher inhibition diameter was observed for the *C.*
267 *coli 873* ($p<0.05$) one more time (Table 1). In sum, the *C. coli 873*, a strain isolated
268 from a faecal sample of a patient with acute gastroenteritis and classified as multidrug
269 resistant (Duarte et al., 2014), was shown to be more susceptible to the coriander oil and
270 linalool than the three other tested strains. The activity of coriander oil against *C. jejuni*
271 strains had already been previously described with inhibition zones ranging from 23 to
272 27 mm of diameter (Rattanachaikunsopon & Phumkhachorn, 2010), results that are
273 dissimilar from the ones obtained in this study. The antibacterial effect of linalool
274 against *C. jejuni* strains was also described, with a total inhibition of growth in the
275 tested plate (diameter >90 mm) (Fisher & Phillips, 2006), which in this case was similar
276 to our results. The differences between the activity of coriander oil from our study to
277 those conducted by others could probably be due to a different composition profile
278 (Nejad Ebrahimi, Hadian, & Ranjbar, 2010). The chemical profile of an essential oil
279 differs according to several factors such as the type of extraction and the climate, soil
280 composition, plant organ, age and vegetative cycle stage of the plant (Bakkali et al.,
281 2008; Nejad Ebrahimi et al., 2010). Essential oils are composed by volatile and non-
282 volatile components, so it is expected that the inhibition zone obtained by the agar
283 diffusion method is a result of the action of both kinds of components (Fisher &
284 Phillips, 2006). Therefore the contribution of the volatile compounds to the overall
285 antibacterial activity of the essential oil was also evaluated by the vapour-phase assay.
286 The inhibition zones obtained by this method are shown in Table 1, and both coriander
287 oil and linalool inhibited the growth of all *Campylobacter* strains through the vapour-

phase activity. The inhibition zones obtained by the vapour diffusion assay were equal to the ones obtained by the agar diffusion method, indicating that the volatile components of the coriander essential oil have an essential role in its antibacterial activity and that linalool, as a pure compound, has the ability to exert its antibacterial activity both by diffusion in agar and through its volatility. In contrast to our results, Fisher and Phillips (2006) found that linalool had a smaller inhibition zone against *C. jejuni* by the vapours when compared to the agar diffusion activity. Thus, it could indicate that the differences in the antibacterial effect obtained by both agar diffusion and vapour-phase methods did not depend only on the compound tested, but also on the bacterial strains (Fisher & Phillips, 2006; Iturriaga, Olabarrieta, & de Marañón, 2012). The antibacterial activity of coriander oil and linalool was also assessed by the broth microdilution method and the MICs obtained are shown in Table 2. The MIC values obtained for the linalool were equal to the ones obtained for the coriander oil and ranged between 0.5 and 1 µL/mL (Table 2). It was also observed that DMSO did not affect the growth of *Campylobacter* in all antibacterial assays (data not shown). All tested strains presented a MIC value of 0.5 µL/mL for both coriander oil and linalool, with the exception of *C. jejuni* 224421 that was resistant with a MIC of 1 µL/mL. Rattanachaikunsopon and Phumkhachorn (2010) studied the effect of coriander essential oil against several strains of *C. jejuni* having obtained MIC values ranging from 0.03% to 0.06% (0.3 to 0.6 µL/mL), which is similar to the present results. Also Fisher and Phillips (2006) showed a MIC value of 0.06% of linalool against *C. jejuni*. Our results are in agreement with the ones described in the literature for *C. jejuni*, however, concerning *C. coli* there are no published studies. It was also observed that the MBC values were equal to the MIC values for all the tested strains, suggesting the bactericidal activity of coriander oil and linalool. Similar results were previously

reported against several microorganisms (Duarte et al., 2013; Silva et al., 2011b), indicating that the primary mechanism of action of coriander oil is membrane damage, leading to cell death (Silva et al., 2011b). Concerning linalool, since it is the major component of the coriander essential oil, its bactericidal action could be explained by the fact that this compound may act on the bacteria cell wall, similarly to coriander oil (Yap et al., 2014). In sum, both coriander oil and linalool were shown to be effective in the inhibition of *C. jejuni* and *C. coli*, increasing its potential to be used to control these foodborne pathogens.

3.2. Anti-biofilm activity

Besides the search for new compounds with antibacterial activity against planktonic cells of foodborne pathogens, new strategies must be developed in order to control and/or eliminate the bacterial biofilms of these pathogens. *Campylobacter* species have the capacity to form biofilms and it has been proposed that the microenvironment created within the biofilm may protect the *Campylobacter* cells from oxygen inactivation and increasing the viability of cells (Buswell et al., 1998). Those biofilms are more resistant to antimicrobials and disinfectants and are becoming a major problem for the food industry, since the formation of bacterial biofilms on the processing equipments increases the possibility of cross-contamination, leading to the subsequent contamination of food and thus contributing to the human infection due to the handling of the contaminated products (Srey et al., 2013). Thus, in this study, we also evaluated the effect of coriander oil and linalool in biofilms of *C. jejuni* and *C. coli* strains. Different concentrations of coriander oil and linalool, ranging from 0.5× to 4× the MIC, were evaluated on the formation (Figure 1) and dispersion (Figure 2) of biofilms of *Campylobacter* spp. through the quantification of the biofilm formed by the CV assay. Concerning the effect of coriander oil on biofilm formation it was observed that the

338 reduction of biofilm biomass increased with the increase in the concentrations of the oil
339 for all the tested strains, however with no significant difference ($p>0.05$) for the *C. coli*
340 ATCC 33559 (Figure 1A). When using 4×MIC, the biofilm formation was inhibited
341 70%, 86%, 75% and 85% for the *C. coli* ATTC 33559, *C. coli* 873, *C. jejuni* ATCC
342 33560 and *C. jejuni 225421*, respectively. With 0.5×MIC the effect was similar for all
343 the tested strains (about 35% of inhibition) with the exception being *C. coli* ATCC
344 33559 with an inhibition of 53% ($p<0.05$). The effect of linalool on biofilm formation
345 was also evaluated (Figure 1B) and a lower effect was observed for all the tested
346 concentrations when compared with the coriander oil. The biofilm inhibition ranged
347 between 43% and 77% when using 4×MIC concentration, and between 14% and 32%
348 with 0.5×MIC. In general, biofilms formed by the reference strains seemed to be less
349 susceptible to the effects of both coriander oil and linalool when using 4× and 2×MIC
350 ($p<0.05$). Regarding the effect against established biofilms, coriander oil (Figure 2A) at
351 the highest tested concentration decreased between 77% to 87% the biofilm biomass for
352 all the *Campylobacter* strains; and linalool (Figure 2B) reduced the biofilm biomass by
353 about 75% for all tested strains. Even at sub-inhibitory concentrations, it was possible to
354 observe a reduction of the established biofilm. As expected, coriander oil had a higher
355 inhibitory effect than linalool, since coriander oil is a mix of different compounds which
356 may act synergistically, increasing its activity (Silva et al., 2011a). In contrast to what
357 was observed for the effect on biofilm formation, the tested compounds presented a
358 similar effect on biofilms already established by all the strains. Szczepanski and Lipski
359 (2014), studied the effect of four essential oils against several pathogens and observed
360 an inhibitory effect on biofilm formation when using concentrations below the minimal
361 inhibitory concentration, similarly to the results obtained in the current study. However,
362 studies regarding the anti-biofilm activity of coriander essential oil or linalool are

limited and there are no published studies concerning this effect on *Campylobacter* biofilms. Furletti et al. (2011) evaluated the action of coriander oil on biofilms formation by *Candida albicans*, showing an inhibitory effect of the oil on the formation of biofilms. Also, Duarte et al. (2013) evaluated the ability of coriander oil to inhibit the formation of *Acinetobacter baumannii* biofilms, and showed that the oil was able to inhibit biofilm formation and promoted its eradication at concentrations ranging from two to four times the MIC value. In contrast to these results, a previous work showed that when *Listeria monocytogenes* biofilms were treated with linalool, biofilm formation increased from 0 to 18 h and then remained constant, despite the reduction in metabolic activity after 6 h of exposure (Sandasi, Leonard, & Viljoen, 2008). In sum, our results suggest that both tested compounds could be used to control the biofilms of *C. coli* and *C. jejuni*. Although various mechanisms are described for inhibiting biofilm formation by essential oils (Issac Abraham, Palani, Ramaswamy, Shunmugiah, & Arumugam, 2011; Nostro et al., 2007; Szczepanski & Lipski, 2014), we found, in this study, that the anti-biofilm activity of coriander oil and linalool could be also due to the effect on QS, since this process has been documented to play a role in the formation and maintenance of *Campylobacter* biofilms (Gölz et al., 2012). However, more studies should be performed to clearly establish the effect of coriander oil and linalool against *Campylobacter*.

3.3. Anti-QS activity

Intercellular communication mechanisms like quorum sensing (QS) enable bacteria to regulate some physiological activities, such as biofilm formation and antibiotic resistance (Gölz et al., 2012). The pathogenesis of bacteria linked to food spoilage has also been associated to QS and the regulation or inhibition of QS can be a good strategy for food preservation (Alvarez et al., 2012; Alvarez et al., 2014). So, in this study, the

influence of coriander oil and linalool in the QS system was evaluated by the disc diffusion test using the *Chromobacterium violaceum* ATCC 12472 as biosensor strain (Table 3). *C. violaceum* ATCC 12472 is a Gram-negative bacterium which synthesizes violacein, a purple pigment, in response to QS regulated by N-acylhomoserine lactones (AHL) (Alvarez et al., 2012). AHL are the major inductor molecules involved in QS in Gram-negative bacteria, being also described for some *Campylobacter* species (Plummer, 2012). So, the biosensor strain *C. violaceum* ATCC 12472 is a widely used strategy to do a first screening of the anti-QS activity of the compounds. In this study, it was observed that both coriander oil and linalool inhibited the violacein production by the biosensor strain. Both pure compounds presented similar anti-QS activity, since the diameter of violacein inhibition was equal ($p>0.05$) (Table 3). As expected, when using the pure compounds the inhibition diameter was higher than when using the compounds at a concentration of 50% ($p<0.05$). In order to confirm the anti-QS activity, the quantification of the violacein production by the *C. violaceum* ATCC 12472 in the absence and presence of coriander oil and linalool was also performed (Figure 3). It was observed that all the tested concentrations of coriander oil and linalool were able to reduce the violacein production, with higher concentrations (0.25-5 µL/mL) showing inhibition percentages higher than 90%. Concerning the lower concentration tested (0.1 µL/mL), the effect of linalool was more pronounced than coriander oil ($p<0.05$), showing an inhibition of 70% and 43%, respectively. DMSO was used as control and it was only possible to observe an inhibition of the violacein production lower than 10%. The loss of purple pigment in *C. violaceum* is an indication of QS inhibition by bioactive products (Alvarez et al., 2014) and our results showed the potential of coriander oil and linalool as promising QS inhibitors (QSI). The potential of the natural compounds as QSI has been extensively described, with authors suggesting their use for

controlling foodborne pathogens and for food preservation (Ahmad, Viljoen, & Chenia, 2015; Alvarez et al., 2012; Castillo, Heredia, Arechiga-Carvajal, & García, 2014; Nazzaro et al., 2013).

3.4. Antioxidant activity

The potential of natural compounds and essential oils as natural antioxidants has been described, and they are considered to be a good alternative to synthetic antioxidants in the food industry (Calo et al., 2015; Falowo et al., 2014; Sacchetti et al., 2005). In this work, the antioxidant activity of coriander essential oil and linalool was evaluated by two different methods: DPPH scavenging assay and β-carotene bleaching test (Table 4). In the DPPH assay, the antioxidants react with the stable free radical with deep violet color and convert it to a compound with no coloration, so the degree of discoloration indicates the free radical scavenging potentials of the tested sample (Samojlik et al., 2010). It was possible to observe that coriander essential oil has better antioxidant activity than linalool since that the IC_{50} value of coriander oil is lower than the one of linalool (Table 4). Although both compounds possess antioxidant activity, this is significantly lower than the reference compounds (gallic acid and quercetin). Coriander oil possesses only moderate activity and linalool has poor activity when looking at the AAI values. The antioxidant properties of coriander oil had previously been described by several authors (Laribi et al., 2015; Samojlik et al., 2010; Teixeira et al., 2013; Wangensteen, Samuelsen, & Malterud, 2004), however we observed relevant discrepancies. For example Wangensteen et al. (2004) described that the essential oil from coriander seeds showed weak inhibitory activity and linalool was inactive and Teixeira et al. (2013) described that coriander oil had almost no antioxidant activity. In contrast, Samojlik et al. (2010) described results similar to ours, with coriander oil

showing an IC_{50} value of 5.35% and linalool proving to be a leading component in the stabilization of the DPPH radical.

The β-carotene bleaching test was used to determine the ability of coriander oil and linalool to inhibit lipid peroxidation. It is a useful test because it is performed in an emulsion, conditions similar to those found in the food industry (Nikolić et al., 2014). The results obtained with this method are listed in Table 4 and it is possible to observe that coriander oil and linalool presented an IC_{50} lower than that of the synthetic antioxidant used as reference (BHT). The lower IC_{50} values indicate the high potential of both compounds to inhibit the lipid peroxidation, even better than BHT. Coriander oil and linalool showed a potential to inhibit the lipid peroxidation, being higher than the BHT inhibitory potential. Taking in account the overall results in antioxidant activity, it is possible to conclude that coriander oil and linalool had relevant radical scavenging properties and an exceptional capacity to inhibit the lipid peroxidation. This result is very promising for the food preservation industry, since these natural products could be used as alternatives to synthetic ones, while having a better protective effect.

4. Conclusions

The present study demonstrated the antibacterial, anti-biofilm and anti-QS potentials of the coriander essential oil and its major compound, linalool. Both compounds showed anti-*Campylobacter* activity and for the first time we described the antibacterial activity against *C. coli*, an emergent foodborne pathogen in addition to *C. jejuni*. Coriander oil and linalool also inhibited *in vitro* biofilm formation and promoted biofilm dispersion even at sub-MIC concentrations, while inhibiting the violacein production through the QS inhibition. In addition, the essential oil and linalool could be considered as potential substitutes of synthetic antioxidants due to their radical scavenging properties and lipid peroxidation inhibition ability. In sum, our results strongly suggest that coriander oil

462 and linalool could be considered as new anti-biofilm agents, QS inhibitors and
463 antioxidants to enhance shelf life of food products and increase food safety without the
464 use of chemical additives or preservatives.

465

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631

632

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634

635 **Figure 1.** Effect of different concentrations of coriander essential oil (A) an linalool (B)
636 on biofilm formation by *Campylobacter* spp. Biofilm formed was evaluated by the CV
637 assay and the results are expressed as percentage of biofilm inhibition. Bars labeled
638 with different letters indicate significant differences at $p<0.05$.

639

640 **Figure 2.** Effect of different concentrations of coriander essential oil (A) an linalool (B)
641 on a pre-established biofilm by *Campylobacter* spp. The results are expressed as
642 percentage of biofilm inhibition and the biofilm formed was evaluated by the CV assay.
643 Bars labeled with different letters indicate significant differences at $p<0.05$.

644

645 **Figure 3.** Anti-quorum sensing activity of coriander essential oil and linalool by the
646 inhibition of violacein production using *Chromobacterium violaceum* ATCC 12472 as
647 biosensor strain. Values are given as mean ± standard deviation. Bars labeled with
648 different letters indicate significant differences at $p<0.05$. Values are all significantly
649 different from control ($p<0.05$).

Table 1. Antibacterial activity of coriander essential oil and linalool against two *Campylobacter* isolates (*C. jejuni* 225421 and *C. coli* 873) and two reference strains (*C. jejuni* ATCC 33560 and *C. coli* ATCC 33559) by disc diffusion and vapour-phase assays. Diameters (mm) of inhibition zones of the compounds (Mean ± Standard Deviation).

***Campylobacter* strains**	**Disc diffusion (diameter in mm)**				**Vapour-phase assay (diameter in mm)**	
	Coriander (100%)	**Coriander (50%)**	**Linalool (100%)**	**Linalool (50%)**	**Coriander (100%)**	**Linalool (100%)**
C. jejuni ATCC 33560	>85	17.00±1.41^{ab}	>85	19.50±0.71^{a}	>85	>85
C. jejuni 225421	>85	17.00±1.00^{a}	>85	17.50±0.71^{a}	>85	>85
C. coli ATCC 33559	>85	16.50±0.71^{a}	>85	17.50±0.71^{a}	>85	>85
C. coli 873	>85	22.33±2.52^{b}	>85	38.00±2.83^{b}	>85	>85

Different letters in the same column indicate significant difference ($p < 0.05$).

Table 2 . Minimal inhibitory concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of Coriander essential oil and linalool against two *Campylobacter* isolates (*C. jejuni* 225421 and *C. coli* 873) and two reference strains (*C. jejuni* ATCC 33560 and *C. coli* ATCC 33559).

***Campylobacter* strains**	**MIC (MBC) (µL/mL)**	
	Coriander	**Linalool**
C. jejuni ATCC 33560	0.5 (0.5)	0.5 (0.5)
C. jejuni 225421	1 (1)	1 (1)
C. coli ATCC 33559	0.5 (0.5)	0.5 (0.5)
C. coli 873	0.5 (0.5)	0.5 (0.5)

Table 3. Diameter (mm) of inhibition of violacein production by coriander essential oil and linalool for the *Chromobacterium violaceum* ATCC 12472 strain (mean ± standard deviation).

	Compounds			
	Coriander (100%)	**Coriander (50%)**	**Linalool (100%)**	**Linalool (50%)**
QS inhibition (diameter in mm)	63.50 ± 2.12[a]	35.00 ± 2.83[b]	69.50±0.71[a]	44.00±1.41[c]

Different letters indicate significant difference ($p < 0.05$).

Table 4. Antioxidant properties of coriander essential oil and linalool by the DPPH scavenging assay and β-Carotene bleaching test. Results are presented as mean ± standard deviation of three replicates.

Antioxidant activity	**DPPH scavenging assay**			**β-Carotene bleaching test**
	IC_{50} (%)	**AAI**	**Antioxidant Activity**	**IC_{50} (%)**
Coriander	5.84 ± 1.17	0.95 ± 0.17	Moderate	0.23 ± 0.01
Linalool	22.66 ± 5.30	0.25 ± 0.03	Poor	1.52 ± 0.03
Gallic acid	0.22 ± 0.01	22.77 ± 0.25	Very Strong	-
Quercetin	0.43 ± 0.04	12.17 ± 1.71	Very Strong	-
Butylated hydroxytoluene (BHT)	-	-	-	3.58 ± 0.02

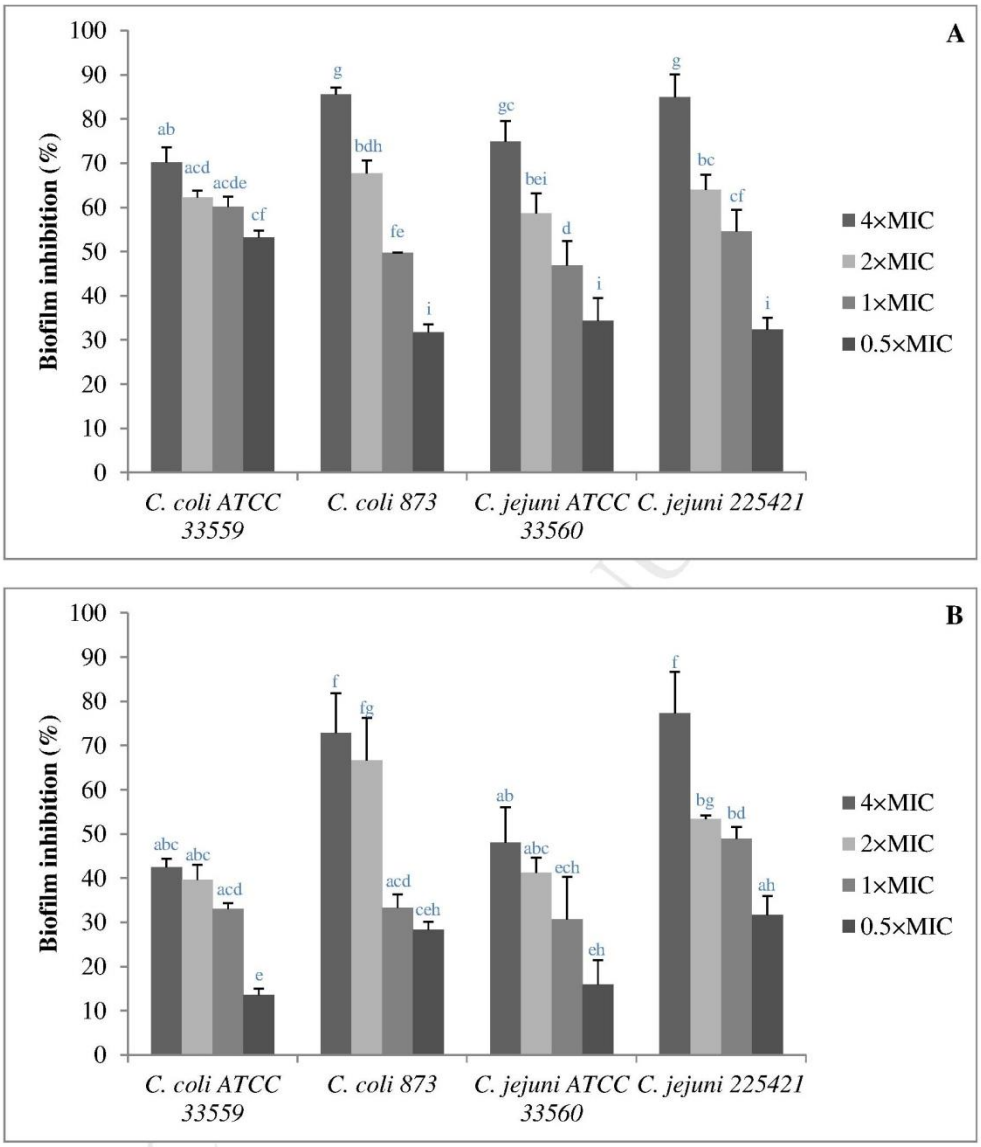

Figure 1.

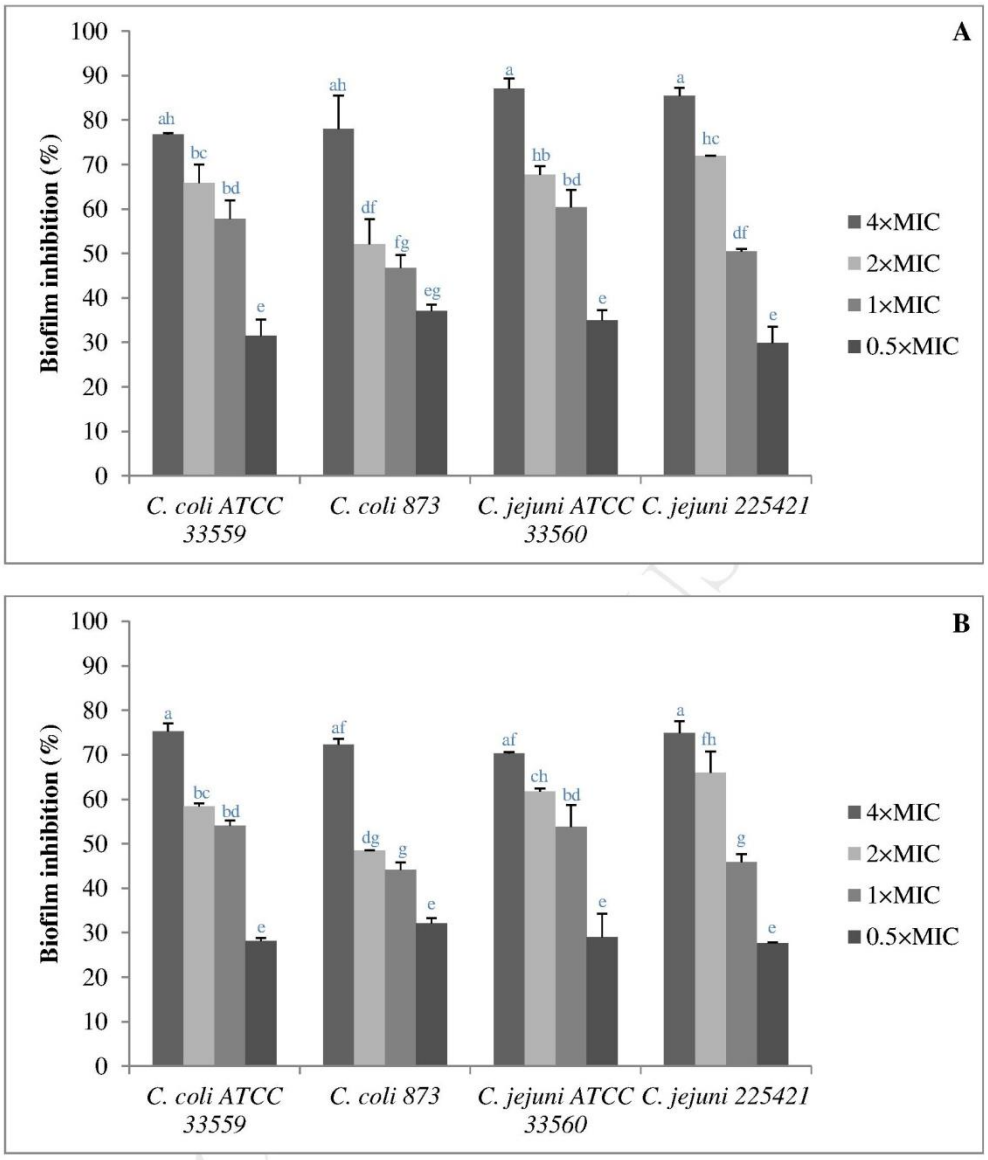

Figure 2.

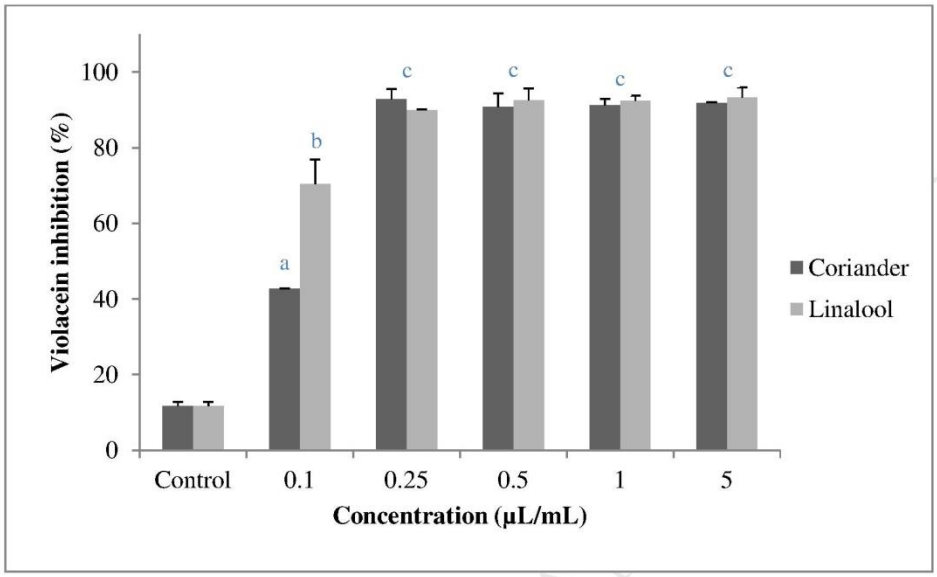

Figure 3.

Highlights

- Coriander essential oil and linalool have anti-*Campylobacter* activity.
- Bactericidal activity of coriander oil and linalool with MIC values of 0.5 and 1 µL/mL.
- Both compounds inhibited biofilm formation and promoted biofilm dispersion.
- The compounds interfered in the quorum sensing by inhibiting the violacein production.
- Both compounds showed an exceptional ability to inhibit lipid peroxidation.

Chapter 8 – Conclusions and Future Perspectives

Chapter 8 – Conclusions and Future Perspectives

Campylobacter jejuni and *Campylobacter coli* are well known foodborne pathogens and are described to cause several gastrointestinal and extragastrointestinal complications in humans. Since *Campylobacter* has developed resistance to several antibiotics and possesses mechanism to survive and persist under hostile environments, the search of new antibacterials to control this pathogen is crucial.

In order to understand the epidemiology of *Campylobacter* in Portugal, the first step in our research was the genotypic and phenotypic characterization of *Campylobacter* strains, isolated from different sources. For this first task we choose 125 *Campylobacter* isolates from humans, 39 from retail food and 32 from animals, and the strains were characterized by MLST and *flaA* typing. We found that the *C. coli* isolates were genetically more conserved than *C. jejuni*. From MLST data we observed that within each species, genetically related isolates were recovered from different sources, suggesting that *Campylobacter* infections in humans arise from different sources, with food and animals being the main source of transmission to humans. With the *flaA* typing results we observed a high variability for both species, while some *flaA* types comprised both *C. jejuni* and *C. coli* isolates, indicating frequent interspecies recombination. Then we studied the resistance phenotype to several antibiotics and we demonstrated high resistance rates to several antibiotics, including the ones used in the treatment of severe campylobacteriosis. In addition, we observed a multidrug resistance phenotype in 86% of the isolates. The high resistance rates for fluoroquinolones, tetracyclines and macrolides that we found, are likely to be related with the widespread use of these antibiotics in food-producing animals, since Portugal is one of the countries with the highest consumption rate of these antibiotics for veterinary treatment. Once we observed that the resistance to antibiotics was higher than the expected, we decided to study the underlying molecular mechanisms. The Thr-86-Ile mutation in the QRDR of the *gyrA* gene was found in all the ciprofloxacin resistant isolates, with no other mutation being associated with this resistant phenotype. For the erythromycin resistance only the mutation A2075G in the 23S rRNA gene was detected and we observed that the efflux pump mechanism did not contribute to this phenotype, unlike the results obtained in other studies. Concerning gentamicin resistance, we found the three gentamicin-resistant isolates harboured the *aphA-3* aminoglycoside resistance marker, and one strain had a non-synonymous point mutation. In addition, we showed that efflux pumps may also play a role in the gentamicin resistance. In sum, the results obtained in this first step of our research allowed us to establish a relationship between strains isolated from different sources in Portugal, and to correlate phenotypic and genetic profiles. Some molecular mechanisms of antibiotic resistance were also elucidated and a worrying antibiotic multi-resistance rate and the emergence of *Campylobacter* strains resistant to antibiotics of human use were highlighted. Then, we select a group of strains representing the highest diversity to be used in the subsequent steps of our work.

In the next step of our work, we decided to study the potential of resveratrol to control *Campylobacter*. However, even though resveratrol has several biological properties, it possesses low aqueous solubility and high instability. So, to address those shortcomings, we studied the encapsulation of resveratrol with a methyl-β-cyclodextrin in order to increase its solubility and stability. We found that resveratrol complexation caused a 400 fold improve in its solubility. Further, the inclusion complex formation was confirmed by Fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), X-ray diffraction (XRD) and scanning electron microscopy (SEM). After the formation of the inclusion complex, we showed that both, resveratrol and inclusion complex, had very strong antioxidant activity, low toxicity and had the ability to reduce the viability of cancerous cells. In addition, we found that resveratrol had antibacterial activity against the previously selected *Campylobacter* strains. These results suggest that resveratrol and its inclusion complex can be used to control *Campylobacter*, while maintaining its biological activities.

Taking in account the work developed by our research group with *Arcobacter*, which is closely related to *Campylobacter* and is also known to be a foodborne pathogen, we further studied the potential of resveratrol against these two organisms. After screening the antibacterial activity of several resveratrol inclusion complexes (data not shown) against *Campylobacter* and *Arcobacter*, we choose a resveratrol-hydroxypropyl-γ-cyclodextrin inclusion complex for further studies. We found that both resveratrol and hydroxypropyl-γ-cyclodextrin inclusion complex have a bactericidal effect against the two microorganisms, and inclusion complex may act by inducing membrane depolarization and by affecting the metabolic activity of the cells. In addition, we have also shown that resveratrol and the inclusion complex inhibit biofilm formation and diminish established biofilms, even at sub-inhibitory concentrations. This anti-biofilm effect could be due to the ability of both compounds to inhibit the QS system, and consequently, affecting the biofilm formation and development. With these results, we have shown that resveratrol could be used in the food industry as antibacterial agent, where the bacterial biofilms are an emerging problem, allowing an enhance shelf life and an increase in food safety.

Nowadays, there has been a growing interest in the use of natural compounds for application in food products, mainly due to the preference of consumers for natural ingredients and the concerns about the toxic effects of synthetic compounds. So, in addition to resveratrol, and given our experience with coriander EO, we also evaluated the potential of coriander EO and its major compound linalool to control *Campylobacter*. Both compound showed a bactericidal effect against all the tested strains and we also observed that the volatile compounds of the coriander EO also inhibited the growth of *Campylobacter*. Coriander EO and linalool also inhibited biofilm formation and promoted biofilm dispersion. The effect on the QS system was also studied and both compounds had anti-QS activity by inhibiting the violacein production. This anti-QS activity could be associated with the anti-biofilm activity, since QS has been

described to regulate the biofilm formation and development. Moreover, in addition to the antibacterial and anti-biofilm potential, coriander EO and linalool showed an exceptional ability to inhibit lipid peroxidation. Once again, we showed that these natural compounds could be used to control this foodborne pathogen and as antioxidant to enhance food quality without the use of chemical additives.

In conclusion, in this thesis, we showed the genetic diversity of *Campylobacter* isolates isolated from different sources in Portugal, as well as worrying antibiotic resistance rates. Therefore, one of the main goals of this work was to search for new antibacterials to control this emergent foodborne pathogen. We found that resveratrol and coriander essential oil, both natural compounds, which have several biological activities, such as antioxidant potential, also have the ability to reduce planktonic cells and biofilms of *Campylobacter*, being potential agents to be used in the food industry to increase the food safety and diminish the human infections by *Campylobacter*.

In a further work, it could be interesting to study the mechanism of action of resveratrol and coriander essential oil on biofilms of *Campylobacter*, by evaluating the effect of these compounds in proteins involved in the process of biofilm formation, such as flagellins (FlaA and FlaB), the filament-associated protein (FlaG), the chemotaxis protein CheA, heat stress proteins, the adhesin FlaC, etc. Since the QS system was described to play a role in the formation and growth of biofilms and since in this thesis we showed the anti-QS activity of these natural compounds, it would be also important to further evaluate the role of the QS in the biofilm formation in *Campylobacter* and how these compounds act in the QS system. For example, using a proteomic approach it would be possible to identify the main proteins involved in the QS, and to correlate the biofilm forming ability with the QS process. Then, in a next step and to clarify the mechanism of QS inhibition, the expression of genes associated with AHLs synthesis, *luxS* and others identified by the proteomic approach, could be evaluated by quantitative real time PCR using samples treated with the compounds. Also, the effective use of resveratrol and coriander essential oil as food preservatives or food active ingredients will require future investigation namely concerning its potential on food model systems and to determine their effective concentrations in food matrices.